UNIVERSITY OF SOUTHAMPTON

Ecophysiology and Genetics of Drought Tolerance in *Populus*

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Thesis presented for the degree of Doctor of Philosophy

School of Biological Sciences

November, 2006

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES
SCHOOL OF BIOLOGICAL SCIENCES
Doctor of Philosophy

ECOPHYSIOLOGY AND GENETICS OF DROUGHT TOLERANCE IN *POPULUS*

By Maricela Rodriguez-Acosta

The experimental work presented here on the physiology and morphology of *P. trichocarpa* and *P. deltoides* provides a good insight into the ecophysiology and the genetic basis of drought tolerance in *Populus* and in particular, in their F_2 hybrid Family. Studies of these and other genotypes have identified their remarkable yields when grown in short rotation coppice, and intensive work has concentrated on finding the genetic and molecular basis of how biomass can be increased at individual and species levels with the aim of finding new sources of biofuel and energy, especially in marginal agricultural lands.

The study of gas exchange and stomatal dynamics revealed the different strategies that the parental species develop when they encounter drought conditions. The results showed the existence of contrasting patterns in stomatal regulation linked to the ontogenetic and developmental changes in the leaf, and an internal leaf blade structure linked to the response to drought. Important variations in stomatal conductance during daytime, leaf age and level of insertion are significantly different between *P. trichocarpa* and *P. deltoides*. The complete lack of response to ABA in mature leaves of *P. trichocarpa* was detected by exogenous application and thermal imaging, and it seems to be linked to developmental processes.

Trichome presence and development of intercellular spaces represent some of the most morphologically distinct features of the two grandparents influencing the differential response to CO_2. The responses of the F_2 showed the negative effect of drought on leaf growth, but also that while leaf traits seem genetically more robust, cell traits were more affected by the environment. The contrasting responses in the two environmental conditions (UK vs Italy) make it possible, through QTL analysis, to propose 42 candidate genes that could potentially explain these responses and contribute to the understanding of the responses to the complex phenomenon of drought.

This work raises several questions regarding the role of night conductance in *P. trichocarpa* and stomata evolution in *Populus*.

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Acknowledgements

I would like to thank Prof. Gail Taylor and Dr. Peter Freer-Smith for their supervision, support and advice during this Ph.D. I am also grateful with the support of the Benemerita Universidad Autonoma de Puebla in Mexico, together with PROMEP for the studentship granted.

I would like to thank to all those who have participated in different stages of this research. Firstly I want to thank Dr. Anne M. Rae who was responsible for the QTL analysis included here and also for her help with the field work in the UK and in Italy. I also want to thank Dr. Nathaniel Street for his participation in the drought experiments and for his help in the use of the *Populus trichocarpa* genome databases.
Special thanks to Dr. James Morison of the University of Essex, for his support in the thermographic experimental phase and Dr. Rainer Hedrich of the Julius von Planck Institute at the University of Würzburg, Germany for his support in the guard cell impalement work and Dr. David Williams, of Lancaster University, for ABA determinations.

Thanks to Harriet Trewin for her support in the co-location of candidate genes in the physical map of *P. trichocarpa*; to Fang Zhang and Dr. Penny Tricker for training in the use of laboratory equipment and for their constant help, to Dr. Carol Westarf for her support in the use of the Instron and to Matthew Pinnel for his support in the field work in the UK and Italy.

Special thanks to Caroline Dixon for her assistance in various aspects of this research in the laboratory, field sites and greenhouse and to Mike Cotton and Dave Badens for their assistance in the growth and care of the plant material used.

I am grateful to the students who helped in various activities necessary for this research, especially to Megan Powis who helped with the stomatal conductance work in Headley, and to Adam Smith and Kathy Cherry. Special thanks to Allen J. Coombes who helped in the work in Boldrewood and Chilworth, and also helped me to correct my English.

Declaration

The contents of this thesis are the results of original work by the author. I hereby declare that this thesis has not been submitted, either in the same or different form to this or any university for a degree and where a publication has been derived, this has been indicated.

List of abbreviations and symbols

Abbreviation	Definition
Ψ	Water potential
Ψ_S	Solute potential
Ψcrit	Critical water potential (at wilting point)
Ψp	Turgor pressure
A	Photosynthetic assimilation rate
ABA	Abscisic acid
ABRE	ABA response element
Amax	Maximum rate of photosynthesis
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
GLM	General lineal model
bp	Base pair
BspA	Boiling stable protein
°C	Degrees Celsius
C_3	Refers to photosynthesis pathway. Form phosphogliceric acid (PGA)
Ci	Carbon intercellular
C_4	Refers to photosynthesis pathway. Form a 4 organic acid (oxaloacetic acid)
CAM	Refers to photosynthesis pathway. Crassulacean acid metabolism
cDNA	Complementary DNA
CE	Carboxylacion efficiency
cM	Centi-Morgans
cv	Cultivar
D	*P. deltoides*
DAT	Days after treatment
DOE	US Department of Energy
DN	*P. deltoides* x *P. nigra*
DRE	Dehydration response element
E	Transpiration rate
ECA	Epidermal cell area
ECD	Epidermal cell density
ECN	Epidermal cell number
eCO2	Elevated CO_2
EST	Expressed sequence tag
g_s	Stomatal conductance

Abbreviation	Definition
G	Genotype
g_{max}	Stomatal conductance maxima
GR	Growth rate
G x E	Genotype per Environment interactions
IRGA	Infra-red gas analyser
J_{max}	Maximum rate of RuBP regeneration
Kx	Xylem conductivity
LEA	Late embryogenesis abundant
LG	Linkage group
Lp	Leaf plastochron
LPI	Leaf plastochron index
Ls or ls	Stomatal limitation
m	Cell wall extensibility
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MAS	Marker assisted selection
min	Minutes
MPa	Megapascals
mRNA	Messenger RNA
$NADP^+$	Nicotinamide adenosine dinucleotide phosphate
ns	No significant
O	Osmolality
OA	Osmotic adjustment
P	Plasticity
p	Probability
Pa	Pascals
PA/PAs	Polyamine (s)
PAR	Photosynthetic active radiation
P_{max}	Maximum photosynthesis
PI	Plastochrome index
PPFR	Photosynthetic photon flux radiation
PU	Putative unit
QTL	Quantitative trait loci
RAPD	Randomly amplified polymorphism
R day	Respiration day

Abbreviation	Definition
RFLP	Restrict fragment length polymorphism
ROS	Reactive oxygen substances
RuBP	Ribulose biphosphate carboxylase/oxygenase
s	seconds
sd	Standard deviation
SD	Stomatal density
SEM	Scanning electron microscope
SI	Stomatal index
SLA	Specific leaf area
SN	Stomatal number
SRWC	Short rotation woody crops
STS	Sequence tagged site
T	*P. trichocarpa*
TD	*P. trichocarpa* x *P. deltoides*
TPU	Triose Phosphate Utilization
TEM	Transmission electron microscope
Vc_{max}	Maximum velocity of carboxylation
WT	Wild type
WUE	Water use efficiency
Y	Cell wall yield threshold
XET	Xyloglucan endotransglucosylase
XTH	Xyloglucan endotransglucosylase/hydrolase

Gene names are shown in CAPITAL and abbreviations in *CAPS*

General Introduction

Populus, is one of the most abundant woody plant genera in temperate forests, with more than 60 species world wide (Flora of China 2006, Hillier and Coombes 2002) and is accepted as a model tree in forestry (Bradshaw et al. 2000, Taylor 2002). Its intense culture and study started in the early1970s, shortly after the U.S. Department of Energy (DOE) embraced the concept of SRWC (short rotation woody crops), as a way of supplying biomass for conversion to liquid transportation fuels. At present, *Populus* is the main choice of a group of model species for SRWC which includes sycamore (*Platanus occidentalis* L.), silver maple (*Acer saccharum* Marshall), and hybrid willow (*Salix* spp.), all suitable for the Pacific Northwest, north central and southeastern regions of the U.S. Genetic improvement programs, silvicultural studies and basic research were initiated and continue today throughout the world (Tuskan 1998).

Poplars have three main properties that make them excellent for short-rotation intensive-culture management: rapid juvenile growth, immediate response to cultural practices and their coppicing property (Bradshaw et al. 2000). They have also a great tendency to hybridization in their natural habit, giving rise to a wide range of genetically diverse populations. In addition to their high yields, it has been observed that hybrid poplars are more drought tolerant than native cottonwoods (Tschaplinski et al. 1998, Wyckoff and Zasada 2002). Because of this there are major selection and breeding programs, with the objectives of extending crops to marginal agricultural lands and supplementing the diminishing supply of natural hardwoods. One of the most successful hybrids created is the intersectional cross between *P. trichocarpa* and *P. deltoides* (Fig. 1A,C), which belong to the *Populus* sections Tacamahaca and Aigeiros respectively. The first of these contrasting species was selected from a maritime and wet climate in the Pacific Coast, the second from a more continental and less humid area in the eastern United States (Figure 1B, D). These two species show marked differences in both stomatal behavior and photosynthetic patterns (Hinckley et al. 1989, Dunlap et al. 1993).

An important feature in poplar hybrids and species linked to their high productivity, is their high rate of stomatal conductance (g_{max} are near 600 mmol m^{-2} s^{-1}), which suggests the transpiration of large volumes of water (U.S. Enviromental Protection Agency, 1999).

Therefore, considerable attention has been given to the study of water regulation in several species, hybrids and cultivars that exhibit a wide range of variation. Of particular interest are the pioneering studies of poplar water relations of Reich (1984), Schulte and Hinckley (1987), Bassman and Zwier (1991), followed by others on physiological processes such as gas exchange (Dickmann et al. 1992, Dunlap et al. 1993, Ridolfi et al. 1996, Liu and Dickmann 1992c and 1996, Schulte et al. 1987, Tschaplinski and Blake 1989b, Tschaplinski et al. 1994, Ceulemans et al. 1988, Mazzoleni and Dickmann 1988), and on growth and biomass rates in large scale plantations (Tschaplinski et al. 1998b, Bunn et al. 2004, Rae et al. 2004).

Whole plant and leaf studies have revealed that hybrid poplars close their stomata rapidly in response to atmospheric and soil water deficit, resulting in lower transpiration rates and a greater drought resistance compared to the parental species, a feature which allows hybrids to maintain higher leaf areas for longer periods (U.S. Environmental Protection Agency, 1999). Consequently the question of how poplars tolerate increasing drought stress without an impact on productivity has been raised and investigated by several authors. This issue is of particular relevance to increasing the use of marginal agricultural land and in achieving an increase in the energy conversion to liquid transportation fuel in temperate regions (Mazzoleni and Dickmann 1988, Tschaplinski and Blake 1989a, 1989b; Tschaplinski et al. 1994, 1998b, 1999).

Of considerable importance is the until now unexplained poor stomatal regulation in *P. trichocarpa* (Dunlap et al. 1993, Dunlap and Stettler 2001, Bassman and Zwier 1991). This contrasts with the very good stomatal regulation of *P. deltoides* and the hybrids *P. trichocarpa* x *P. deltoides*. It has been found that a strong relationship between stomatal conductance and leaf surface stomatal density in *Populus* cultivars (Reich 1984). As these two parameters have a direct effect on gas exchange and therefore on productivity, a better understanding of the variation in stomatal dynamics is important (Ceulemans et al. 1988).

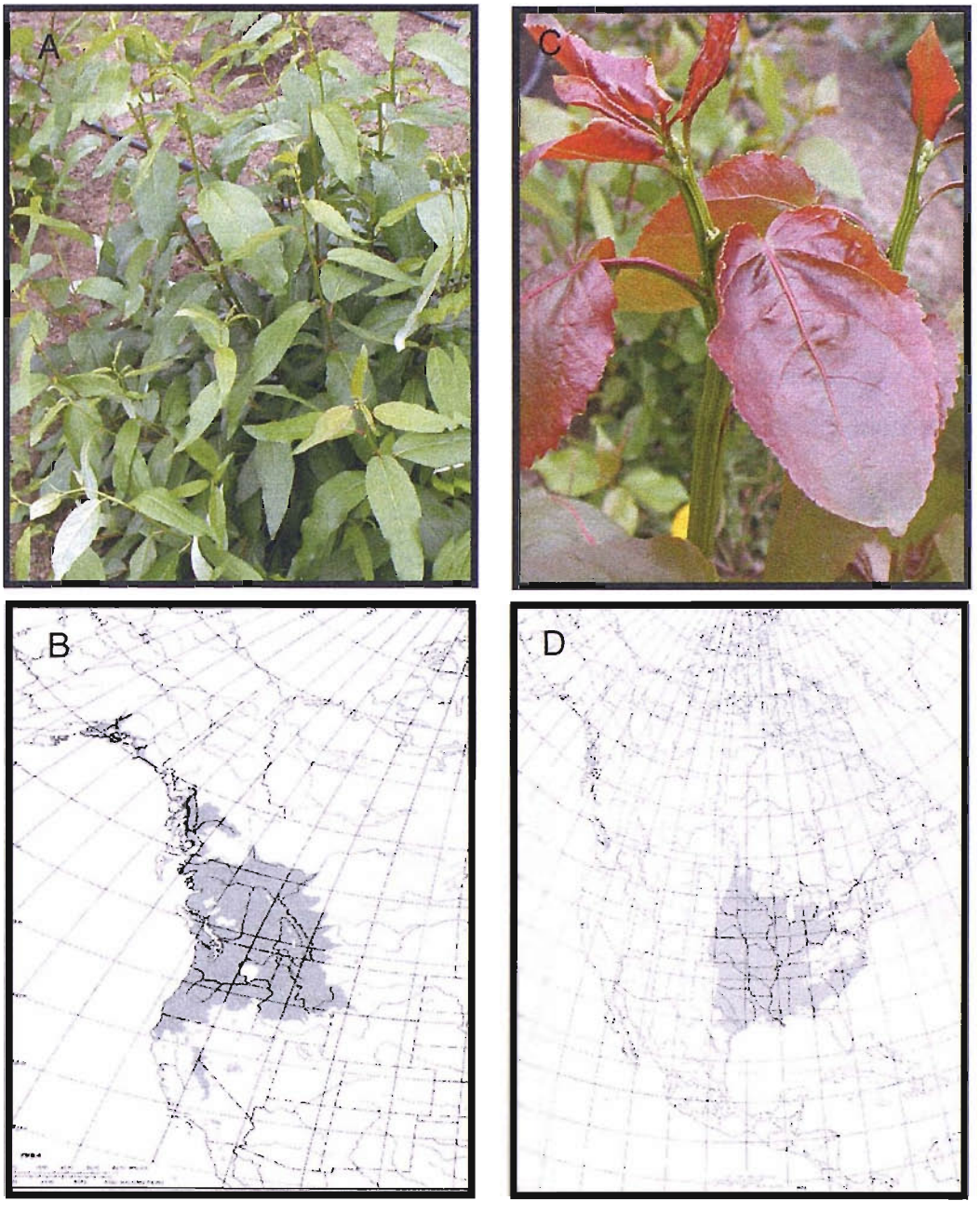


Figure 1 Young foliage of *Populus* species growing in the UK showing their American distribution. (A) *Populus trichocarpa* is one of the fastest growing tree species in the riparian communities in the (B) Pacific Northwest region of North America. It grows at high elevations and latitudes in the humid Coastal Forest in the Pacific Northwest, where the precipitation ranges from 250 to 3050 mm and temperature varies between 47 and -47 °C. (C) *P. deltoides* is native to drier habitats in the D) East of America is considered as intolerant of shade. Precipitation in its natural range varies between 380 and 1400 mm and temperatures between 46 and -45 °C. Its habitat is close to rivers or within 100 m of them and its root is phreatophytic.

A major aim of this work is to understand the morphological and physiological responses to drought of the original parental species of the hybrid F_2 Family 331 *P. trichocarpa* and *P. deltoides* (Figure 1A and C) and of hybrid F_2 generation, when they are submitted to conditions of water deficit. As part of the work, stomatal function has been examined because of its importance in the control of gas exchange, drought tolerance and productivity. The study of leaves in the F_2 progeny, at a micro and macro structural level, will show the genetic variation in the traits evaluated, and consequently allow the detection of traits that can assist in interpreting the adaptation of a plant to its environment (Wu et al., 1997). This morphological integration increases as the environment becomes more stressful (Wu and Stettler 1998).

Chapter 1

Introduction

1.1 Drought and its implications in plants

Water is both the most abundant and limiting resource for the productivity of agricultural systems, ecosystems and individual plants. The effects of drought are rapidly seen as wilting of leaves and are strongly linked to plant productivity. Thus understanding the uptake and loss of water by plants is very important and has been widely studied (Beadle et al. 1993, Coder 1999, Bray 2002a, Taiz and Zeiger 2002).

The term "drought" denotes a period during which the water content of the soil is reduced to such an extent that plants can no longer extract sufficient water for normal life processes (Augé et al. 1998, Coder 1999, Taiz and Zeiger 2002). Drought can also be produced by: heat shocks, low air moisture, insolation and salinity (Monneveux and Belhassen 1996) and causes a diverse set of physiological, morphological and developmental changes that occur at different levels of life organization (molecule, cell, organ, plant etc.), impacting metabolism, growth and rigidity (Bray 1997, Monneveux and Belhassen 1996).

Plants have evolved many physiological, biochemical, morphological, anatomical, and phenological characteristics for responding to and resisting drought stress according to the particular situation faced by the plant (Augé et al. 1998, Coder 1999, Taiz and Zeiger 2002). Examples are the metabolic adaptations present in CAM and C_4 plants, an alternative to what occurs in C_3 plants. Transpiration is 10 to 20 times higher in C_3 species and this has a direct impact on photosynthetic efficiency. A plant that has higher water-use efficiency will resist drought better. Resurrection plants show the most extreme adaptation to drought. They can remain alive in a dried state for several years and then return to complete function when they are again in contact with water (Brown and Hettersley 1989, Taiz and Zeiger 2002, Scott 2000).

When water deficit develops slowly enough to allow changes in developmental processes, it has several effects on growth, one of the most sensitive processes to drought. Leaf area affects directly photosynthesis, and rapid expansion of leaf area can adversely affect water availability and therefore affecting also photosynthesis (Taiz and Zeiger 2002).

1.1.1 Morphological and physiological diversity of drought tolerance

Different biological plant types such as herbaceous, woody and epiphytic species exhibit various adaptation strategies to drought. Different organs in the plants also show modification. Root extension, decrease of leaf area, leaf abscission stimulation, stomatal closure and limitation of photosynthesis are among the first responses to water deficit (Coder 1999, Arndt et al. 2000, Taiz and Zeiger 2002). For roots, attributes relevant for adaptation to environmental conditions are length, depth in the soil and resistance to water variations. There is no specific root pattern for drought resistance, but the intraspecific variability in xylem vessel diameter could be linked with water uptake and transfer (Monneveux and Belhassen 1996). In addition to water deficit perception by roots and the signalling to shoots (Sauter et al. 2001, Sauter and Hartung 2002), the ratio of root-to-shoot biomass is regulated by a functional balance between water uptake by the root and photosynthesis by the shoot (Taiz and Zeiger 2002).

Leaves and stems are the evaporative areas and they contain the various organs through which gas exchange occurs: stomata, cuticle and lenticels. In vascular plants stomatal transpiration represents approximately 90% of the total water loss. Therefore stomatal density, position and closure are important in controlling water loss and carbon gain, i.e. biomass production. Carbon dioxide has a direct effect on photosynthesis through its role as a substrate, and indirect effects on both photosynthesis and transpiration through its effect on stomatal conductance (Beadle et al, 1993).

Water movement through cuticle and lenticels is not controllable by the plant and represents only 10 % of water loss. The hydraulic permeability of the cuticle depends on its thickness, and is affected by the presence and nature of cuticular waxes and lenticels. Other morphological leaf adaptations that can modulate transpiration are pilosity, size and width, colour that could influence reflectance and leaf heating, and some mechanisms such as leaf rolling induced by loss of turgor and leaf falling when the dry season is beginning (Monneveux and Belhassen, 1996).

1.1.2 Stomatal conductance and its impact on photosynthesis

Stomatal conductance depends on several variables such as light quality and quantity, temperature, atmospheric vapour pressure deficit and plant water status, hydraulic conductance of the soil-leaf pathway, soil moisture and evaporative demand (Beadle et al. 1993, Buckley et al. 1997). The extent of the response to these variables depends on species, age, and any adaptive response. Its determination is important since stomata play an important role in maintenance of the balance between water loss and carbon gain. Their functioning is vital in the case of crop plants where it is important to maximize water-use efficiency (WUE), defined as the mass of CO_2 assimilated (or dry weight gained) per unit of mass of water transpired (Beadle et al. 1993).

Stomatal conductance is more related to soil water status than to leaf water status, and the only plant part that can be directly affected by soil water status is the root system. pH and inorganic ion redistribution appear to play a role in long distance signalling between the roots and the shoots (Croker et al. 1998, Hartung et al. 1998, Liu et al. 2001, Sauter et al. 2001, Sauter and Hartung 2002, Davies et al. 2002). Bond and Kavanagh (1999) postulate that species differences in stomatal responses to soil and atmospheric water deficits can be explained by two parameters, 1) leaf-specific conductance of the vascular system and 2) a threshold value of leaf water potential.

Uptake and loss of water by guard cells result in changes of turgor and, according to intensity, modulate stomatal opening and closing. Solute loss from guard cells can be triggered by a decrease in the water content of the leaf in which abscisic acid (ABA) plays an important role in two ways.1) ABA is released from the chloroplast to the apoplast in the mesophyll cells, where pH changes and alterations of membrane permeability occur, and 2) The increase in concentration of ABA enhances or prolongs the initial closing effect of the stored ABA. When, stomata start to close, water–use efficiency (WUE) may increase in the first stages of drought, as water deficit increases. The dehydration of mesophyll cells also inhibits photosynthesis and decreases photosynthates exported from leaves because of the inhibition of movements of these assimilates in the phloem (Taiz and Zeiger 2002).

1.1.2.1 Stomatal regulation in drought

In drought conditions, stomatal regulation (and longer-term leaf area regulation) of gas exchange is necessary to preserve hydraulic continuity of the soil-leaf continuum. Without such regulation, the advantages of vascular tissue and root systems for acquiring soil water could not be fully exploited (Sperry et al. 2002). The stomata function like a pressure regulator controlling flow rate, and they limit the variation in plant water potential (Ψ) with soil moisture and evaporative demand by controlling transpiration, avoiding a damaging decrease in Ψ. To function as a pressure regulator, the stomata must sense or predict plant water potential as it changes with ambient conditions and it is inevitable that changes will drive changes in stomatal conductance and transpiration. Stomatal conductance is not the same in all part of the leaf or a plant. It is patchy as are its effect on gas-exchange calculations (Buckley et al. 1997).

During drought, a striking relationship has been found between stomatal function and plant hydraulics (Sansing et al. 2003). Stomatal resistance to the water flow increases very sharply, particularly near the permanent wilting point (Ψ_{crit} = -1.5 MPa), below which plants cannot regain turgor pressure even if all transpiration stops. Water flow from soil to roots is very slow to allow the overnight rehydration of plants that have wilted during the day. The shrinking and suberization of the cell root hairs and cavitation (breakage of water columns in xylem) are the process that contribuite to that resistance (Taiz and Zeiger 2002). Cavitation can occur when the water potential is between -1 and -2 MPa and the largest vessels cavitate first (Tyree et al. 1994, Hacke and Sauter 1995, Hinckley et al. 1999, Taiz and Zeiger 2002). The pressure at which xylem conductivity (Kx) declines depends on the permeability of the pits rather than on the dimensions of the conduit lumina; cavitation-resistant xylem has less permeable pits than cavitation-susceptible xylem.

Plants could achieve coordination between the stomatal regulation of Ψ and the hydraulic capabilities of the soil-canopy supply line, by natural selection for the midday leaf Ψ that maximizes gas exchange: a Ψ at a safe distance above Ψ_{crit}. In a physiological sense coordination may be achieved more directly via a physiological link between cavitation and

the stomatal signalling system (Nardini and Salleo 2000, Sperry et al. 2002). This shows that some stem cavitation inducing small and/or transient changes in stem hydraulic conductance might represent a signal for the stomatal control of transpiration.
It is often difficult or impossible to distinguish between particular sensitivity to drought, and high drought responsiveness, and this must be remembered when choosing indicators of drought stress (Pelah et al. 1997a,b, Price at al. 2002, Taiz and Zeiger 2002).

On the other hand, many aspects of plant water use may have, as their basis, the alteration of hydraulic conductance from soil to canopy. Large differences in water use between species can be attributed in part to differences in their 'hydraulic equipment' that is optimized for drawing water from a particular temporal and spatial niche in the soil environment. A number of studies have identified hydraulic limits as the cause of partial or complete foliar dieback in response to drought (Sperry et al. 2002).

When soil dries, an alteration of hydraulic conductance from soil to canopy occurs. Modelling the changes in hydraulic conductance with pressure gradients in the continuum allows the prediction of water use as a function of soil environment and plant architectural and xylem traits. The interactions between root:shoot ratio, rooting depth, xylem properties, and soil properties in influencing the limits to canopy water supply can be used to predict which combination should optimize water use in a given circumstance.

1.1.3 Cellular responses to water deficit

Cellular water deficit can result in a concentration of solutes, changes in cell volume and membrane shape, disruption of water potential gradients, loss of turgor, disruption of membrane integrity and denaturation of proteins. Complete loss of free water will result in desiccation or dehydration (Bray 1997). Resistance to water deficit occurs when a plant tolerates the imposed stress. The response depends on the species and genotype, the length and severity of water loss, the age and stage of development, the organ and cell type, and the sub-cellular compartment, and involves mechanisms to avoid water loss, and to protect

the cellular machinery and repair damage (Bray 1997). The ability of the plant to respond to, and survive, cellular water deficit depends on whole-plant mechanisms that can integrate the cellular responses. It can occur in seconds, minutes or hours, and can involve changes in phosporylation or gene expression (Bray 2002a, b).

One important cellular response to water deficit is the accumulation of osmotically active compounds that allow cells to re-establish turgor and to extract additional water from the soil. This accumulation can occur through uptake of solutes or the breakdown of osmotically active compounds (Mullet and Whitsitt 1996). Osmotic adjustment (OA) - limited to 0.2-0.8 MPa- has been recognized as a major mechanism in drought tolerance and promising strategies for improving drought resistance lie in molecular technology that allows genes or QTL controlling OA to be tagged and isolated (Tschaplinski et al. 2005). These genes can be expressed in transgenic plants, and efficiency of breeding via marker-assisted selection can be improved (Zhang et al. 1999). It is considered that osmotic adjustment is developed slowly as a response to tissue dehydration and could be a result of a decrease in growth rate. The amount of water acquired by this method is very small and is neutralized by diminishing returns in terms of water availability to the plant, which can be interpreted as a promotion of dehydration tolerance but does not have a major effect on productivity (Arndt et al. 2000).

The decrease in plant cell volume as a result of the loss of turgor and the increase in solute concentration in the cell affect growth. Leaf expansion and root elongation are turgor-dependent activities very sensitive to water stress. This relationship is described in the equation:

$$GR = m (\Psi_p - Y) \quad (\text{Lockart 1965})$$

Where GR equals growth rate, m wall extensibility and Y yield threshold. Ψ_p (turgor) only needs to decrease to the value of Y (yield threshold) to eliminate expansion. This equation only holds if cell wall is limiting growth. Slight decreases in water content and turgor can slow down or completely stop growth. Water stress not only decreases turgor, but also decreases m (wall extensibility) and increases Y. Wall extensibility is normally greatest

when the cell wall solution is slightly acidic, decreasing when cell wall pH increases during stress (Taiz and Zeiger 2002). However, cell and leaf expansion also depends on other biochemical and molecular factors like cell wall and membrane biosynthesis, cell division, and protein synthesis (Pelah et al. 1997a, b; Burssens et al. 2000, Bray 1997, Pandey and Agarwal 1998, Taiz and Zeiger 2002).

1.1.4 Molecular perception of drought

Loss of water from the cell triggers a cellular signal transduction pathway. In this way, a physical stress is converted into a biochemical response. The stress can be recognized by decrease in or loss of turgor, change in volume, membrane area, loss of membrane 'stretch', change in water activity or solute content, and alteration in conformation of cellular macromolecules (Bray 1997, 2002a). However some of the disruption in physiological and metabolic processes may be interpreted as an injury response such as reduced growth rate (Bray 2002a).

The osmotic stress recognition mechanism in plants is not clear; however it is more advanced in yeast (*Saccharomyces cerevisiae*) where it involves the work of two transmembrane proteins, SHO and SLN1, as sensors. Mutations affecting *SLN1* (*sln1* or *sln-ts*) which encodes a two component histidine kinase, are lethal because of constitutive activation of a specific mitogen-activated kinase cascade (HOG1) which regulates adaptive responses such as an increase in glycerol content promoting the cellular adaptation process. *Arabidopsis thaliana* gene *ATHK1*, encodes a "two component" histidine kinase domain structurally similar to SLN1 and a receiver domain that is similar to response regulator, speculated as their osmosensor (Bray 1997, Riera et al. 2005). However, ATHK1 mRNA accumulation is unregulated in response to water-deficit stress in *Arabidopsis*, so it would not be a requirement for sensing mechanisms, although it could serve to up- regulate the response (Bray 2002b).

Following cellular perception a signalling mechanism must be activated to induce specific genes at different times and conditions. One of the major signals operating during drought stress is the plant hormone abscisic acid (ABA) which is also involved in many other abiotic stresses (Bray 1997, Finkelstein et al. 2002), the induction of tolerance of water, salt, hypoxic, and cold stress, and wound or pathogen responses. In addition, recent studies have demonstrated interactions between signalling by ABA and ethylene, brassinosteroids, light, or sugars (Finkelstein et al. 2002, Bertrand et al. 1994). However, not all water deficit-induced genes are regulated by ABA. Bray (2002b) showed that the regulation of ABA synthesis derives from carotenoid biosynthesis and many of the genes involved are members of multi-gene families. The general pathway leading to ABA biosynthesis is now well established (Bray 2002b). It can be drafted in three steps:

1) Action of zeaxanthin epoxidase (ZEP), which converts zeaxanthin to violaxanthin, a two-step epoxidation occurring in the chloroplast.
2) 9-cis-epoxycarotenoid dioxygenase (NCED) is the enzyme that cleaves the carotenoid, 9-cis-violaxanthin or 9'-cis-neoxanthin, to result in xanthoxin, the first C15 precursors of ABA. It is present in the chloroplast and up-regulated by water deficit.
3) The final step is catalyzed by abscisic aldehyde oxidase (AAO), and is not considered to be a rate limiting step. Four genes have been cloned from Arabidopsis AAO1-4 and none of them are induced in the roots.
A mechanism for sensing loss of water from the cell resulting in the accumulation of ABA has been proposed by Bray (2002b), where histidine (H) and aspartate (D) are phosphorylated in the phosphorelay system (Bray 2002b, Tang et al. 2000) (Fig 1.1).

1.1.5 Perception of ABA by the cell

The recognition of ABA needs the presence of both extracellular and intracellular reception sites for ABA (Allan and Trewavas 1994, Schwartz et al. 1994, Jensen et al 1996). Increasing evidence suggests that ABA signal transduction involves increases in cytosolic Ca^{2+}, suggesting that ABA induction of guard cell closure is transduced through increases

in intracellular cytosolic calcium, resulting from increased opening of calcium channels (McAinsh et al. 1990). Release of Ca^{2+} inside guard cells initiates closure. ABA and Ca^{2+} appear to act synergistically to induce stomatal closure by inhibition of inward-rectifying K^+ channels, activation of outward-rectifying K^+ channels and activation of voltage dependent anion channels. ABA and temperature seem to interact at the level of Ca^{2+} response in guard cells (Jensen et al. 1996). Some genes have been found in plants under water-deficit conditions: phospholipase C to generate secondary messengers, calcium dependent protein kinase, a MAP kinase and ribosomal S6 kinase (Allan and Trewavas 1994, Schwartz et al. 1994). It has become clearer that during stress, the ABA signal transduction pathway interacts with other signalling pathways that have not been described, like the expression of LEA (Late embryogenesis abundant protein).

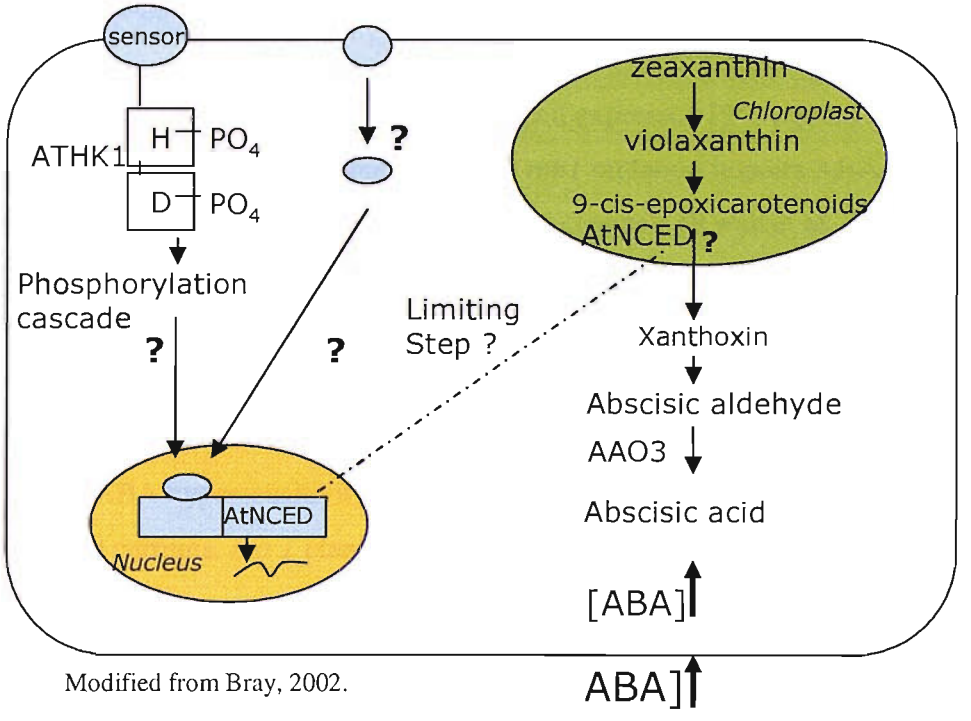


Figure 1.1 Model of water deficit perception in *Arabidopsis thaliana*. The proposed model is based on ABA biosynthesis and accumulation. H and D represent Histidine and Aspartate respectively that participate in the phosphorelay system. AtNCED could be a limiting step as mRNA would be produced by water deficit.

From the study of *Arabidopsis* mutants, several possibilities of disruption in the early ABA signal transduction have been reported. The corresponding genes, *ABI1* and *ABI2*, both encode type 2C protein phosphatases and the dominant mutant alleles *abi1* and *abi2* have point mutations altering a conserved amino acid. ABA-induced $[Ca^{2+}]_{cyt}$ increases were significantly reduced but not abolished in guard cells of the ABA-insensitive protein phosphatase mutants *abi1* and *abi2* (Allen et al. 1999, Pei et al. 1997, Schroeder et al. 2001b). In guard cells of the V-ATPase mutant det 3, external calcium and oxidative stress elicited prolonged calcium increases which did not oscillate, and stomatal closure was abolished (Allen et al. 2000). Activation of Ca^{2+} channels by H_2O_2 and ABA-induced stomatal closing are disrupted in the recessive ABA-insensitive mutant *gca2*. These data indicate that ABA-induced H_2O_2 –activated Ca^{2+} channels are important mechanisms for ABA-induced stomatal closing (Pei et al. 2000).

AT-DNA disruption mutation in *RCN1* (a guard cell expressed PP2A gene) confers recessive ABA insensitivity to *Arabidopsis*. The *rcn1* mutation impairs ABA-induced stomatal closing and ABA activation of slow anion channels (Kwak et al. 2002). *OPEN STOMATA1* (*OST1*) gene, encoding a serine-threonine protein kinase, renders *Arabidopsis thaliana* guard cells insensitive to ABA, so that stomata remain open in the presence of this phytohormone (Assman 2003). Loss-of-function mutations in the *Arabidopsis* ERA 1 farnesyltransferase β subunit cause an enhanced response to ABA in seeds. The *era 1* mutant shows ABA hypersensitivity stomatal closing and ABA hypersensitivity activation of S-type anion currents. *era 1* plants show reduced water loss during drought (Schroeder et al. 2001b).

Use of novel creative genetic screens will lead to identification of many new mechanisms affecting guard cell signalling. An example of this is the isolation of new *Arabidopsis* guard cell signalling mutants through small differences in leaf temperature. These signalling mutants in stomatal responses could be isolated based on variation in responses among *Arabidopsis* ecotypes and use of recombinant inbred lines to map quantitative trait loci that affect guard cell signalling (Schroeder et al. 2001a). Future research in this area combined

with inducible cell-specific gene expression or cell-specific gene silencing will lead to identification of mechanisms for engineering improved gas exchange in response to drought, elevated atmospheric CO_2, and other environmental stresses (Schroeder et al. 2001a,b).

1.1.6 Gene expression during water deficit

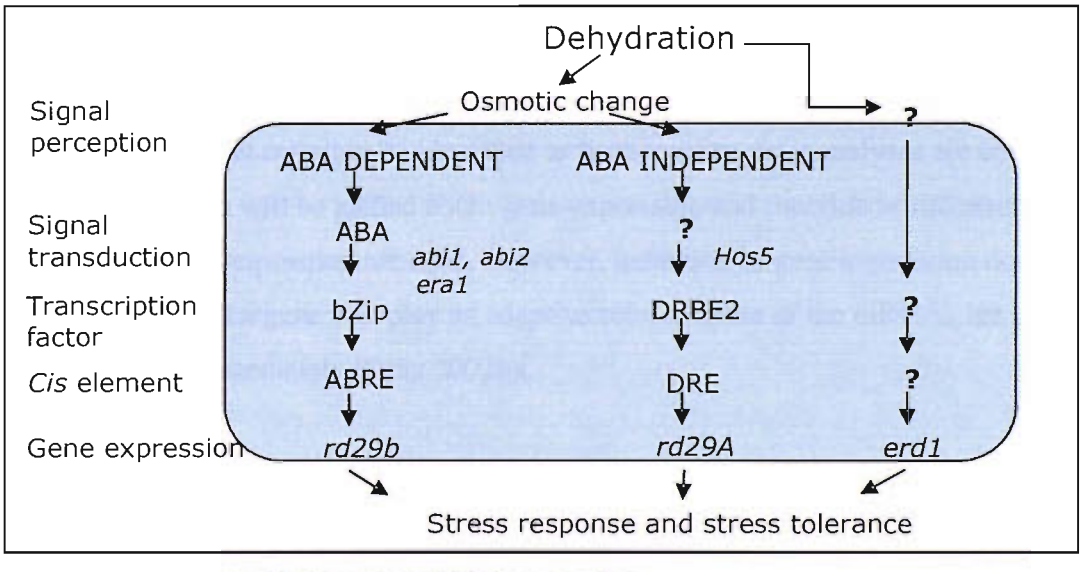
Two classes of DNA elements, ABA-response element (ABRE) and the dehydration responsive-element (DRE) as well as additional sequence-specific DNA elements, have been linked with the control of gene expression during water deficit (Bray 1997). The ABRE has been shown to be sufficient for ABA-regulated gene expression, but in some genes must be associated with a coupling element which is still not clear. ABRE contains the core sequence ACGT-containing G box which confers ABA responses upon a reporter gene and several b-zip type proteins binding to this motif have been cloned.

However, the ABRE is also present in a variety of genes that are not induced by ABA suggesting that this element is not always ABA responsive. A possible explanation is that the bases flanking the ACGT-core sequence are important for specifity. Therefore it was proposed that in general the specificity for ABA response is determined by the ABRE and a coupling element (Jensen et al 1996).The coupling element CE1 was discovered in barley gene *HVA22* and CE3 was discovered in another ABA induced gene of barley *HVA1*- a group 3 *lea* . Other sequence-specific DNA-binding proteins are also required. One of these is the transcriptional activator VP1, first shown to be involved in the regulation of C1 (a gene encoding a Myb-like DNA-binding protein involved in anthocyanin biosynthesis) in maize seeds. The presence of VP1 has a synergistic effect with ABA on *HVA1* (but not *HVA22*) expression (Bray 1997).

The DRE (TACCGACAT) has been shown to be involved in this gene regulation by an ABA-independent pathway induced by water deficit, low temperature and salinity (drought and osmotic stress). The regulation of this gene is independent of ABA in the first few

hours after dehydration, but becomes dependent on ABA in the later stages. ABRE is present in the DNA sequence, but it does not appear during the first few hours of water deficit). A study of the promoters led to the identification of specific regulatory sequences for genes involved in different stresses (the *RD29A* gene is an example) (Bray 1997, Jensen et al. 1996). A gene encoding the transcription factor CBP1, which binds the DRE, has been isolated from *Arabidopsis* (Bray 1997).

At least two signalling pathways have been implicated in the regulation of gene expression in an ABA-independent manner. Transacting transcription factors (called DREB1 and DREB2) that bind to the DRE elements in the promoters of osmotic stress-responsive genes are apparently activated by an ABA-independent signalling cascade and are directly controlled by the so called MAP kinase signalling cascade of protein kinases. Other changes in gene expression appear to be mediated via other mechanisms not involving DREBs. The complexity of these two mechanisms and the communication between them reflect the wealth of interactions between gene expression and the physiological processes mediating adaptation to osmotic stress (Mantyla et al. 1995, Bray 1997, Shinozaki and Yamahuchi-Shinozaki 2000, Taiz and Zeiger 2002). Figure 1.2 shows a model proposing the cellular transduction pathways in water deficit.



Modified from Shinozaki and Yamaguchi-Shinozaki, 2000.

Figure 1.2 Cellular transduction pathways and gene expression in *Arabidopsis*. *aba1*, *abi2* and *era 1* are involved in ABA signalling. *hos5* functions in DREB2-related dehydration signalling.

The determination of the function of an observed response is very complex as many genes are involved. The accumulation of mRNA during water deficit may indicate gene induction, but additional regulatory mechanisms, such as translational regulation and posttranslational modification, may be required for a fully functional gene product. The complexity increases when the plant is submitted to multistressful conditions in unfavourable environments and plants must withstand a wide range of abiotic and biotic stresses (Bray 1997).

Changes in gene expression in response to water deficit stress may promote the ability of the plant to respond appropriately, furthering the ability of the plant to survive or function during water-deficit stress (Bray 2002a). As many gene expression patterns are influenced by the severity, extent and rate or application of the stress, gene expression can be altered at the initial step or at subsequent steps that control specific mRNA levels or the translation of a specific mRNA. Consequently, a complex pattern of gene expression is established that is a result of the specific stress conditions, therefore the important challenge is to understand which genes function to promote cellular and whole plant tolerance of water–deficit stress (Bray 2002b).

An effective way of showing gene expression is provided by Microarray analysis, a powerful method to study the many changes in gene expression that occur in an organism in response to developmental and environmental cues (Bray 2002b). Until now, genes induced by water deficit stress have been allocated to 11 different functional categories. More genes will almost certainly be identified as further microarray analyses are completed and more information will be gained about gene expression and function as different techniques for stress imposition are used. However, induction of gene expression does not necessarily imply that a gene will play an adaptive role, as some of the mRNAs are stored and not translated immediately (Bray 2002b).

1.2 Morphology, physiology and molecular responses of *Populus* to drought

With nearly 60 species around the world (Flora of China 2006, Hillier and Coombes 2002) and several hybrids and cultivars, *Populus* is considered one of the more abundant woody plant genera in temperate forests. Poplars, cottonwoods or aspens are the common names for these species which have a tendency to hybridization in their natural habit, especially where different species overlap, which cause some taxonomic problems. Poplar species are divided into 5 sections: Aigeiros, Leucoides, Populus (syn. Leuce), Tacamahaca and Turanga (Wyckoff and Zasada 2003, U.S. Environmental Protection Agency 1999, Bradshaw et al. 2000, Taylor 2002). The hybrids of section Tacamahaca are considered to have a particularly high water use efficiency (Mazzoleni and Dickmann 1988) and this property can be estimated by carbon isotope discrimination and others techniques (Donovan and Ehleringer 1994). As has been mentioned before, hybrids are more drought tolerant than native cottonwoods (Hinckley et al.1989, Wyckoff and Zasada, 2003) and it is precisely this characteristic that makes them more attractive as a crop to supplement the diminishing supply of natural hardwoods.

1.2.1 Water relations in *Populus* species and hybrids

It has been reported that the hybrids between *Populus trichocarpa* and *P. deltoides* in Washington and Oregon in the United States had a volume growth two or three times that of the best growing parent species (Heilman and Stettler 1985, Stettler et al. 1988, Dickmann et al. 1992). Nine species of poplar: *P. angustifolia, P. balsamifera, P. deltoides, P. euphratica, P. fremontii, P. tremula, P. tremuloides, P. tomentosa, P. trichocarpa* (Table 1.1) are the most used in experiments related with drought. Research using poplar species has been focused on gas exchange and water relations, metabolite concentration and protein determination and characterization, as an answer to water and heat stress and to the responses of callus submitted to drought and desiccation. So far *Populus tremula* has been

studied most. However, *P. trichocarpa* and *P. deltoides* and their hybrids have been used in a large number of studies. The research on *Populus* hybrids related to drought tolerance is more focused on gas exchange, photosynthetic rate, WUE and leaf osmotic potential behaviour under mild and severe drought and flooding with and without nitrogen interaction at the laboratory and field planting level. A summary of all the research on drought in Poplar hybrids is presented in Table 1.2.

The morphology and physiology in *Populus* species varies between geographically distinct populations and is strongly linked with the environment. It follows that any responses to drought tolerance will depend not only on the species but also on the particular genotype (Weber et al. 1985, Rogers et al. 1989, Braatne et al. 1992, Dunlap and Stettler 2001, Rowland et al. 2001). For example, work on poplars growing in different river valleys found that there was variation of physiological processes like photosynthesis both within and between locations (Braatne et al. 1992, Dunlap et al. 1993).

The cultivars which have been investigated most are 'Eugenei' and 'Tristis' and there are some major differences between them. 'Eugenei' has a remarkable tendency to a higher leaf area production, lower root/leaf weight ratio and higher tolerance of flooding; while 'Tristis' has a higher allocation of dry weight to the stem and the roots showing that it has a higher root/shoot ratio and is less tolerant of flooding (Mazzoleni and Dickmann 1988, Liu and Dickmann 1992a,b,c). Mazzoleni and Dickmann (1988) showed the importance of more physiological studies of growth patterns analyzed under changing conditions of water stress. Physiological responses were associated with stomatal function, the influence of plant growth regulations and growth of leaves and roots.

Table 1.1 *Populus* species studied with reference to drought. This table is a summary of the research in water stress done in several species of *Populus*. It includes the most common physiological, cellular and molecular mechanisms and responses observed in several species of Poplars.

Species	Studies focused on	Findings	Reference
P. angustifolia	Water relations	Shoot growth reduction	Rood et al. 2003
P. balsamifera	Water relations	Shoot growth reduction	Rood et al. 2003
P. deltoides	Tissue culture	Callus response to drought	Tschaplinski et al. 1995a
	Water deficit	Gas exchange reduction	Taylor 1998
		Leaf development, genomics	Street et al. 2006.
	Gas exchange	Adaptation to the environment	Bassman and Zwier 1991
	Water stress	Reduction in photosynthesis	Gebre and Kuhns 1993
	Metabolites	Increase in osmotic adjustment	Tschaplinski and Blake 1989 a,b,c Tschaplinski and Tuskan 1994 Gebre et al. 1994 Gebre and Kuhns 1991 Gebre and Tschaplinski 2000
	Cell expansion	Leaf growth reduction	Mohiuddin et al. 1997 Van Volkenburgh and Taylor 1996
P. euphratica	Water relations	Shoot growth reduction and dieback	Rood et al. 2003
P. fremontii	Water relations	Shoot growth reduction and dieback	Rood et al. 2003
P. tomentosa	Leaf	Protein accumulation	Wang et al. 1997 Pelah et al. 1997b
	Rewatering and sampling	Solute accumulation	Gebre et al. 1997
P. tremula	Shoot proteins in water stress	BspA presence	Pelah et al. 1995
	Abscisic acid	Genotypic variation	Chen et al. 1997
	Protein presence	SP1 characterization	Wang et al. 2002
		Solute accumulation	Wang et al. 1997 Pelah et al. 1997b
P. tremuloides	Amino acids	Clonal variation	Griffin et al. 1991
	Intermittent root drying	Increased tolerance	Griffin et al. 1991
P. trichocarpa	Tissue culture	Callus response to drought	Tschaplinski et al. 1995a
	Water deficit	Gas exchange variations	Taylor 1998, Schulte 1986
		Leaf development, genomics	Street et al. 2006
		Root morphology	Pezeshki and Hinckley 1988
	Stomatal traits and productivity	Clonal variation	Dunlap et al. 1994, 1995 Dunlap and Stettler 2001 Dunlap and Stettler 1998
	Cell expansion	Leaf growth reduction	Mohiuddin et al. 1997 Van Volkenburgh and Taylor 1996
	Water regulation	Stomatal control in xylem cavitation	Sparks and Black 1999
	Gas exchange	Adaptation to the environment	Bassman and Zwier 1991
	Metabolites	Increase in osmotic adjustment	Tschaplisnki and Blake 1989 a,b,c Tschaplinski and Tuskan 1994 Gebre and Tschaplinski 2000
	Gene isolation and expression	ABA-insensitive 3 (ABI3) homologue	Rohde et al. 1998
	Stomatal opening and hydraulic conductance	ABA relations	Aasamaa et al. 2002

Table 1.2 Summary of research studies in Poplar hybrids related with drought. Included are the most common physiological, cellular and molecular mechanisms and responses observed in several hybrids of Poplars.

Hybrids	Studies focused on	Findings	Reference
Populus x *euramericana* 'Eugenei'	Gas exchange with and without N_2 supply	Loss of sensitivity in stomata, less adapted to drought	Liu and Dickmann 1992a,b,c Bassman and Zwier 1991
	Water-N_2 interaction	Drought induced stomatal closure WUE increased in high N/severe drought	Liu and Dickmann 1996
	WUE	Low WUE More final biomass	Dickmann et al. 1992
	Leaf expansion	No effect on leaf initiation rate, Reduced leaf expansion. Stem reduction	Liu and Dickmann 1992b
	Patterns of growth	Short term: Reduction of growth, stomatal conductance and leaf water potential. Long term: more susceptible to damage	Mazzoleni and Dickmann 1988
	Leaf abscission	Xylem ABA accelerated leaf abscission	Chen et al. 2002
	ABA	Genotypic variation in drought tolerance	Chen et al. 1997
	Leaf osmotic potential	Osmotic potential decrease over growing season and age	Gebre et al. 1998
Populus x *euramericana* 'Laura Avanzo'	Rewatering	Water potential, stomatal conductance, leaf sugar concentration	Marron et al. 2002
Populus x *euramericana* 'Dorskamp'	Rewatering	Water potential, stomatal conductance, leaf sugar concentration	Marron et al. 2002
P. tristis x *P. balsamifera* 'Tristis'	Gas exchange with N_2 supply	Adapted to drought	Liu and Dickmann 1992c
	Water-N_2 interaction	Drought induced stomatal closure	Liu and Dickmann 1996
	Leaf expansion	No effect on leaf initiation rate	Liu and Dickmann 1992b
	Patterns of growth	Short term: Reduced growth, stomatal conductance and leaf water potential. Long term: little susceptible to damage	Mazzoleni and Dickmann 1988
	WUE	Low WUE Less final biomass than 'Eugenei'	Dickmann et al. 1992
P. deltoides x *P. balsamifera*	Gas exchange, Water relations	Late season accumulation	Tschaplinski and Blake 1989 a,b,c
	Growth	Root growth decreases	
	Organic solutes	Delayed senescence	
	Root growth	Decrease with solutes concentration	
	Osmotic potential		
P. maximowiczii x *P. nigra*	Shoot defoliation	Organic solutes concentration in roots	Tschaplinski and Blake 1994, 1995
	Shoot decapitation	Decreasing of Carbohydrates	
P. trichocarpa x *P. deltoides*	Cytokinin effect in hypoxic and aerated roots	No differences found	Neuman et al. 1990
	Stem diameter and water relations	Osmotic potential no changes in treatment	Tschaplinski et al. 1998a,b
	Stomatal response	Leaf growth reduction	Mohiuddin et al. 1997 Van Volkenburgh and Taylor 1996
	Growth, water relations, gas exchange,	Osmotic adjustment helps in drought tolerance	Tschaplinski et al. 1994
	Root growth	Delayed senescence	Tschaplisnki and
	Osmotic potential	Decrease with solutes concentration	Blake 1989a,b.c
	Grown in a large scale plantation	Stomatal control at midday	Tschaplinski et al. 1998b
	Gas exchange	Adaptation to the environment	Bassman and Zwier 1991
	Nutrition and xylem cavitation	Fertilization can influence the response to drought	Harvey and Van Den Driessche 1997
	Stomatal responses	Rewatering effects on conductance	Schulte and Hinckley 1987 Schulte et al. 1987

Hybrids	Studies focused on	Findings	Reference
	Stomatal traits and productivity	Clonal variation	Dunlap et al. 1994, 1995 Dunlap and Stettler 1998
	Metabolites	Increase in osmotic adjustment	Tschaplinski and Tuskan 1994 Gebre and Tschaplinski 2000
	Carbon allocation	Restriction in preferential carbon allocation	Tschaplinski et al. 1998b
P. koreana x *trichocarpa* 'Peace'	Stomatal function	Leaf age dependance responses	Ridolfi et al. 1996
	Hydraulic properties	Xylem embolism	Cochard et al. 1996
	Growth and stomatal response	Role of ABA	Mohiuddin et al. 1997
P. deltoides x *P. nigra*	Stem diameter and water relations	Osmotic potential no changes in treatment. Greater drought resistance More favorable water balance by stomatal regulation	Tschaplinski et al. 1998a,b,c
	Leaf osmotic potential	Declining of osmotic potential over the growing season and age	Gebre et al. 1998
Populus nigra 'Charkowiensis' x *P. nigra incrassata*	Effect of near lethal heat stress	Response of bud break, heat-shock proteins and ubiquitin	Wisniewski et al. 1997
P. popularis	Leaf	Protein accumulation	Pelah et al. 1997a,b

1.2.2 Stomatal responses

One of the main features of poplars is their high rates of stomatal conductance, (g_{max} are near 600 mmol m^{-2} s^{-1}) suggesting the transpiration of large volumes of water and making them comparable to other agricultural crops in water use. However, the measurement of a transpiration rate of 4.92 mm^3 d^{-1} in a four-year old hybrid over a wide range of atmospheric conditions shows the existence of other mechanisms in poplars which help stomata to avoid water loss. These mechanisms include biophysical (size and density of stomata, degree of stomatal opening, hydraulics of water conducting tissues) and environmental factors such as: solar radiation, temperature, humidity, soil moisture and canopy boundary-layer conditions (wind speed, airflow patterns). In a poplar stand, stomatal conductance is high, but their canopy results in a low boundary-layer conductance (U.S. Environmental Protection Agency 1999). The question of how poplars can tolerate increasing drought stress without an impact of productivity was raised many years ago (Tschaplinski and Blake 1989 a,b,c, Tschaplinski et al. 1998b, c, 1999) and the most recent results indicate that it is possible to find drought tolerance adaptations in *Populus* (Tschaplinski et al. 2005).

Stomatal behaviour in *Populus* has been widely studied, especially the effect of water deficit on biomass production expressed as height and dry weight growth, leaf growth and water potential. Stomatal behaviour influences growth patterns which in *Populus* is also convenient to study in intermittent periods of water deficit (Dickmann 1971, Dickmann et al. 1992, Mazzoleni and Dickmann 1988, Rood et al. 2003). It has been proved that growth is strongly affected by drought and the responses seem to be similar between species and clones; for example in *P. deltoides* morphological and physiological traits vary according to habitat (Rowland et al. 2001), however these traits also vary with the application of cyclic water deficit, showing better adaptation based on a more favorable plant water status (Mazzoleni and Dickmann 1988).

Studies of gas exchange have shown that stomatal behaviour in all *Populus* species is not the same. There are several reports that *P. trichocarpa* and some of its hybrids show an abnormal behaviour in response to water deficit, not observed in most *Populus* species. This abnormal behaviour includes the lack of stomatal closure in water deficit conditions (Bassman and Zwier 1991, Ceulemans et al. 1988) and similar abnormal stomatal behaviour has been reported in other plants such as barley and potato (Raskin and Ladyman 1988). In addition, a considerable night transpiration has been reported in *P. balsamifera* spp. *trichocarpa* (Snyder et al. 2003) and less hysteresis in the response to solar irradiance (Ceulemans et al. 1988). Night time flux suggests either re-filling of depleted water stores (not likely) or continued night time transpiration (Hinckley et al. 1999) and it could influence predawn disequilibrium between plant and soil water potentials (Donovan et al. 1999).

It has been reported that stomatal behavior in *P. trichocarpa* can be modified after several water deficit and rewatering periods, when the surviving leaves exhibited some response to leaf water potential. Bassman and Zwier (1991) reported that *P. trichocarpa* has stomata insensitive to decreasing xylem pressure potential (XPP) while others species do not show this feature. It seems that some *Populus* cultivars could memorize the drought experience and have a better adaptation to drought conditions through stomatal control (Liu and Dickmann 1992a,b,c), or as if this behaviour is a mirror of the adaptation to a riparian

habitat, where water supply is variable (Rood et al. 2000, 2003). It has also been observed that stomatal responses showed leaf age dependence (Schulte et al. 1987b), not only in their gas exchange and water relation responses, but also in responses to other environmental stresses such as CO_2 enriched atmospheres (Ceulemans et al. 1995) and growth (Van Volkenburgh and Taylor 1996). The adaptation to water deficit in remaining leaves in the shoot, could be related to leaf age and development and may not be an acclimation response.

Here it is important to say that stomatal behaviour can, together with the mesophyll, contribute to the limitation of photosynthesis and can lower water-use efficiency in severe drought (Dickmann et al. 1992). The reduction in stomatal aperture is observed by a decrease in g, E, WUE and the shedding of leaves, however these responses can vary if soil moisture changes or additional fertilizers (nitrogen) are applied (Dickmann et al. 1992, Tschaplinski et al. 1998c). The study of stomata and leaf structure has become important in *Populus* because of the close relationship between stomatal and mesophyll limitation (Scarascia-Mugnozza et al. 1986).

1.2.3 The influence of Abscisic Acid

It has been proved that drought induces alterations in stomatal responses, and Abscisic acid (ABA) has been identified as the chemical signal involved from the root to the leaf (Liu and Dickmann 1992a). This is based on the finding of increased concentrations of ABA in leaves during soil drying (Davies et al. 1990), where photosynthesis and stomatal conductance showed a close and continuous relationship with ABA concentrations, including root concentrations (Liu and Dickmann 1992a, Mohiuddin et al. 1997). ABA has been proposed as an essential factor in triggering stomatal closure in response to water deficit in *Populus*, however this relationship is not always clear (Ridolfi et al. 1996). The variation in response depends not only on the degree of hydration of the plant but also on the particular species, hybrid or clone being studied (Liu and Dickmann 1992a, Chen et al. 1997, Chen et al. 2002). The increases in ABA concentration do not always correspond

to resistance to drought in *Populus*. Chen et al. (1997) studying *Populus* 'Popularis' and *P.* x *euramericana* 'Italica' found differences in the timing and patterns of ABA production in response to the onset of water stress. They suggested that there are two different sensitivity mechanisms in ABA synthesis that trigger adjustments in the shoots that confer drought resistance, concluding that there are drought-tolerant and drought sensitive genotypes. Leaf abscission is strongly related to these mechanisms.

The same clones have been studied by Chen et al. (2002), who investigated the effect of endogenous and exogenous ABA on Polyamines (PAs) and ethylene synthesis on leaf senescense. They reported that an increase of ABA in the xylem decreases PA levels but enhances ethylene synthesis. They conclude that the low levels of PAs promote defoliation, even though the ethylene emission of stressed plants returned to prestress values by day 3, and that this effect is greater in older leaves. The responses found in the genotypes mentioned vary according to the frequency of rewatering and the additional supply of N, or with the exogenous application of ABA (Liu and Dickmann 1992a, Chen et al. 2002).

Recently it has been found that interspecific variability in endogenous ABA concentration provides an important factor for differences in shoot hydraulic conductance (Lm), stomatal conductance (g_s) and P_{max} (maximum photosynthesis) (Aasamaa et al. 2002). This has also been mentioned by Liu et al. (2001) refering to the 4-fold increase in xylem sap ABA concentration following the root drying treatment. Apart from ABA, ethylene is considered the most dominant hormonal factor promoting leaf senescense but its correlation to ABA is still contradictory (Chen et al. 2002). The reduction of PAs in the leaf increased its sensitivity to ethylene, accelerating defoliation in two hybrid poplars. Another hormone which has been reported to vary in concentration during soil drying is cytokinin in xylem sap, however, its role is not yet clear (Neuman et al. 1990).

1.2.4 Drought impact on leaf and root growth

It has been shown that water stress affects plant physiology and morphology, causing retarded leaf growth, stomatal closing and lowered photosynthetic capacity (Ceulemans and

Impens 1980, Liu and Dickmann 1992b, c, Van Volkenburgh and Taylor 1996). The reduction in active photosynthetic leaf surface is probably the most important factor affecting overall plant growth rate and leaf expansion (Mazzoleni and Dickmann 1988). In some species it is possible to increase drought tolerance when the roots are acclimated to short periods of water deficit (Griffin et al. 1991). This has been also proved in TD (*Populus trichocarpa* x *P. deltoides*) and DN (*P. deltoides* x *P. nigra*) hybrids (Tschaplinski and Blake 1989a,b; Tschaplinski et al. 1998b).

Clones of DN typically maintained higher midday leaf water potentials, suggesting better stomatal control of water loss and differences cannot be observed with leaves at full turgor. Greater drought resistance of clone DN compared with clone TD was the result of the maintenance of a more favorable water balance by stomatal regulation and greater carbon allocation to roots during early stages of drought. Severe water limitation restricted the preferential allocation of carbon to belowground tissues, so that both root and shoot growth were constrained by severe drought (Tschaplinski et al. 1998a). Root density and root density to stem volume ratio can modify drought tolerance (Tschaplinski et al. 1998b, Tschaplinski and Blake 1989a,b) which will be reflected in a high stem volume growth rate under moderate drought conditions. *P. trichocarpa* has excellent and prolific rooting which differs from that of other species such as *P. deltoides* (Bradshaw and Villar 1996).

Minerals affect the root hydraulic conductivity (Harvey and Van Den Driessche 1997) and have shown a positive correlation with phosphorus concentrations. Mineral nutrition can significantly affect cavitation resistance in *P. trichocarpa* and *P. deltoides*. The root hydraulic conductivity (Lp) and gas exchange processes can be affected when roots are in contact with sodium naphtenates (NAs) which metabolically inhibited Lp probably by affecting water channel activity. This inhibition could be responsible for the reduction in gas exchange and leaf growth (Kamaluddin and Zwiazek 2002). Cochard et al. (1996) concluded that efficient stomatal regulation is essential for maintaining the integrity of xylem sap flow under drought conditions and that *Populus* 'Peace' shows an exception to the general rule of stomatal control of embolism. Sensitivity of poplar roots to variation in

soil water content varies according to clone and a rapid short-term accumulation of ABA in shoots in response to water stress may contribute to drought tolerance (Chen et al. 1997).

1.2.5 Biochemical responses to drought in *Populus*

In spite of the importance of drought survival by poplars the molecular basis of drought tolerance in *Populus* species is not yet known (Pelah et al. 1995). The study of the biochemistry of the responses to drought in *Populus* is patchy and mainly focussed on the mechanism by which osmotic potential and ABA interact with stomata to respond to water deficit.

Leaf osmotic potential at full turgor has been studied in several hybrids and clones of *Populus* (Tschaplinski and Tuskan 1994, Griffin et al. 1991, Tschaplisnki and Blake 1989a,b,c, Wang et al. 1997). Osmotic potential studies in TD and DN growing under field conditions in USA have shown that the osmotic potential in clone TD was increased by sucrose as the main constituent (70%) of the total organic solutes during severe water deficit (Gebre et al. 1998). These findings opened the possibility of studying sugar accumulation in *Populus* species, which could be used as molecular markers in studies of drought resistance and tolerance. Even though the role of sugars in poplars is not yet understood, it is possible that they are involved in leaf abscission or senescence, as in *Arabidopsis* (Yoshida 2003), and not just to protect the cell stabilizing membrane and proteins to maintain the cell integrity during dehydration (Scott 2000).

It has also been mentioned that some of the responses to drought are triggered by the primary osmotic stress signals, whereas others result from the secondary stress-signals which can be phytohormones (ABA and ethylene), reactive oxygen species (ROS) and intracellular second messengers (e.g. phospholipids). The study of the biochemistry of the mechanisms of drought avoidance and tolerance in Poplars is made more difficult because there a series of cascade signals derived from the primary and secondary stress signals. For example, the existence of a clear inhibitory effect of drought on ABA on PAs biosynthesis

has been proved, and also decreased levels of PAs concentration are strongly linked with leaf abscission in Poplars (Chen et al. 2002).

It is extremely important to realize that the plant response to PAs concentration varies depending on the degree of water stress in leaves and their age as well as the stage of growth (Gebre et al. 1998, Chen et al. 2002). An example of this is that young leaves produce more ethylene than old leaves and it is believed that they are able to maintain high levels of PAs due to high concentrations of endogenous growth regulator (Chen et al. 2002). This difference between young and old leaves shows the importance of making comparisons with plants and leaves of the same age.

Apart from the sugars reported in the cytosol (Gebre et al. 1998), other substances have been isolated in poplars in response to gradual water stress. Pelah et al. (1995) characterized a major Boiling stable protein (BspA) in aspen (*Populus tremula*), and two years later reported the presence of two homologues of dehydrin accumulated in roots and shoots of aspen, a sucrose synthase homologue and GAPDH accumulated in shoots in response to water stress (Pelah et al. 1997a,b). The same BspA as well as sucrose synthase and the constitutive presence of dehydrin-like protein were expressed in *P. popularis*, but low expression of BspA was found in *P. tomentosa*, decreasing its tolerance to drought (Pelah et al.1997a,b). They concluded that 1) desiccation tolerance can be attributed to soluble sugars and 2) that the accumulation of water stress related proteins and sucrose synthase confers increased water stress tolerance in *P. popularis*. Care must be taken in the sampling for solute accumulation as its content can vary according to the time of sampling and rewatering, and these factors can affect the results (Gebre et al. 1997). It has also been proved in *P. deltoides* that male clones displayed greater stress tolerance provided by osmotic adjustment than female clones. In contrast, female clones allocate carbon to lateral branches under water stress conditions (Tschaplinski et al. 1994), a feature that must be taken into account when screening clones for drought tolerance. The roles of heat induced protein degradation, HSPs, and ubiquitin in overcoming dormancy by near-lethal heat stress have been studied in *Populus nigra* 'Charkowiensis' x *P. nigra* incrassata (Wisniewski et al. 1997).

In spite of the work mentioned above, the biochemistry of drought in poplars is still not completely understood. It is accepted that reduction in leaf water content or potential results in a decline in growth and stomatal conductance, but the genetic basis for this variation has not been established. There are several homologies between *Arabidopsis* and *Populus* in their drought adaptation but more work must be done on how the osmotic stress is sensed and the signal transduced (Xiong and Zhu 2002). The completion of the genome of *P. trichocarpa,* advances in the studies of drought in *Arabidopsis* and the study of the hybrid F_2 generation of *P. trichocarpa* and *P. deltoides* represent a major opportunity to unravel the genetic basis of drought tolerance in *Populus*.

1.3 Linking morphology and physiology to genetics in *Populus*

Members of the genus *Populus* are becoming more important because of their suitability for genetic and environmental studies of carbon sequestration as they are some of the fastest-growing trees in the world (Stettler et al. 1996, Tuskan et al. 2002). Their ease of propagation and the intensive work done in physiology, biochemistry, agronomics and genomics around the world has led several authors to regard them as excellent models for forest trees (Bradshaw and Stettler 1995, Taylor 2002, Tuskan 2003).
The genetic studies in *Populus* have advanced rapidly, after the completion of the *Arabidopsis* genome, *P. trichocarpa* is the first tree species with a complete genome sequenced in the United States (U.S. Department of Energy (DOE) 2002, Tuskan et al. 2002, Tuskan et al. 2004).

Genomics in other species of *Populus* is also advanced, for example the study of *P. tremuloides* at the University of Agricultural Sciences, in Sweden, where more than 60,000 EST (expressed sequence tags) sequences have been obtained by sequencing more than 14 tissue specific cDNA libraries from *P. tremula* x *tremuloides.* These ESTs have been used as a base to build a unigene set consisting of ca 13,000 sequences for production of DNA microarrays (Nilsson 2002). An integrated genetic map of *Populus deltoides* based on amplified fragment length polymorphisms (AFLPs) in *Populus deltoides* clones in China

(Wu et al. 2000) is also being developed and recently, the poplar root transcriptome where 7000 ESTs were analysed (Kohler et al. 2003). The isolation and expression of some genes related with water deficit and stress have also been reported (Street et al. 2006). The isolation and expression analysis of an Abscisic Acid-Insensitive 3 (ABI3) homologue from *P. trichocarpa* was reported by Rohde et al. (1998).

In order to understand the genetics of adaptation there is a growing body of knowledge about the physiological and molecular genetics of adaptive traits, with an increasing interest in predicting the genetic response of populations to changing climates, and a trend towards incorporating adaptive as well as economic traits in breeding programs (Bradshaw and Strauss 2001, Aitken and Adams 1996). Now, the physiology of *Populus* needs to be accompanied with investigation at a molecular level. Undoubtedly, genomics and transcriptomics will help to unravel the relationship between ABA and guard cells, g_s and between root and shoot.

1.3.1 QTL analysis

One way to link physiology and genomics is the use of Quantitative traits loci (QTL) as a valuable tool that will identify traits in a progeny that can be allocated to specific positions on the chromosome. Several QTL studies have been done in *Populus* on biometrical traits such as growth, biomass production, leaf phenology and morphology, dormancy, stem proportion and height, basal area and volume growth (Bradshaw and Grattapaglia 1994, Bradshaw and Stettler 1995, 1997, Taylor et al. 2001a, Chen et al. 2002, Ferris et al. 2002, Rae et al. 2004). This approach has been possible thanks to the availability of the current linkage maps (Bradshaw and Grattapaglia 1994, Bradshaw and Stettler 1995, Cervera et al. 2001). Family 331 map is one of the best known and used linkage maps and the genetic length of the *Populus* genome has been estimated as 2600cM.

1.3.2 Heterosis or hybrid vigour

Heterosis (heterozygous advantage) for vegetative gowth is characteristic of interspecific hybrids and/or wide crosses in many plant genera; forest trees are no exception (Wright 1976; Zobel and Talbert 1984 in Bradshaw and Grattapaglia 1994). Exploiting the phenomenon of heterosis requires either a simple method for generating large numbers of hybrid seedlings, or the ability to vegetatively (i.e. clonally) propagate desirable hybrid genotypes. In current forestry practice, only clonal propagation has been widely used to capture heterotic combinations for commercial planting, largely limiting the application of interspecific hybridization to those taxa with well-developed vegetative propagation systems, such as *Eucalyptus*, *Populus* and *Salix* (Bradshaw and Grattapaglia 1994).

In order to design and implement genetic improvement strategies of interspecific hybrids in forest trees, basic information is needed on the genetics of variation in commercially important traits. This information can be obtained in F_1 for selection in most hybridization programs (Bradshaw and Grattapaglia 1994). Identifying loci contributing to genetic variance in the F_2 or backcross generations, make it feasible to identify loci responsible for F_1 heterosis and that is what has been done in *Populus*. The contribution (positive or negative) and mode of action of each parental QTL allele to the phenotype of the hybrid may be determined, and used to evaluate the merits of various long-term breeding plans (Bradshaw and Grattapaglia 1994). QTL mapping in *Populus* also aims to give insights into the genetic basis of hybrid vigor (Bradshaw and Stettler 1995, Bradshaw and Grattapaglia 1994).

1.3.3 The construction of a *Populus* pedigree map

The *Populus* QTL mapping pedigree used in this study was constructed by hybridization between *P. trichocarpa* and *P. deltoides*. The predictable heterosis in this hybrid combination is the basis of applied genetic improvement programs for *Populus* in much of western North America. Mating of two F_1 siblings was performed to produce the F_2. Fifty-

four F_2 clones, along with the F_1 and parental trees, were established in a pilot-scale replicated clonal trial (6 ramets per clone) in 1990. Phenotypic data from the clonal trial were used to calculate broad-sense heritabilities for all the traits, allowing the estimation of the proportion of genetic variance accounted for by each mapped QTL (Bradshaw and Grattapaglia, 1994). More than 100 phenotypic traits were measured and the current linkage map for this F_2 family consists of 343 RFLP, STS, and RAPS markers, covering approximately half of the genome (Bradshaw et al. 1994, Bradshaw and Grattapaglia 1994).

The *Populus* map was constructed from marker segregation in the F_2 by treating the parental species as homozygous inbred lines and the F_2 sib-mating as a self-fertilization. Only two alleles, derived either from *P. trichocarpa* (T) or *P. deltoides* (D), were recognized at any locus, which has the potential to downwardly bias estimates of QTL magnitude (Bradshaw and Stettler 1995). The tacit assumption is that most of the genetic variance for the traits of interest is partitioned between the parental species rather than among individuals within the species. The theoretical disadvantage of treating the parental clones as inbred lines is clear: *Populus* clones, like most trees, are not homozygous but highly heterozygous. The advantage of treating the two parental species as inbred lines is that any marker which is polymorphic between the two parental species is informative for QTL mapping under the "simplified" model, and such "fixed" polymorphisms are more abundant than the multi-allelic markers necessary for a more thorough analysis of QTL inheritance (Bradshaw and Grattapaglia 1994).

1.3.4 Study of QTL in *Populus*

Interspecific hybrids of poplars or other forest trees have proven to be particularly amenable to map-based QTL analysis. They are fast growing trees and the commercially important phenotypes are revealed soon after planting. Secondly, they grow in a relatively homogeneous environment, reducing the non-genetic variance components of complex traits and facilitate detection of the genetic basis of phenotypic variance. Finally, clonal

propagation allows precise estimation of the broad-sense heritability and an accurate assessment of the magnitude of the effect of QTL on the genetic variance (Bradshaw and Grattapaglia 1994).

Mapping QTL in *Populus* has been widely used. Bradshaw and Stettler (1995) mapped quantitative trait loci for commercially important traits (stem growth and form) and an adaptive trait (spring leaf flush). They found that 30% of the phenotypic variance and 45% of the genetic variance in stem volume after 2 years of growth is controlled by just two QTL in the F_2. These results suggest that the polygenic model is not applicable to this Family F2. QTL governing stem basal area were found clustered with QTL for sylleptic branch leaf area, sharing similar chromosomal positions and modes of action suggesting a pleiotropic effect of QTL responsible for stem diameter growth (Bradshaw and Stettler 1995).

Wu et al. (1997) mapped quantitative trait loci affecting leaf variation in the same population, Family 331, assessing leaf size, shape, orientation, colour, structure petiole size and petiole cross section. They found that the same chromosomal segment of linkage group L (LG VIII in Family 331) seemed to account for 20% of the phenotypic variation of all dimension related traits, leaf size, petiole length, and midrib angle. In all traits, the *P. deltoides* alleles had positive effects and were dominant to the *P. trichocarpa* alleles. Similar relationships were also found for lamina angle, abaxial greenness and petiole flatness. The importance of leaf size, morphology, structure and orientation in influencing tree productivity has been demonstrated in numerous studies and the variation in leaf morphology and display can be helpful in interpreting the adaptation of a plant to environmental factors, including light, water, and wind. Thus it becomes important that little is known about the genetic basis of leaf morphological variation in forest crops (Wu et al. 1997).

In 2000, Frewen et al. (2000) studied the QTL affecting the timing of autumn bud set and spring bud flush, using a linkage map of hybrid poplar family 822. They found three QTL controlling bud set and six QTL controlling bud flush. Additionally, five candidate genes

believed to be involved in perception of photoperiod (PHYB1, PHYB2) or transduction of abscisic acid response signals (ABIIB, ABID, and ABI3) were placed on the QTL map. PHYB2 and ABI1B were found to be coincident with the QTL affecting bud set and bud flush (Frewen et al. 2000). Taylor et al. (2001a) in UK have been studying the *Populus* hybrid Family 331 from the biomass perspective and putative QTL for leaf and stem growth have been described, when this population is growing in UK conditions.

Recently, Yin et al. (2004) reported the most complete genetic map for *Populus* with 544 markers mapped onto 19 linkage groups equivalent to the *Populus* chromosome number. The estimated length is 2300 and 2500 cM observed number of crossovers in the maternal haplotypes. In this research they use the mapping pedigree (Family 13), an interspecific backcross consisting of 180 offspring. An important point in this study is that they detected some markers exhibiting segregation distortion that occurs largely in two linkage groups. They hypothesized that divergent selection has occurred on chromosomal scales among the parental species used to create this pedigree and explored the evolutionary implications.

The most comprehensive analysis of growth, branch, and leaf traits was carried out two years after plantation establishment, one-third of a typical rotation for pulpwood. In this case, for second year-height a single QTL was found accounting for 26% of the phenotypic variance and 32% of the genetic variance, with the T allele having a positive effect dominant to the D allele. Second year basal area is partly controlled by a single QTL explaining 24% of the phenotypic variance, 38% of the genetic variance, having the D allele dominant with a positive effect (Bradshaw and Grattapaglia 1994). Some QTL overlap, and they could be the linkage to explain the phenotypic correlations between radial growth and sylleptic branch leaf area.

The research work on epidermal and gas exchange under atmospheric and CO_2 enriched conditions in the Plant and Environment Laboratory at the University of Southampton has also given valuable information on epidermal and stomatal traits in the genetic quantitative analysis of *Populus* species (Ferris et al. 2002, Taylor et al. 2003, Rae et al. 2006).

It is believed that with the completion of genome analysis in *Populus trichocarpa*, the clarification of several mechanisms in *Populus* will be possible, leading to an advance in the understanding of the response to drought in *Populus*. However, the functional genomic and QTL analysis must be accompanied by more morphological and physiological work, in order to complement the results.

Coincidence of QTL position, magnitude of effect, mode of action, and direction of allelic effect suggest that pleiotropy can explain correlations among anatomical, physiological, and gross morphological traits. The ability to map QTL for morphophysiological traits also suggests that molecular breeders will be able to provide tree physiologists with subsets or clones differing primarily at a single QTL, with other loci held constant. This highly refined material is the forest tree equivalent of near-isogenic lines available in inbreeding crops, and will increase tremendously the power of physiological studies (Bradshaw and Grattapaglia, 1994).

The highest priority in interspecific hybrids is the stability of QTL across time. Stand development in forest trees takes several to many years, and undoubtedly there are different suites of QTL active at various life history stages (e.g. establishment, juvenile growth, crown closure and competition, flowering). This is known to be true in *Populus*. Quantitative genetic studies suggest that patterns of genetic variance change over time (Balocchi et al. 1993 in Bradshaw and Grattapaglia 1994). It should prove interesting to see how these changes in variance components occur at the sub-chromosomal level. Further, by mapping QTLs for both incremental and cumulative traits, such as stem growth, it should be possible to model tree responses to stand development more accurately. In clonally-propagated trees such topics as competition can be investigated by replicating test designs at different spacings, for example (Bradshaw and Grattapaglia 1994).

Provenance trials in forest trees indicate that there will be substantial environmental variance and genotype x environment (GxE) interactions in the expression of most commercial traits. Genome mapping work in crop plants shows that some QTL are

important across all environments and others vary in their magnitude of effect in different environments (Bradshaw and Grattapaglia 1994).

The study of leaf traits from a segregating pedigree, such as an F_2, grown at differing sites should show evidence of traits that are expressed as a consequence of the environmental changes. Apart from the valuable information of traits expressed in an F_2 family, these responses could also show some of the changes that occur in the epidermis, a multifunctional tissue of which the development and morphogenesis has not been completely understood. This also includes other leaf structures such as trichomes, stomata, cuticle and waxes and their differentiation processes (Glover 2000).

QTL studies have yielded valuable insights into plant response to a range of environmental changes including salinity (Koyama et al. 2001) and drought (Lanceras et al. 2004) and have prompted gene-cloning strategies and identified *Arabidopsis* as a valuable model to understand genetic variation in an ecological context (Alonso-Blanco et al. 2000).

The F_2 generation segregates for a wide variety of traits including growth, form and phenological characters making this an ideal pedigree in which to study quantitative traits. In addition, poplar is now recognized as the 'model' forest tree (Taylor 2002, Wullschleger et al. 2002), and has several advantages as a model system, including the availability of several linkage maps and pedigrees, transformation systems and a large genomic resource including EST collections and poplar microarrays (Andersson et al. 2004). The release of the complete genome sequence, the first for a tree, in 2004 (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) will enable significant advances to be made in our understanding of both economically important traits (eg. wood formation), as well as increasing our ability to answer fundamental questions in forest ecology and evolution.

The assessment of genetic variation for physiological traits related to adaptation to environmental stresses includes cold hardiness and phenology, the drought response related traits leaf gas exchange, water-use efficiency, hydraulic architecture and vulnerability to embolism of xylem water columns; disease resistance, root morphology and wood yield

traits (Wu et al. 1992, Aitken and Adams 1996, Taylor 1998, Taylor et al. 2001). Genetic linkage mapping of osmotic potential in mesic versus xeric conditions suggested that there were several putative QTL accounting for about 20% of the phenotypic variation under wet conditions. In addition, a QTL for adjustment was found on linkage group D, with overdominance indicated located in different LG, suggesting that they are controlled by different mechanisms (Tschapklinski et al. 1998c). Currently, 12 QTL for osmotic potential in the same Family 331 are available in the published literature. This suggests that there are possibilities of range extension in drought tolerance of *Populus* hybrids (Tschaplinski et al. 2005).

1.4 Aims

This research aims to identify the physiological, morphological and epidermal cell level responses to water deficit in *Populus* taking as a model the hybrid family 331 (grandparents, parents and progeny) in order to select the traits useful as indicators of resistance or adaptation to drought. Knowledge of the responses in the original parental species (grandparents) is of considerable importance in discerning the relevant traits in the 220 genotypes member of the progeny. In particular this research aims:

1) To investigate the extent to which the gas exchange mechanisms of each of the two original parental species is affected by short periods of water deficit and how the response is related to leaf morphology, structure, developmental stage and water content.

2) To identify the morphological changes of the F_2 progeny when submitted to a drier environment and to determine the genetic information that the morphological and physiological responses to water shortage are based on in poplar.

3) To determine future directions of research which will extend the cultivation area of *Populus* without detriment to biomass production?

To answer these questions, several experiments were planned. The first experiment (Chapter 3) focussed on the response of the carbon assimilation process in the original parental species to different environmental factors: water deficit, light and increasing CO_2 concentration. The second experiment (Chapter 4) was designed to study stomatal function in the original parental species under different environmental conditions in all the Family 331 genotypes. The third experiment (Chapters 5, 6, and 7) was a study of leaf morphology and growth in all the Family 331 (F_2) grown in contrasting environmental conditions in order to identify QTL for drought tolerance. Overall, these experiments were expected to identify the adaptive strategies of *Populus* when water is scarce and to identify the traits which can assist in the selection of more drought tolerant clones of the *P. trichocarpa* x *P. deltoides* F_2 pedigree.

Chapter 2

Materials and Methods

2.1 Plant material

2.1.1 Plant source

All the parental material used in this work was obtained from the maternal parent, *P. trichocarpa* (93-968), a native black cottonwood from Western Washington State and the male parent *P. deltoides* (ILL-129), of the eastern cottonwood from Illinois (Figure 2.1). The two F_1 hybrid parent genotypes (53-246, female), and (53-242, male) plus 300 siblings of Family 331 derived from the cross of the the last two genotypes (53-246 x 53-242). The pedigree derived from a stool bed at the University of Washington, Seattle, USA, and was introduced for the first time into the UK in 1999 as hardwood cuttings of approximately 25 cm length. Since then, plants have been cultivated at the Headley Field Experimental Station of the Forestry Commission, U.K. (51° 07° N, 0°50´ W). Cuttings were always obtained in winter and stored in a cold room at 1 °C until their use. Before planting they were soaked in water for 48 hours.

Black cottonwood (*Populus trichocarpa*) (Figure 1A), is one of the fastest growing tree species in North America. Its natural range is shown in Figure 1B. On mesic sites west of the Cascade mountains it can grow up to 1.5 m in the first year and 6 m in the second year. Its habitat is mainly riparian, even though some genotypes grow in mountainous regions. An important feature of this species is the shallow root system (Heilman and Stettler 1985). The growth of *P. deltoides* (Figure 1C) is relatively fast; 1.5 m per year and with phreatophytic (deep) roots it; is difficult to root from cuttings. Its habitat is drier than that of *P. trichocarpa*, and it is considered to be intolerant of shade. Its habitat is mainly close to or within 100 m of rivers (Dickmann and Stuart 1983, Uddin et al. 1983). Its natural range is shown in Figure 1D.

2.1.2 Planting

Plant material was grown every year in different conditions to have it ready for different research experiments. Every year all the Family 331 progeny, F_1, and grandparental material was cut back and cuttings stored in the fridge at 1 °C for dormancy.

Grandparents

P. trichocarpa **93-968**

♀

X

P. deltoides **ILL-129**

♂

Parents

F_1 Family 53

53-246

♀

X

53-242

♂

Progeny F_2 Family 331 (formed of 300 genotypes)

Figure 2.1 Origin of Family 331. The maternal parent, *P. trichocarpa* (93-968), a native black cottonwood from Western Washington State and the male parent *P. deltoides* (ILL-129), a clone of the eastern cottonwood from Illinois. The two F_1 hybrid parent genotypes (53-246, female), and (53-242, male) gave rise to 300 siblings of Family 331.

2.1.2.1 Pots in control room

20 uniform unrooted cuttings each of *P. trichocarpa* and *P. deltoides* were planted in March 2002 into plastic tubes 75 cm long and 13 cm diameter. The cuttings were planted in the centre of the pot leaving only one bud above the soil. The tubes were filled with 10 kg John Innes No. 3 compost (Figure 2.2). The base of each tube was sealed with a fine mesh to prevent the loss of compost. The end of the tubes were placed on a plate to prevent water spillage. Pots were kept growth in the plant room in the conditions specified in each experiment.

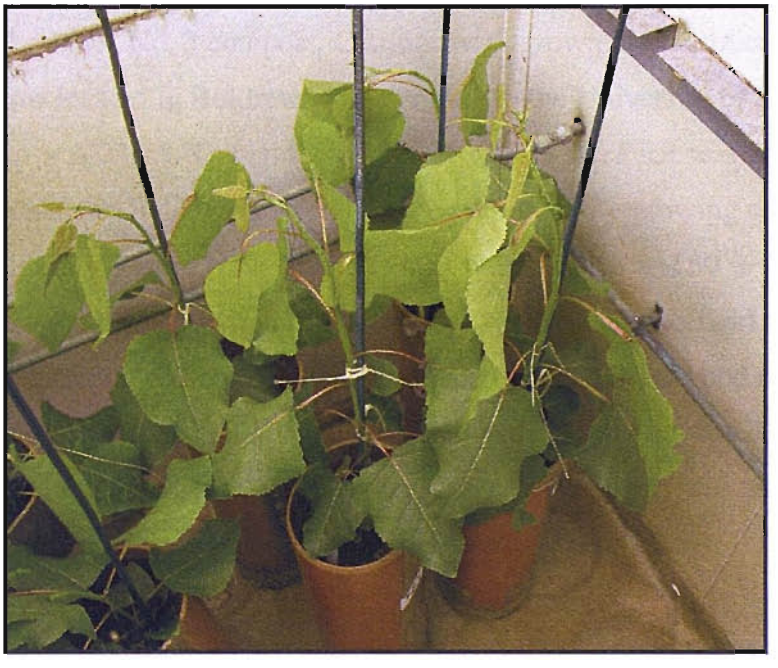


Figure 2.2 *P. deltoides* plants growing in the plant room. Cylindrical tubes were used to give more growing space for roots. Plant height was about 60 cm.

2.1.2.2 Planting at Boldrewood ground

54 cuttings, 27 of *P. trichocarpa* and 27 of *P. deltoides* were planted on 14 March 2003 in a plot next to the greenhouse in the nursery at the University of Southampton. The unrooted cuttings were planted in two blocks, at 40 cm separation. The plot was partially shaded by trees to give conditions with between 200-600 $\mu mol\ m^{-2}\ s^{-1}$ PAR during the day. When planted, cuttings were watered every day until established. During

the summer, a small dose of liquid NPK fertilizer (Osmocote, Scotts Europe B.V., Netherlands [N 14:P 13: K 13]) was applied. Plants did not receive any additional water.

2.1.2.3 Planting in the greenhouse

In the greenhouse at Boldrewood, cuttings of Family 331 were planted in the last week of January 2005 into plastic pots 29 cm diameter and 23 cm length. The cuttings were planted in the centre of the pot leaving only one bud above the soil. The pots were filled with 10 kg John Innes No. 3 compost. Cuttings were grown in controlled conditions at the Greenhouses located in Boldrewood belonging to the University of Southampton, U.K. Day temp was 23-25 °C, night temperature was 18 °C, PAR was 200-300 µmol m^{-2} s^{-1}, supplementary light was given at the beginning of the growing season using high density lighting Vialox Nav-T (son-T) 400 W, humidity was 50 % and day photoperiod was 16 h (6am - 10 pm). All cuttings were watered to field capacity every day during the growing season from the beginning of March to the end of September. Trees were given a balanced NPK fertiliser (Osmocote, Scotts Europe B.V., Netherlands [N 14:P 13: K 13]). The maintenance of these plants was under the supervision of technical personnel in the Greenhouse (Figure 2.3 A).

At the greenhouse at Chilworth, 6 replicates of 167 clones of Family 331 progeny were planted in 50 cm diameter and 48 cm length plastic pots (25 l) using the compacted JI-3 compost described above. Plants were arranged randomly in two greenhouse modules, to protect plants from frost and rain, but there was no supplementary lighting. Plants were grown at Chilworth for 5 months starting on 05 May 2004. Cuttings were watered daily until establishment, and drought treatment initiated 131 days after planting. In half of the replicates watering was withheld for 7 days plants were then watered with 0.5 l, and so on. Soil water content was measured with a Delta-T ML2x ThetaProbe connected to an HH2 Moisture Meter (Delta-T Devices, Cambridge, UK) averaging 27% for control and 15% for drought treatment. The plants used as control received full field capacity watering (Figure 2.3 B). Stomatal conductance measurements in control and treated plants were done at 17 days after drought treatment started. Grandparental

stomatal conductance was measured at day 15 of the treatment. Experimental design for planting and maintenance in Chilworth was under the responsibility of PhD student Nathaniel Street (now graduated) with the collaboration of other PhD students James Tucker and myself, Postdoctoral research fellow Dr. Matthieu Pinnel and the technicians Caroline Dixon and Mike Cotton.

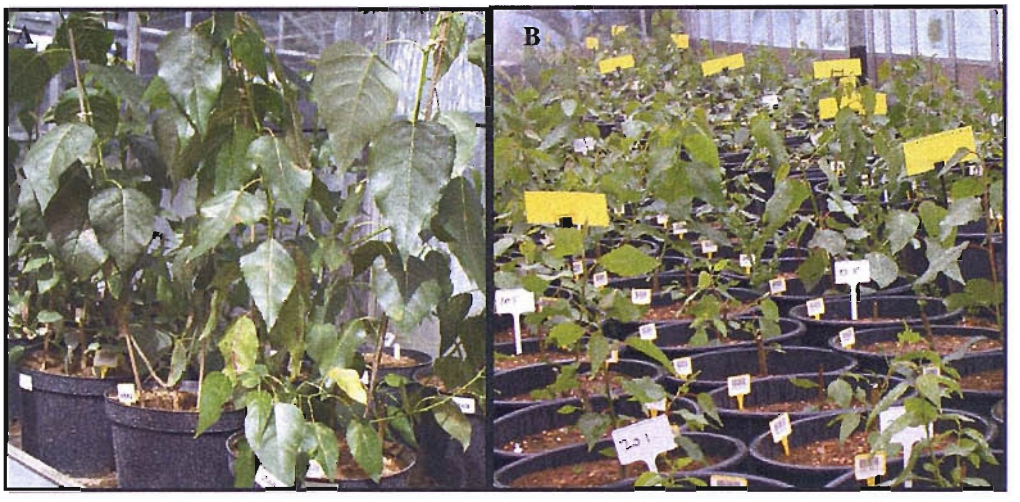


Figure 2.3 Plants growing in the Greenhouses at the University of Southampton. A) Boldrewood and B) Chilworth at ages of 5 and 3 months respectively.

2.1.2.4 Planting in the experimental field in the UK and Italy

The *Populus* F_2 hybrid Family 331 was used in this experiment. A randomized block design was used for the plantations at each field site. For each field site, six blocks were defined and one of each of 210 genotypes of Family 331 was randomly assigned to each block. Each block thus contained one replicate of each grandparent, two F_1 (Clone 242 & 246) and 206 F_2 genotypes. Spacing was 0.75 x 2.0 m. A double row of *Populus* 'Gaver' was planted around the 6 blocks to reduce border effects. Unrooted hardwood cuttings were planted in April 2003 at both sites. The experimental plantings were located in two sites, i.e. United Kingdom (Headley, 51° 07' N, 0° 50' W) and in Italy (Cavallermaggiore, 44° 21' N, 8° 17' E) (Figure 2.4). Plants in the UK received water three times a week during the night and in Italy the blocks were irrigated by flooding on four occasions; June 24th, July 16th and 30th, and August 17th. A complete map for the planting at both sites is given in Appendices 2 and 3. The environmental conditions at

the two field sites are shown in Table 2.1. This planting was done by all members in Professor Gail Taylor's laboratory with the technical support of Caroline Dixon.

Table 2.1 Environmental conditions at experimental field sites in the UK and Italy. Annual temperature differences are just 2 °C, but differences of 3 °C occurred in the maximum temperature reached during the growing season (in brackets), keeping the same 2 degrees difference in the minimum temperature. Rainfall in Italy was 65% of that received in the UK. However rain received during the growing season (in brackets) was 86% of that received in the UK.

Site	Location	Elevation (m)	T annual mean (°C)	Rainfall Annual (mm)
Cavallernaggiore	44° 21' N	285	12.9	470.9
(Ita)	8° 17' E		(15.9/12.9)	(153.7)
Headley (UK)	51° 07' W	60	10.9	729.3
	0° 50' W		(12.6/10.9)	(179.6)

2.2 Leaf age determination

The Leaf Plastochron Index (LPI), is a measurement of the developmental status of the leaf, calculated as suggested by Erickson and Michelini (1957) and Taylor et al. (2003), using the formula below:

$$PI = n + \frac{\log L_{n+1} - \log 10}{\log L_n - \log L_{n+1}}$$

Where L_{n+1} is the length (mm) of a leaf just shorter than 30 mm and L_n is the length of the next leaf that was longer than 30 mm. n is the serial number of the leaf. Data for PI are not shown but were used to calculate LPI. Leaves with the same LPI can be regarded as being of the same developmental status. It is important to say that PI of a plant is based on length measurements of just two emergent leaves, and the LPI or LPIs of additional leaves are derived by computation. That computation is a form of extrapolation which assumes a uniform rate and duration of development for each leaf in the zone of leaf development (Larson and Isebrand 1971).

Figure 2.4 View of the two experimental field sites in A) England (UK) and B) Italy. *Populus trichocarpa* and *P. deltoides* planted together with the F_1 parents and F_2 hybrid progeny.

2.3 Gas exchange responses to drought, light and CO_2

2.3.1 Plant growth conditions

Pots with cuttings were grown in controlled conditions at University of Southampton, U.K. Day temperature was 22 °C, night temperature was 16 °C, recorded PAR (Photosynthetically Active Radiation) was 160 µmol m^{-2} s^{-1}, humidity was 50 % and day photoperiod was 16 h. All cuttings were watered to field capacity for 20 days. On 1 July 2002, drought treatment was initiated. Water was completely withheld to induce acutely stressed trees (n=5), Control plants (n=5) were watered to field capacity each

day. Trees were given a balanced NPK fertiliser (Osmocote, Scotts Europe B.V., Netherlands [N 14:P 13: K 13]). Mature plants were used for the experiment (Figure 2.5 A). In this part of the work I would like to acknowledge the participation of the PhD student Nathaniel Street and the technician Caroline Dixon.

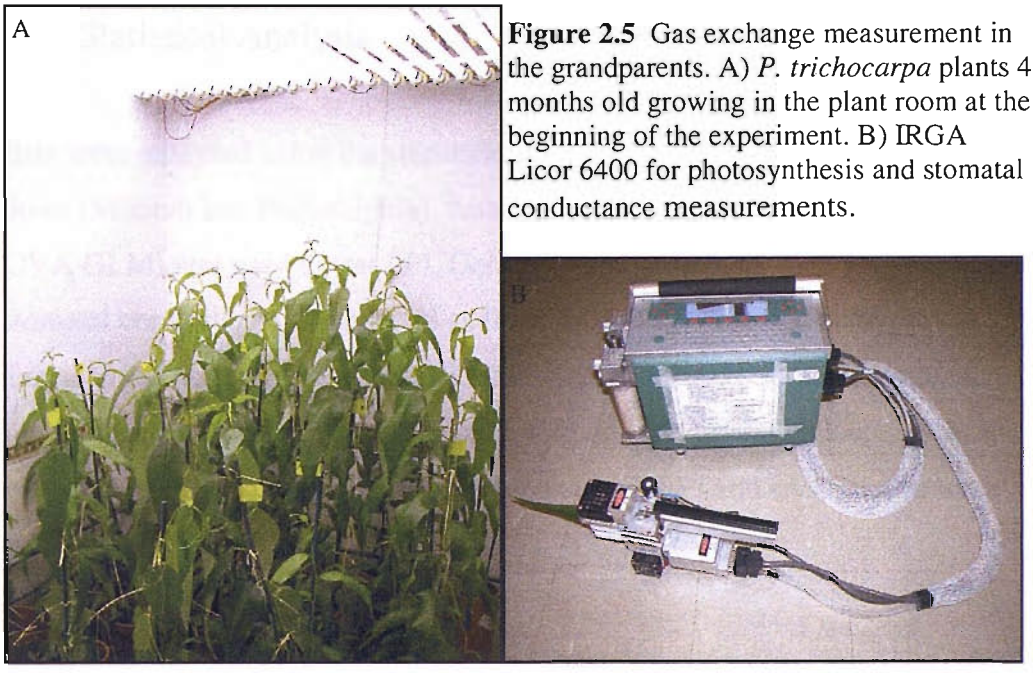


Figure 2.5 Gas exchange measurement in the grandparents. A) *P. trichocarpa* plants 4 months old growing in the plant room at the beginning of the experiment. B) IRGA Licor 6400 for photosynthesis and stomatal conductance measurements.

2.3.2 Gas exchange measurements

Photosynthetic CO_2 assimilation (A) and stomatal conductance (g_s) were assessed every other day in the morning in each species using an infrared gas analyser (Licor 6400) portable photosynthesis system, Lincoln, Nebraska (U.S.A.). This IRGA model is an open or differential system which measures the difference in CO_2 concentration between the chamber entrance (c_e) and outlet (c_o). Here the air streams are dried prior to entering the IRGA, the assimilation rate (A) will approximate to:

$$A = \frac{u\,(c_e - c_o)}{s} \qquad \text{(Long and Hällgren 1993)}$$

Where u is flow rate through the chamber and s= time in seconds.

IRGA used has 3 components: source, cell and detector (Figure 2.5 B).

Conditions in the chamber were: PAR 300 µmol m^{-2} s^{-1}, CO_2 400 µmol mol^{-1} and constant fixed flow of 400 µmol s^{-1}. Leaf temperature was 23°C. Photosynthetic

determination was carried out on the 5 control and treated plants of each of the grandparental genotypes. Each reading was made on leaves of three different ages for every plant, LPI 0, 5 and 9, each one taking two minutes.

2.3.2.1 Statistical Analysis

The data were analyzed using the statistical software package Minitab 13.0 for Windows (Minitab Inc, Philadelphia). Analysis of Variance General Lineal Model (ANOVA GLM) was used to test LPI, Genotype and Treatment for CO_2 assimilation and stomatal conductance at day 9, all as fixed factors. A post-hoc analysis was conducted to test genotypes at each LPI. Statistical differences in photosynthetic assimilation and stomatal conductance rates with drought treatment were tested with one-way analyses of variance (Student's t-test) at each LPI and each measurement date for each genotype.

2.3.3 Light response

Leaves LPI 7, from mature plants growing in the planting field at Boldrewood, were cut at midday near the base of the petiole, recut in water (Sperry et al. 1988) and kept in a semi-dark and constant temperature room in the laboratory until the time of the experiment (Robinson et al. 2004).

A light response curve was constructed according to the guidelines given in the LI-COR manual Book 1, Part 1. A known 6 cm^2 area of the leaf was clamped into the chamber of an infrared gas analyser (Licor 6400) portable photosynthesis system, Lincoln, Nebraska (U.S.A.) and exposed to the following conditions in the chamber: 1500 PAR ($\mu mol\ m^{-2}\ s^{-1}$), 400 $\mu mol\ CO_2\ mol^{-1}$ and a constant flow of 500 $\mu mol\ s^{-1}$. The curve was started from light at 1500, 1250, 1000, 800. 600, 400, 300, 200, 100, 50 PAR ($\mu mol\ m^{-2}\ s^{-1}$). Using Photosynthesis Assistant, software for analysis of photosynthesis (version 1.1, Parsons and Ogston 1998), the responses of leaf net photosynthesis (A) to light level (Q), the Apparent Quantum Efficiency (ϕ), Light Compensation point and

respiration are estimated from the axis intercepts and Light Saturation (Amax) estimates were determined using the equation given by Prioul and Chartier (1977).

$$A = \frac{\phi .Q + A_{max} - \sqrt{(\phi .Q + A_{max})^2 - 4.\phi .Q .k .A_{max}}}{2k} - R_{day}$$

All values, including k (convexity) were calculated by a linear regression of half of the data to estimate ϕ and R_{day} (Day respiration) and taking a value slightly above the maximum as A_{max}. The best fit is given automatically by the model produced by the software mentioned above. The light curves were done during September 24-29, 2003.

2.3.4 A/*Ci* response

Leaves LPI 7 were collected at dawn by cutting the base of the petiole from the stem. Once in the laboratory, the petioles were recut under water. The leaves were stored in a constant temperature room in the laboratory until the time of their use in the experiment and were kept in water all the time. A known 6 cm^2 area of the leaf was clamped into the chamber of an infrared gas analyser (Licor 6400) portable photosynthesis system, Lincoln, Nebraska (U.S.A.) and exposed to varying levels of carbon dioxide at 20°C of leaf temperature and 600 µmol s^{-1} from red and blue LEDs. The CO_2 concentrations started at 400 µmol mol^{-1}, and then 360, 300, 200, 100 and 50 µmol mol^{-1}, going back to 400 and then steadily raising to 2000 µmol mol^{-1}.

This response uses the following equation:

$$A = (CE*Ci*Amax)/(CE*Ci+Amax) - Resp$$

Where *A* is assimilation, *CE* is carboxylation efficiency, *Ci* is internal concentration of CO_2, *Amax* is the assimilation at saturating CO_2 and in the respiratory processes (dark and light). The data were analysed by the software Photosyn Assistant (Version 1.1, Dundee Scientific, Dundee) (Parsons and Ogston).

Mechanistic *A/Ci* curve to describe photosynthetic characteristics from the leaves; A/*Ci* (CO_2 assimilation rate vs internal leaf CO_2 concentration), leaf respiration

Vc_{max} (an in vivo measurement of Rubisco activity) and J_{max} (the electron capacity of the thylakoid during the regeneration of RubP from Fructose 6-Phosphate) and triose phosphate utilization (TPU), were estimated following the equation:

$$A = \left(1 - \frac{0.5\,O}{\tau Ci}\right) x \min(\ Wc\ , Wj\ , Wp\ ) - Rday$$

(Brooks and Farquhar 1985).

This equation can be estimated using the following modeling equations:

For Carboxylation limited by Rubisco,

$$Wc = \frac{Vc_{max}.Ci}{[Ci + Kc\ (1 + O\ /\ Ko\ )]}$$ (Brooks and Farquhar 1985)

Where Kc and Ko are Michaelis-Menten constants for CO_2 and O_2, O is stroma O_2 concentration .

For photosynthesis limited by electron transport,

$$Wj = \frac{J.Ci}{4\,(Ci + O\ /\ \tau)}$$ (Brooks and Farquhar 1985)

Where Tau (τ) is specificity factor for Rubisco, 4 represents the four electrons needed to regenerate RuBP.

The potential rate of electron transport is calculated using the empirical expression,

$$J = \alpha.I \div \sqrt{1 + (\frac{\alpha.I}{J_{max}})^2}$$ (Smith 1937, Harley et al. 1992)

Where α is the efficiency of light conversion, J_{max} is the light saturated rate of electron transport and I is the incident radiation.

For Carboxylation limited by the regeneration of inorganic P,

$$Wp = 3\,(TPU\ ) + \frac{0.5\,xVoxO}{Cix\ \ \tau}$$ (Farquhar et al. 1980)

Where *Vo* represents the rate of oxygenation of Rubisco.

2.3.5 Separation of stomatal and mesophyll limitations

Assimilation rate at atmospheric CO_2 concentration (Ca= 360 µmol mol^{-1}), is subtracted from Ao, (the rate with no stomatal limitation) which is the value of A interpolated from the response curve at ambient Ci. The relative limitations (l) which the stomata impose is calculated with the following equation: $l = (A_o\text{-}A)/A_o$ (Farqhuar and Sharkey 1982, Hall et al. 1993). The separation of limitations due to carboxylation and to capacity for regeneration of RuBP is determined by the initial linear response where the efficiency of carboxylation (i.e. amount of active ribulose-1,5-biphosphate carboxylase-oxigenase,

Rubisco) determines the slope $\delta A/\delta Ci$, followed by an inflection to a slower rise where it approaches zero because A is limited by the supply of RuBP for carboxylation (Long and Hällgren 1993).

2.4 Stomatal conductance

2.4.1 Leaf stomatal conductance measurements

All stomatal conductance (g_s) measurements were taken with a portable Steady State porometer Li-1600 (LI-COR inc. Lincoln, Nebraska), with manual recording of data. A sample is placed onto the cuvette aperture and clamped. The relative humidity of the cuvette, in contact with the sample is held constant. The water loss as transpiration is determined as humidity change in the chamber (Li-COR, inc. 1984. Steady State Porometer. Instruction manual). Reading times increase as conductance decrease, so readings were taken 30-60 seconds after clamping (Figure 2.6). It is desirable to make measurements in controlled conditions to minimize the impact of the instrument on stomatal conductance. The instrument has been shown to be accurate for measuring gas in the field when used in this way (Beadle et al. 1993).

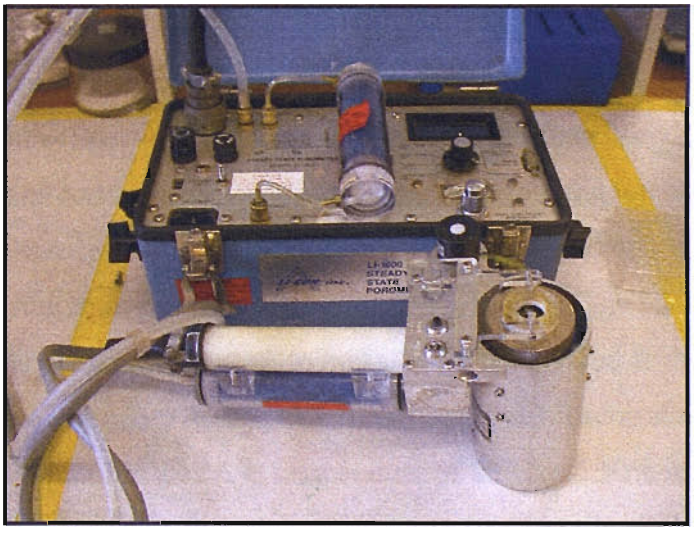


Figure 2.6 Portable Licor 1600 for leaf conductance measurements. The console and chamber are shown.

The Licor 1600 permits rapid measurement of water loss and diffusive resistance. Its theory is based in three steps: 1) the empirical measurement of the mass flow rate of water vapor entering the cuvette from all sources (m_{wi}), the relationship between the

mass flow rate (m_{wi}) and the physiological definition of stomatal resistance and 3) the effect of the mass flow rate measurement process upon stomata and stomatal resistance.

2.4.1.1 Diurnal stomatal conductance in the grandparents

Stomatal conductance in the grandparents was measured over a period of 22 hours, in midsummer 2004. Three plants of each grandparent were grown in greenhouse conditions at Chilworth with daily watering (see 2.1.2.3). Conductance and transpiration on the abaxial side of a mature leaf was determined at ambient temperature every one or two hours. Stomatal conductance at midday and midnight also was also measured using the plants growing on the ground at Boldrewood. Graphs were constructed to illustrate stomatal conductance during the day. This was done with the help of Allen J. Coombes.

2.4.1.2 Stomatal conductance in the Family 331

Stomatal conductance of all Family 331 was measured in the Experimental Field Station in Headley in midsummer 2004. Leaf 7 of the main stem from each genotype was removed and cut under water using a size 24 surgical blade and then recut under water to prevent air entry (Sperry et al. 1988). The petiole was transferred to a test tube and about $1cm^2$ area of cotton wool sheet was then placed into the top of the test tube to act as a bung. This ensures that the leaf will stay in the tube (and that the petiole remains under water) during transportation, especially with short petiole leaves. Then they were transported to the plant room, put on to racks, the tubes refilled with water to a fixed volume and left to acclimate to the plant room conditions. Environmental conditions were: light of 300 $\mu mol\ m^2s^{-1}$ PAR, temperature of 25°C and 50% relative humidity (+/- 10%). Three replicates of each genotype were measured in a period not exceding 4 hours after excision (Davies et al. 1993). Data were recorded in a Microsoft® Excel spreadsheet. This experimental part was undertaken with the project student Megan Powis from Biological School of Sciences in the University of Southampton (Figure 2.7 A-D).

2.4.1.3 Stomatal conductance in selected genotypes

After stomatal conductance had been measured in all the population, average values were calculated and, from these values, a sample of extreme clones with high and low stomatal conductance was selected. The clones were grown in 10 L pots and irrigated daily in greenhouse conditions at Boldrewood, Southampton during summer 2005. Measurements were made on four consecutive days from 8.30 to space 10.30 in the morning and 13.00 to 14.30 in the afternoon.

Figure 2.7 Sampling and measurement of stomatal conductance in Family 331. A) Leaf selection and sampling, B) Leaf transportation, C) Leaf acclimatization in D) Leaf conductance measurement using Licor 1600

2.5 Cell wall properties: cell extensibility and osmolality in leaves of the grandparents

2.5.1 Sample preparation for cell extensibility

Leaf numbers 1-12 from four mature plants of both *P. trichocarpa* and *P. deltoides* were sampled and stored in 20 ml vials of methanol solution at 4°C. These samples were for determination of the biophysical properties of the leaf cell walls using an Instron equipment (see below). Before biophysical tests, leaves were fully re-hydrated in 100 ml distilled water for 20 minutes and then passed to a new container with 250 ml distilled water and put for 10 min on a shaker (Mini Orbital Shaker, SO5, Stuart Scientific) at 50 rev/min, as described by Ferris et al. (2001). Following removal from the water, leaves were blotted dry, and a strip of known area (5 x 15mm) was cut from the right side, parallel to the mid-rib at the second intervein section, avoiding major veins. Strips were prepared sequentially for all 12 leaves of each grandparental clone of *P. trichocarpa* and *P. deltoides* and properties were determined using an Instron equipment (Instron 5542, Instron Ltd., UK) (Figure 2.8).

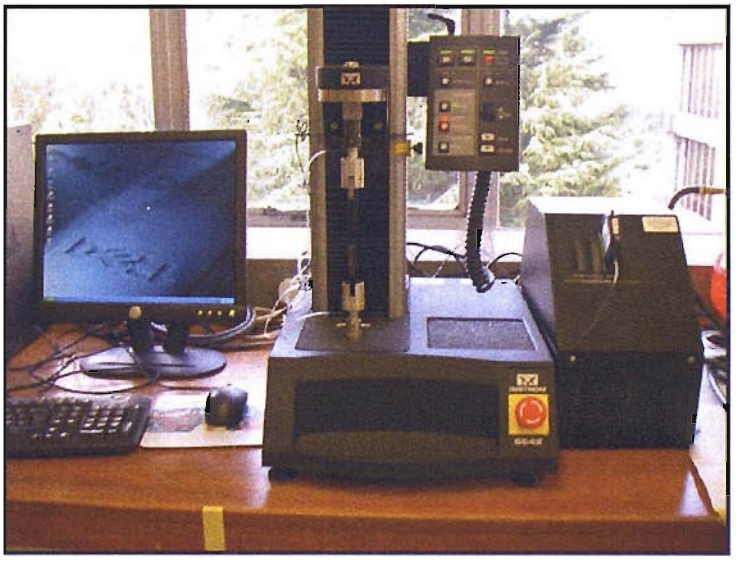


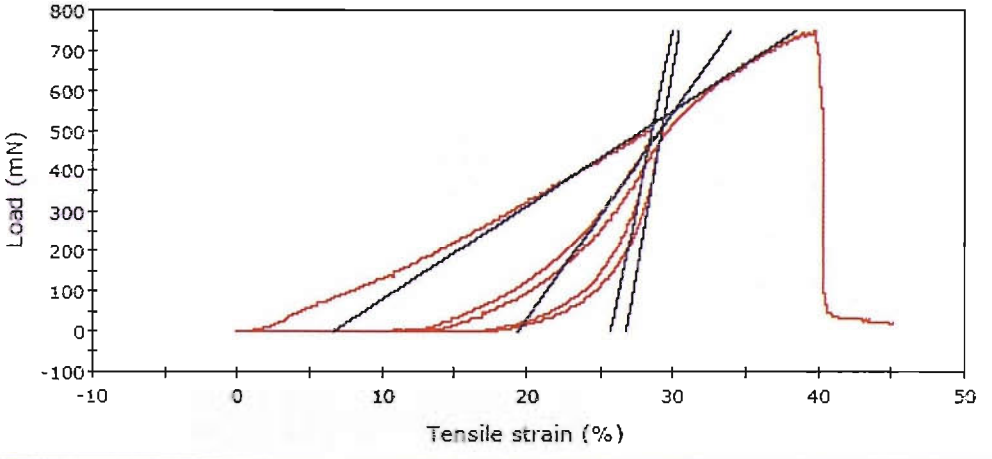
Figure 2.8 Instron 5542 for measuring cell extensibility. Clamp, load transducer, drive controller and computer are shown

The use of Instron and associated methods was with the assistance of Dr. Carol Waffstag and the PhD student Fang Zhang.

2.5.2 Plasticity, elasticity and maximum load measurements

Leaf strips (5x15mm) were attached between two clamps set 7 mm apart and stretched twice between two small brass clamps to a load of 500 mN. To obtain the measurements of plasticity and elasticity, the leaf strips were stretched twice, the second up to breaking point to obtain the maximum load value of the leaf material (ML). The results were expressed as percentage plasticity (% P; % irreversible extension per 0.5 N loads) and percentage elasticity (% E; % reversible extension per 0.5 N loads), % total extension, and maximum load (ML). The gradient of the first slope is equivalent to the total extensibility of the leaf sample, including plasticity and elasticity (P+E) (Figure 2.9).

A



B

Specimen label	Load at Absolute Peak (N)	X-Intercept at Modulus (Chord 0.40N - 0.43N) Load to 0.5N 1 (mm/mm)	X-Intercept at Modulus (Chord 0.43N - 0.40N) Unload to 0.5N 1 (mm/mm)	X-Intercept at Modulus (Chord 0.40N - 0.43N) Load to 0.5N 2 (mm/mm)	X-Intercept at Modulus (Chord 0.43N - 0.40N) Unload to 0.5N 2 (mm/mm)	P+E	E	p
T1-1	0.743	0.06577	0.25791	0.19268	0.26765	19.21472	7.497	11.71817

Figure 2.9 Cell extensibility in a young leaf in *P. trichocarpa*. Graphs and data produced by Instron 5542, operated with the software Blue Hill. Graphs and data list are presented in PDF files. A) The load extension curves and X-intercept and B) Interception values for each stretch, total cell extensibility, elasticity and plasticity are indicated in the table.

2.5.3 Measurement of cell sap osmolality

Osmolality is defined as an expression of the total concentration of dissolved particles in a solution (without taking into consideration particle size, density, configuration, or electrical charge). The measurement of osmolality (total solution concentration) is

through comparison of solution colligative properties in a pure solvent. Vapour pressure is a colligative property of a solution, (but not a cardinal property of the solvent) that can be measured directly in a semi-permeable membrane and is expressed in mmHg. The Wescor vapor pressure osmometer uses a passive technique of measurement. A small sample (8-10 µl) is inserted into a chamber, sealed and a sensitive thermocouple hygrometer in the chamber measures the dewpoint temperature depression and this colligative property of the solution is an explicit function of solution vapour pressure (Instruction Manual Vapor pressure osmometer model 5100C) (Figure 2.10).

In September 2005, leaf samples from well hydrated mature plants growing in greenhouse conditions at Boldrewood were taken. From four plants of each grandparent, leaves 1-12 were wrapped in aluminium foil, immediately

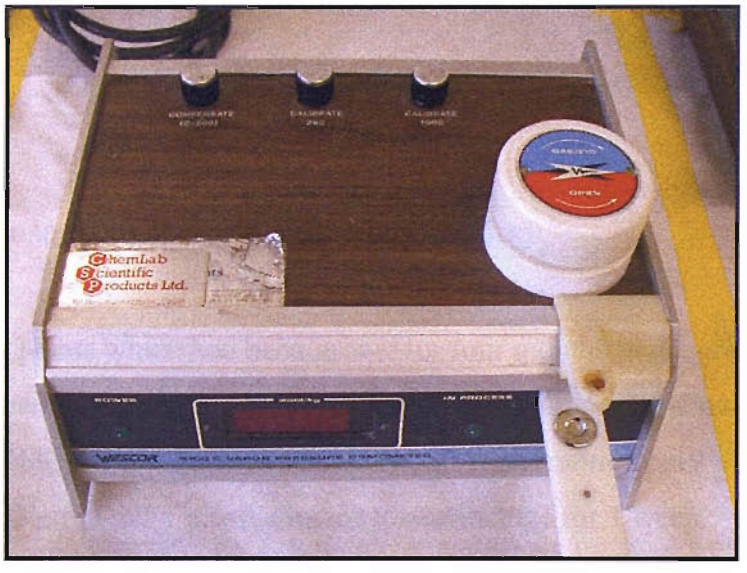


Figure 2.10 Vapour pressure osmometer 5100C used for osmolarity measurement. The sample is put onto a paper disk in the sample holder (botton right).

frozen and stored at - 80°C freezer for sap analysis. The sap was collected from the stored leaves (1-12) by placing them in a 0.5 ml centrifuge tube with a needle hole in the bottom and centrifuged for 15 s at 16,000 x g. The centrifuge tubes were placed on ice after spinning. Then, a 10 µl sap sample was taken from the top of the supernatant and tested following the manufacturer's instructions of Wescor 5100C vapour pressure osmometer (Wescor Inc., Logan, USA). Osmotic potential was calculated from the cell sap osmolarity using the conversion factor 40 mmol kg^{-1} = -0.1 MPa (Van Volkenberg and Cleland, 1986).

2.6 ABA responsiveness

2.6.1 ABA concentration in the grandparents

One recently mature leaf (LPI 8-9) was cut at the base of the petiole, folded in foil and immersed in liquid nitrogen. Once frozen, it was stored in dry ice until its storage in the freezer for further analysis. Leaves were samples in the UK, Italy and at the glasshouse in Boldrewood.
Samples were freeze dried and sent for ABA quantification in Professor William Davies' laboratory, using the procedure described by S. Wilkinson, with modifications by J. Theobald (Appendix 4), from The Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK. This procedure is based on the method described by Quarrie et al. (1988).

2.6.2 Response to exogenous ABA application

In March 2004, plants which had been grown for four months in the greenhouse at Boldrewood, were transferred to a climate chamber with the following day/night conditions: 14/10h; relative humidity 60-65/%; air temperature 25/19°C; PAR at the top of the plants 300 μmol m^{-2} s^{-1}, and allowed to acclimatize for 1 week.
Stomatal sensitivity to exogenous ABA was measured in detached leaves of two physiological ages, young (LPI 3) and old (LPI 9).

Leaf conductance to water vapour (g_w) was recorded in the chamber with a gas analyzer Licor 6400 with the following conditions in the chamber: 23°C temperature, 300 μmol mol $^{-1}$ PAR, 400 μmol s^{-1} flux and CO_2 ambient concentration 400 μmol mol^{-1}.
Detached leaves were fed with exogenous ABA (±2-cis, 4-trans-abscisic acid, MW 264.32, Aldrich # 862169) via the transpiration stream. Each leaf was cut under water and after 1 h irradiance; the petiole was transferred, still under water, to a tube containing a solution of ABA at two recommended concentrations: 10^{-3} and 10^{-6} M (Ridolfi et al. 1996). Readings were taken every 20 minutes for 240 min.

2.7 Thermal imaging to detect hormonal and mechanical stress responses

Thermal imaging is a tool for estimating leaf temperature as an indicator of stomatal closure and water deficit stress (Leinonen and Jones 2004). Leaf temperature is important to plants because of the subtle effects of small temperature changes on the rates of key physiological processes such as a biochemical reactions and cell growth and division, and because of the damaging effects of extreme temperature (Jones 1999, Merlot 2002).

The principle is that transpiration leads to reduction of leaf temperatures due to latent heat loss (Riera et al. 2005), and this approach has been shown to be useful in several ABA mutans of *Arabidopsis* (Merlot et al. 2002) and barley (Raskin and Ladyman 1988). This work was supported by Dr. James Morison from Biology School at the University of Essex.

2.7.1 Thermal imaging in the grandparents and parents of Family 331

Mature Leaves LPI 9 from plants growing in the greenhouse at Boldrewood were cut under water and transported to the growth-room set at 25 °C and 65-75% of RH, recut and then transferred to an ependorf tube with distilled water, to allow stabilization of stomatal conductance. The thermal camera was set up on a tripod, connected to a laptop computer external to the growth room in preparation for recording.

A tray of water was put under a fine nylon gardening net to support the leaves and a wet wick was prepared and mounted to be used as wet reference. Three leaves of each grandparental and parental genotype were placed on the fine nylon net in their tubes to give support without allowing the leaves to touch any surface. Samples were positioned in rows according to the genotype and treatment (Figure 2.11 A)

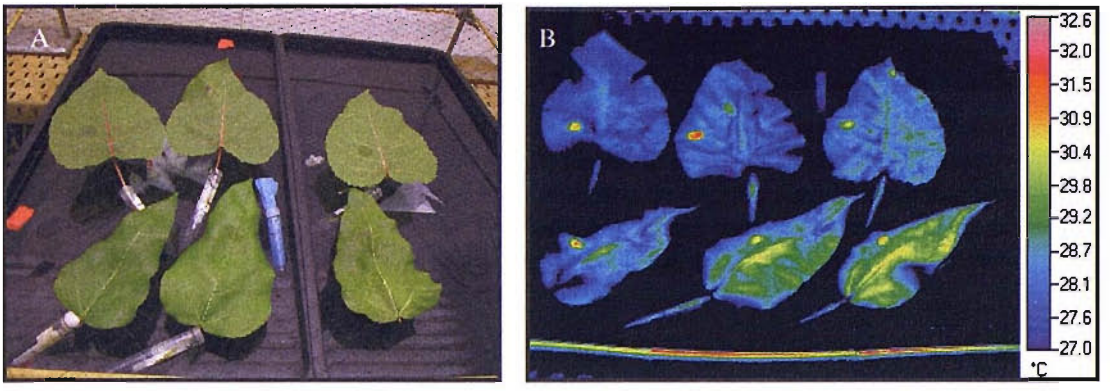


Figure 2.11 Poplar leaves arranged for thermal imaging analysis. A) *P. deltoides* (above) and *P. trichocarpa* (below) in three rows: left, leaves in distilled water used as control, middle, leaves in ABA 10^{-4} M and right, leaves with petiole excised simulating acute dehydration. B) View with the Infrared camera when leaves had stable transpiration rates. The colour of the leaves equals the colour of the wet reference as it can be compared on the colour scale bar.

Using the infrared camera (IR Camera NEC Thermovision), leaves were monitored until transpiration and temperature had stabilized (Figure 2.11 B) and a grease spot was applied to the leaf as a dry reference. After this (approximately one hour), the camera was set to record every 30s and then one leaf was transferred to the solution ABA 10^{-4} M and the petiole of a second excised as quickly as possible, simulating acute dehydration. The remaining leaf was kept as a control.

A digital image was recorded every 30 seconds for two hours, after which no more changes occurred. Instructions for the infrared camera were provided by Dr. James Morison from the University of Essex and the software used was THT1RX.

2.7.2 Calculating mean leaf temperature from IRT image analysis

Once all images had been taken, the number and time for every image was recorded and any unusable images were eliminated. After this, images were analysed using the software Image J which allows the import of SVT files to determine mean leaf temperature. For this, the perimeters of the leaves, grease spot and wet reference which were traced using polygon tool and the data were saved in TIFF format for later use (Figure 2.12). The images were 320 x 240 pixels in size and were used to analyse stacks, that included all the images (slices) obtained in the specified file. Data were copied into Excel, the sheet was duplicated and then used to calculate the average

temperature for each selection using the formula by 117 and add 40, add times and plot as required.

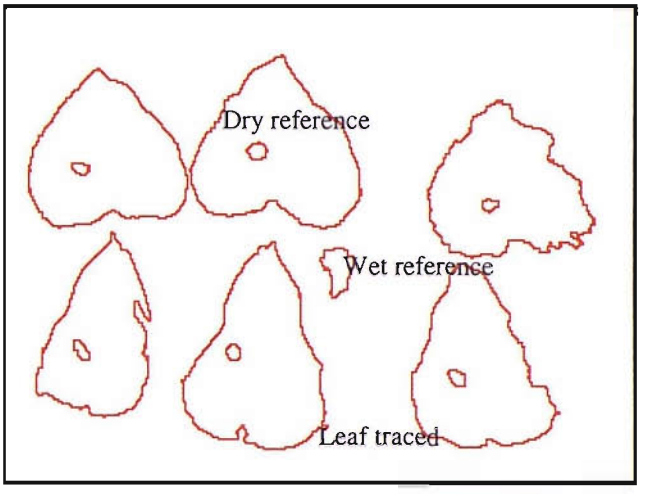


Figure 2.12 Leaf perimeter selection using Image J.Tracing with polygon tool showing every component of the selection.

2.7.3 Relative leaf conductance

From leaf temperature, leaf conductance (g_l) can be calculated according to Lyn Jones (1999), who proposed that $g_l = \{(Tdry - Tleaf)/(Tleaf-Twet)\} x\ G$ where G is l/[the mean boundary layer resistance for water (r_a) and the radiative resistance, r_r]. Here G was assumed be a constant, wet leaf temperature (Twet) is taken from the filter paper and dry temperature is the grease spot temperature. Therefore, an index of conductance was calculated as $g' = \{(Tdry - Tleaf)/(Tleaf-Twet)\}$ (Jones 1999 modified by Morison 2006, per. comm.). Relative leaf conductance data were plotted for one leaf each of the young, mature and F_1 parents.

2.8 Characterization of leaf morphology in the grandparents: *P. trichocarpa* and *P. deltoides,* growing in contrasting environmental conditions

2.8.1 Leaf and sample collection

The first mature leaf (LPI 7-10) was sampled in all cases. Plastochron index was calculated as previously with the equation suggested by Erickson and Michelini (1957). Every genotype was sampled for the traits of individual leaf shape (Leaf area, and specific leaf area [SLA (mm^2 g^{-1})]). From the same leaf, cell imprints were taken for stomatal and epidermal counts (stomatal density, stomatal index, epidermal cell area and epidermal cell number). All the field work in the UK and in Italy had the support of Dr. Anne Rae, Dr. Matthieu Pinnel, the technician Caroline Dixon and the undergraduate student Adam Stewart.

2.8.2 Leaf Traits

One fully expanded mature leaf from each genotype of three blocks was scanned into Corell Photopaint (Corell Inc.). Leaf samples were dried for 72 hours at 85°C with petioles removed, and dry weights obtained. Specific leaf area (area per unit dry weight in mm^2 g^{-1}) was calculated using the formula:

$$SLA = \text{leaf area} / \text{leaf dry weight}$$

and the area, length and width were calculated by using MethaMorph Imaging System (Westchester, Philadelphia, U.S.A.) Version 4.5, calibrated in μm.

2.8.3 Leaf microstructure

2.8.3.1 Transverse leaf sections

Two small leaf discs (10 mm diameter) from an attached, recently mature leaf of each parent were collected from 5 replicates and fixed in a main fixative buffer pH 7.2 (formalin:glacial acetic acid: 70% (v/v) ethanol [1:1:18, v/v]) (Taylor et al., 2003). The samples were stored at 5 °C until the time of final preparation. For light microscopy, the leaf discs were cut into 1–2 mm^2 and fixed in buffered osmium tetroxide (1 hour), 2 buffer rinses (10 min each), different concentrations of ethanol (30, 50, 70 and 95 %) for 10 min each, two stages in absolute ethanol for 20 min each and acetonitrile (10 min) followed by 6 hours overnight in acetonitrile resin. After a resin inclusion (6 hours) they were embedded in fresh resin and polymerised at 60 °C for 20-24 h. A section of about 0.5 µm was cut using an OMU 3 Ultramicrotome (Lecia Microsystems, Milton Keynes, UK), mounted on a slide and stained with 1% (w/v) toluidine blue in 1% borax (w/v). Images were then captured at x200, x400 and immersion with a digital camera attached to a light microscope (Zeiss axiophot 2). Leaf thickness (µm) was measured in 'Scion Image' with 6 replicates of each parent.

2.8.3.2 Scanning electron microscope (SEM) sections

A leaf disc (10 mm diameter) was collected from mature leaf 9 in three individual plants of the two grandparental species *P. trichocarpa* and *P. deltoides* growing at the field experimental sites in the UK and Italy. The leaf samples were stored in the primary fixative buffer (3% glutaraldehyde in 0.1 M phosphate buffer pH 7.2). The specimens were then rinsed in 0.1 M phosphate buffer (pH 7.2) and dehydrated in an ethanol series. A section was cut using an OMU 3 Ultramicrotome (Lecia Microsystems, Milton Keynes, UK), mounted on stubs and embedded in spur resin. The section was viewed on a Hitachi 5800 scanning electron microscope (SEM). Stomata and waxy cuticule deposition was observed for the two leaf surfaces.

For particularly dense tissue the main fixative time can be extended to 2 - 3 hours and the resin infiltration times can be extended to several days. It is important that it does not polymerise in the vials. See Appendix 1 for safety procedures and the SEM fixation technique. SEM and transverse sections were prepared by Dr. Anton Page from the Biomedical Imaging Unit at Southampton Hospital. The complete technique used by Dr. Anton Page is included in the Appendix 1.

2.8.4 Cellular traits

2.8.4.1 Stomatal and epidermal countings

Impressions of adaxial and abaxial leaf epidermis were made from each of the parents in replicates of 3. Cell imprints were taken from the first young fully developed leaf at the second interveinal section, near the main vein using transparent nail varnish (Boots no. 17) over the leaf, allowed to dry for 15 min and then removed with a piece of sellotape and mounted on a microscope slide (Ferris and Taylor 1994, Ceulemans et al. 1995, Taylor et al. 2003). From every imprint, six images were taken with an Upright Differential Interference Contrast Nikon microscope at x400 in the grandparental species. The images were then were imported into MetaMorph version 14 program for analysis (MetaMorph Imaging System, Westchester, Philadelphia, U.S.A.).

For the calculation of cell dimensions, ten cells per image were randomly chosen, (subsidiary guard cells were excluded) and epidermal cell area (ECA) calculated using MetaMorph software mentioned above. Epidermal cell number (ECN) was counted directly on the field view and after that, converted to epidermal cell density per mm^2 (ECD). Total epidermal cell number (ECN) per leaf was calculated by dividing leaf area by the mean epidermal cell area.

The number of whole stomata (SN) per field of view was counted, and converted to the number of stomata per mm^2 to form stomatal density (SD). From these data, stomatal index (SI), the number of stomata as a proportion of epidermal cells expressed as a percentage, was calculated using the formula:

$$SI = (SD/(SD + ECD)) * 100$$

(Ferris and Taylor 1994, Ceulemans et al. 1995, Ferris et al. 2002).

2.8.4.2 Stomatal pore length and width in the parental species

From each of the 6 images, the length and width of the stomatal pore was measured using digital imaging software, MetaMorph Imaging System (Westchester, Philadelphia, U.S.A.) calibrated in µm. The numbers were averaged and considered as the value for the replicate.

2.8.4.3 Trichome Density and Index

From the same images used for stomatal density and cell number, the number of trichomes on the abaxial and adaxial sides of leaves of *P. trichocarpa* was counted. Trichome density (TD) was calculated as for stomatal density from the number of trichomes and area of epidermis observed. The trichomes were not counted in *P. deltoides* as none were observed on the images analysed. Trichome index (TI), the proportion of trichomes per epidermal cell, was calculated using the same formula as in the case of Stomatal Index.

2.9 Leaf growth in the grandparents, parents and the F_2 hybrid Family 331 growing in contrasting environmental conditions

2.9.1 Leaf and sample collection

The first mature leaf (at insertion level 7-9) was selected for each genotype. Cell imprints for microscopic analysis and whole leaves were taken for further analysis. All the grandparents, parents and progeny in Family 331 were studied for 30 traits of which ten were the leaf blade and petiole characteristics: individual leaf area, leaf length, leaf

width, leaf width/length ratio, dry weight, specific leaf area, leaf extension rate, petiole length, height and width. 20 cell traits, ten for each side of the leaf were also evaluated as follows: epidermal cell area; epidermal cell number; total number of epidermal cells, stomata and trichomes; cell number per leaf; stomatal number, density and index; and finally, trichome number, density and index.

Petiole length was scored on the fresh petiole to the nearest mm using a ruler, and two measurements of petiole diameter, width and height, were made using a digital micrometer. Leaf growth was assessed non-destructively by measuring leaf length to the nearest millimetre using a ruler made of graph paper, preventing damage of young leaf tissue. The youngest growing leaf was labelled and measurements made until fully expanded (Taylor et al. 2001a).

2.9.2 Leaf area and specific leaf area

One mature leaf from each genotype in each of three blocks was scanned into Corell Photopaint (Corell Inc.) and the area, length and width, were calculated by MetaMorph Imaging System (Westchester, Philadelphia, U.S.A.) Version 4.5, calibrated in μm. Leaf samples were dried for 48 hours at 85°C with petioles removed, and dry weights obtained. Each value for leaf area was divided by leaf dry weight to calculate specific area (SLA, area per unit dry weight in mm^2g^{-1}). Petiole length was scored on the fresh petiole to the nearest mm using a ruler, and two measurements of petiole; width (parallel to the leaf blade) and height (perpendicular to the leaf blade) were made using a digital micrometer. Petiole widths and heights were measured 0.5 cm from the base of the leaf blade. Leaf extension rate (LER) was measured using the first fully unfurled leaf was selected and labelled. Leaf growth rate was assessed non-destructively by measuring leaf length to the nearest millimetre using a ruler made of graph paper, this prevents damage to young leaf tissue. Measurements were repeated 2-5 days later to allow calculation of leaf extension rate (Taylor et al. 2001a, b).

2.9.3 Stomatal and epidermal counts

Impressions of adaxial and abaxial leaf epidermis were made for three replicates of each individual. Cell imprints were taken from the first mature leaf at the second intervein section, near the main vein, using nail varnish over the leaf, allowed to dry for 20 min and removed with a piece of sellotape over the dried varnish (Ferris and Taylor 1994, Ceulemans et al. 1995, Taylor et al. 2003). From every imprint, 1 image were taken with an Upright Differential Interference Contrast Nikon microscope at x200 magnification. Stomatal density (SD) and stomatal index (SI), epidermal cell number (ECN), Trichome number (TN) and trichome density (TD) were calculated as in the grandparents in Chapter 2.

Means for all the traits in the F_2 were calculated and their distribution was graphically represented

2.10 QTL analysis

All measurements of traits evaluated in the progeny of Family 331 growing in the two experimental field sites in the UK and Italy were summarized in a table of raw data, to proceed with the QTL analysis. This analysis was performed by Dr. Anne Rae, Postdoctoral research fellow working in the POPYOMICS project in the Plants and Environment Laboratory in Biology School at Southampton University.

2.10.1 QTL mapping

The genetic linkage map for this pedigree was produced by Tuskan et al. (comm.. pers. [http://www.ornl.gov/sci/ipgc/ssr_resource.htm]) consisting of 91 SSRs genotyped on 350 individuals and 92 fully informative AFLPs genotyped on 165 individuals. The primer sequence of SSR markers were blasted against the poplar genome sequence and linkage groups orientated based on the physical sequence (ie 3' to 5'). An updated map

based on this, with 637 SSRs and 533 AFLPs , has been used for the recent publication on osmotic potential traits (Tschaplinski et al. 2006).

Trait data were tested for normal distribution using the Andersson-Darling test. In cases where data were non-normally distributed Box-Cox transformations were carried out. The data were analysed for QTL using the linear regression approach (Haley and Knott 1992). The analysis was carried out using the option for outbred large single full-sib families with the software QTLExpress (Seaton et al. 2002).

One-way ANOVA were carried out for each trait to test for variation between genotypes. The error mean square (MS_E) from this was taken as environmental variance, V_E, and the genetic variance, V_G, was calculated as:

$$V_G = \frac{(MS_G - MS_E)}{r} \quad (1)$$

where, MS_G is the mean square between genotypes and r is the number of replicates. Within family broad-sense heritability was calculated by dividing the genetic variance by the total or phenotypic variance ($V_P = V_G+V_E$) for each trait (Falconer and Mackay 1996).

2.10.2 Data analysis

For the comparison between the UK and Italian sites, Two-way ANOVA were carried out using R-script for all traits scored at each site. If significant variation was seen between blocks, within the field sites, Papadakis spatial correction (Papadakis 1984), based on a 7x3 grid was used to remove small spatial effects. In cases where this was found to decrease block effects the smoothed data were used in all further analyses. Having removed block effects, one way ANOVA was used to estimate environmental variance, V_E from the error mean square (MS_E), and the genetic variance, V_G, was calculated as;

$$V_G = \frac{(MS_G - MS_E)}{r}$$

(Falconer and Mackay 1996)

where MS_G is the mean square between genotypes and r is the mean number of replicates. Individual broad sense heritability (h^2) and clonal heritability (h^2_c) were calculated for each trait at each site using

$$h^2 = \frac{V_G}{V_G + V_E} \quad \text{and} \quad h^2{}_c = \frac{V_G}{V_G + (V_E/r)} \quad \text{respectively.}$$

Positive and negative heterosis was tested for in the F_1 and F_2 generations using a one tailed t-test to compare the progeny to the highest and lowest parent respectively. Two-way ANOVA was then used to test for genotype and site interactions.

2.11 Candidate genes

Throughout the work reported here the literature has been continually reviewed in order to identify genes which could be responsible for the morphological and physiological variation being observed. After the placement of QTL in the linkage map, a number of candidate genes were selected based on our understanding of the physiology and biochemistry of the traits of interest identified in previous studies. Each candidate gene selected was searched in the Arabidopsis database (http://www.arabidopsis.org/) or NCBI Sequence viewer (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=NM_118584), and all the sequences were saved in separate files as word documents.

Every sequence of the candidate genes was blasted in the *Populus trichocarpa* database (http://genome.jgipsf.org/Poptr1/Poptr1.home.htmland) and the DB (http://www.populus.db.umu.se/) from Sweden, to select those with the highest scores. The protocol used for the selection of the best candidates genes was prepared by the PhD student Harriet Trewin (protocol prepared in Dr. Gail Taylor's laboratory, 2006).

The selection of the final candidate genes was made using the criteria of sequence alignment which assigns a score for aligning any possible pair of residues by a "substitution matrix". Only scores above 70 for every sequence blasted in the *P. trichocarpa* database were chosen discarding all those that did not reach this level.

Once the list of candidate genes was complete, ratios were calculated and PU numbers were searched in the DB database in order to find their position in the physical map of *P. trichocarpa*. QTL collocation was done with the assistance of the PhD students Harriet Trewin and Nathaniel Street.

Chapter 3

Gas exchange in *Populus* in response to drought, light and CO_2

3.1 Introduction

3.1.1 Gas exchange in *Populus*

Gas exchange studies in *Populus trichocarpa*, *P. deltoides* and their hybrids have proved that a better stomatal control occurs in *P. deltoides* while an abnormal stomatal behaviour has been found in *P. trichocarpa*. This behaviour has also been found in other plant species such as barley (Raskin and Ladyman 1988). A great variation of gas exchange and water relationship responses has been found at the intraspecific level in natural populations of *P. trichocarpa* and *P. deltoides*. For these parameters there is a high interdependency between the clone and the natural habitat in which they live (Dunlap et al. 1993, Dunlap and Stettler 2001, Rowland et al. 2001, Tschaplinski et al. 1994).

The adaptation of these two species to different environments will result in the expression of key features and help us to understand the complete function of both species. In this context, the study of the physiology of gas exchange (photosynthesis and stomatal conductance) plays a key role in the understanding of growth and water deficit tolerance.

P. trichocarpa was reported to have stomata insensitive to decreasing xylem pressure potential (XPP) while other clones did not shown this feature (Bassman and Zwier 1991). These responses indicate that biotypes in this genus may vary qualitatively and quantitatively in how they deal with water shortage (Scaragsia-Mugnoza et al. 1986). A similar behaviour has also been observed in a hybrid, *P. trichocarpa* x *koreana* 'Peace', which has stomata that do not close in response to ABA (Ridolfi et al. 1996). However, the mechanistic explanation for this type of response has not yet been found. The fact that there are clones of *P. trichocarpa* which exhibit a better stomatal control supports the idea that their stomatal response could reflect an adaptation to environmental conditions. This applies to other hybrid *Populus* clones such as 'Tristis' and 'Eugenei' which show clonal variations in the relationship between gas exchange and leaf ABA concentration (Dickmann et al. 1992, Liu and Dickmann 1992a).

In contrast to the stomatal behaviour reported for some clones of *P. trichocarpa*, *P. trichocarpa* x *P. deltoides* hybrids showed very good signalling for stomatal conductance and shoot growth originating from the root environment (Mohiuddin et al. 1997).

3.1.2 Light and carbon assimilation in *Populus*

Terrestrial plants have found a balance between CO_2 gain and H_2O loss through diffusion, which has enabled adaptation to many habitats. It is considered that stomatal limitation of photosynthesis is a common characteristic in plants and as a consequence, lower photosynthetic rates are obtained than those expected if the stomata were open as wide as possible. It has also been suggested that this trade-off has influenced characteristics of stomatal behaviour (Geber and Dawson 1997).

Studies of CO_2 exchange can prove that apart from stomatal limitation, biochemical limitation of photosynthetic carbon assimilation also occurs. From simultaneous measurements of CO_2 and water vapour exchange rates it is possible to separate effects on the light-limiting and CO_2- limiting phases of photosynthesis (Long and Hällgren 1993, Geber and Dawson 1990).

Photosynthetic CO_2 assimilation will also be affected by other factors such as light regime, leaf age, water stress and other physiological parameters (Von Caemmerer and Farquhar 1981). So it is assumed that the net rate of CO_2 assimilation reflects changes in stomatal conductance and mesophyll capacity for photosynthesis.

To calculate CO_2 exchange, the mechanistic model proposed by Farquhar and Sharkey (1982), later modified by Sharkey (1985), Harley et al. (1991) and Sharkey (1991) has been used. This model makes it possible to know to what extent species differ in their gas exchange characteristics. The Farquhar model quantifies three important components of gas exchange: 1) the activity of ribulose -1,5-bisphosphate (RuBP) carboxylase - oxygenase, 2) the rate of ribulose 1,5-bisphosphate regeneration via electron transport and 3) the rate of Triose phosphate utilization (Wullschleger 1993).

At high C_i, photosynthesis is limited principally by the leaf's ability to regenerate RuBP. Theory predicts that C_3 plants should operate at a *Ci* at which photosynthesis is co-limited with the chloroplast by RuBP consumption and regeneration (Farquhar and Sharkey 1982).

Current global warming theories predict an increased warming in the Northern Hemisphere, therefore phenology, growth, reproduction and hence distribution of plants will be affected by changing environmental factors. The response to this could affect photosynthesis and therefore would highly affect net primary production (Muraoka et al. 2002). Environmental stresses, such as drought, reduce conductance to CO_2 diffusion in the leaf mesophyll (mesophyll conductance) which is often linked to changes in leaf anatomy that can be permanent when leaf thickness is involved (Delfine et al. 1999).

Work carried out on *Populus* to determine stomatal and non-stomatal limitation of photosynthesis in elevated CO_2 and O_3 concentrations showed that the relative stomatal limitation to photosynthesis (l_s) is about 10% under control and elevated O_3. This percentage is reduced to 5% when the CO_2 increases. The results indicate that differences between *Populus* clones were primarily triggered by altered mesophyll processes. The changes in stomatal conductance seem to be a secondary response, maintaining stable C_i under the treatment given, that indicates close coupling between stomata and mesophyll (Noormets et al. 2001).

Mott and Woodrow (1993) have shown that the Rubisco activation state is responsive to C_i, therefore a higher Rubisco activity is expected under elevated CO_2 and so it can be assumed that total Rubisco activity is proportional to its content under a given CO_2 concentration. In this context, the initial response to different concentrations of CO_2 would induce stimulation of photosynthesis in a way that will allow us to know the relationship between the stomatal and biochemical limitation in the mesophyll in the two species of *Populus* studied in this experiment: *P. trichocarpa* and *P. deltoides*.

In this experiment we address the question of how the gas exchange process will be affected when two different clonal species are submitted to drought stress. It is known that as gas exchange is directly related to water supply, it will be affected in both of the species studied. However it is unknow the extent to which this effect will occur nor

which visible traits will be altered. It will also be valuable to know more about the stomatal function under these conditions. The aim of this experiment is to answer these questions.

In order to differentiate the light-saturated photosynthesis (A), and parameters from A-C_i curves in the two *Populus* species studied, Light and CO_2 responses were studied. This experiment aims to show the response of *P. trichocarpa* and *P. deltoides* to increased concentrations of CO_2 to determine the limitation this can have on photosynthesis. The response in A/C_i has two applications: 1) an alternative method of separating stomatal from mesophyll limitation and 2) separating *in vivo* carboxylation from electron transport limitation within the mesophyll (Long and Hällgren 1993). In C_3 plants the initial slope of the A/C_i curve is limited or determined by the activitation of Rubisco and saturated by RuBP, but beyond the inflection of the A/Ci response, A is constrained by the capacity of the leaf to regenerate RuBP for carboxylation.

The results obtained will be analyzed with the leaf structure data of the same species growing in contrasting environmental conditions and with the responses to conditions of water deficit. It is expected also that the knowledge of stomatal and mesophyll limitation would help in the complete understanding of how gas exchange occurs in the two grandparental species of Family 331, and how these responses could be affected by increasing concentrations of CO_2 and variations in light intensity.

3.2 Aims

This experiment had two aims: 1) To investigate the effect of water stress on gas exchange (photosynthesis and stomatal conductance) in *P. trichocarpa* and *P. deltoides* under water stress conditions, and 2) to study the responses of the parental species to light and CO_2 in order to understand the stomatal and mesophyll limitation in *P. trichocarpa* and *P. deltoides*.

The particular objectives are:

- To measure the impact of water stress conditions on photosynthesis and stomatal conductance in the two original parental species of *Populus*.

- To determine the extension of water deficit tolerance in days in environmentally controlled conditions in order to establish the limits of drought tolerance in the two *Populus* species.

- To observe dehydration signs in the two parent species that can be traced in their progeny to assist in the selection of drought tolerant clones.

- To determine the responses of the two species studied to different light gradients.

- To determine the responses of the two species studied to variable CO_2 concentration at a given irradiance.

- To link CO_2 responses to stomata, the responses in gas exchange to drought and leaf structure in the two species studied.

3.3 Materials and Methods

3.3.1 Planting

10 uniform plants in pots, each of *P. trichocarpa* and *P. deltoides* were chosen to be used in this experiment. On 1 July 2002, drought treatment was initiated. Water was completely withheld to induce acutely stressed trees (n=5), Control plants (n=5) were watered to field capacity each day. Trees were given a balanced NPK fertiliser (Osmocote, Gsotts Europe B.V., Netherlands [N 14:P 13: K 13]).

3.3.2 Gas Exchange determinations

Gas exchange measurement, photosynthetic CO_2 assimilation (A) and stomatal conductance (g_s) were assessed every other day in the morning in each species using an infrared gas analyser (Licor 6400) portable photosynthesis system, Lincoln, Nebraska (USA) and exposed to the following conditions in the chamber: PAR 300 µmol m^{-2} s^{-1}, CO_2 400 µmol mol^{-1} and constant fixed flow of 400 µmol s^{-1}. Leaf temperature was 23°C.

3.3.3 Leaf Age determination

Leaf age was determined in the same plants used for this experiment according to the LPI calculation mentioned in Chapter 2.

3.4 Results

3.4.1 Photosynthetic CO_2 assimilation

CO_2 assimilation differed between *P. trichocarpa* and *P. deltoides*. The assimilation pattern showed a leaf age dependence for each parental species. In *P. trichocarpa*, control and treated plants maintained similar assimilation rates during the experiment, although CO_2 assimilation tended to be slightly lower in the drought treatment with the largest differences at day 12 of the treatment, when, in all leaf ages, the responses of the control and treated plants were significantly different (Figure 3.1). Young and juvenile leaves in *P. trichocarpa* maintained higher photosynthetic rates than the oldest leaves.

Photosynthetic rates in the controls were very similar in the two genotypes studied. Values steadily increased in young leaves and were constant in juvenile and mature leaves in *P. trichocarpa.* The variation observed in *P. deltoides* leaves can be explained by differences in leaf maturation. The main difference between the two genotypes is that while drought caused a pronounced decrease in CO_2 assimilation in *P. deltoides*, in *P. trichocarpa* it remained more constant. For the older leaves of *P. deltoides* drought caused a statistically significant effect on assimilation as early as day 6 ($P< 0.05$ – Figure 3.1 bottom right).

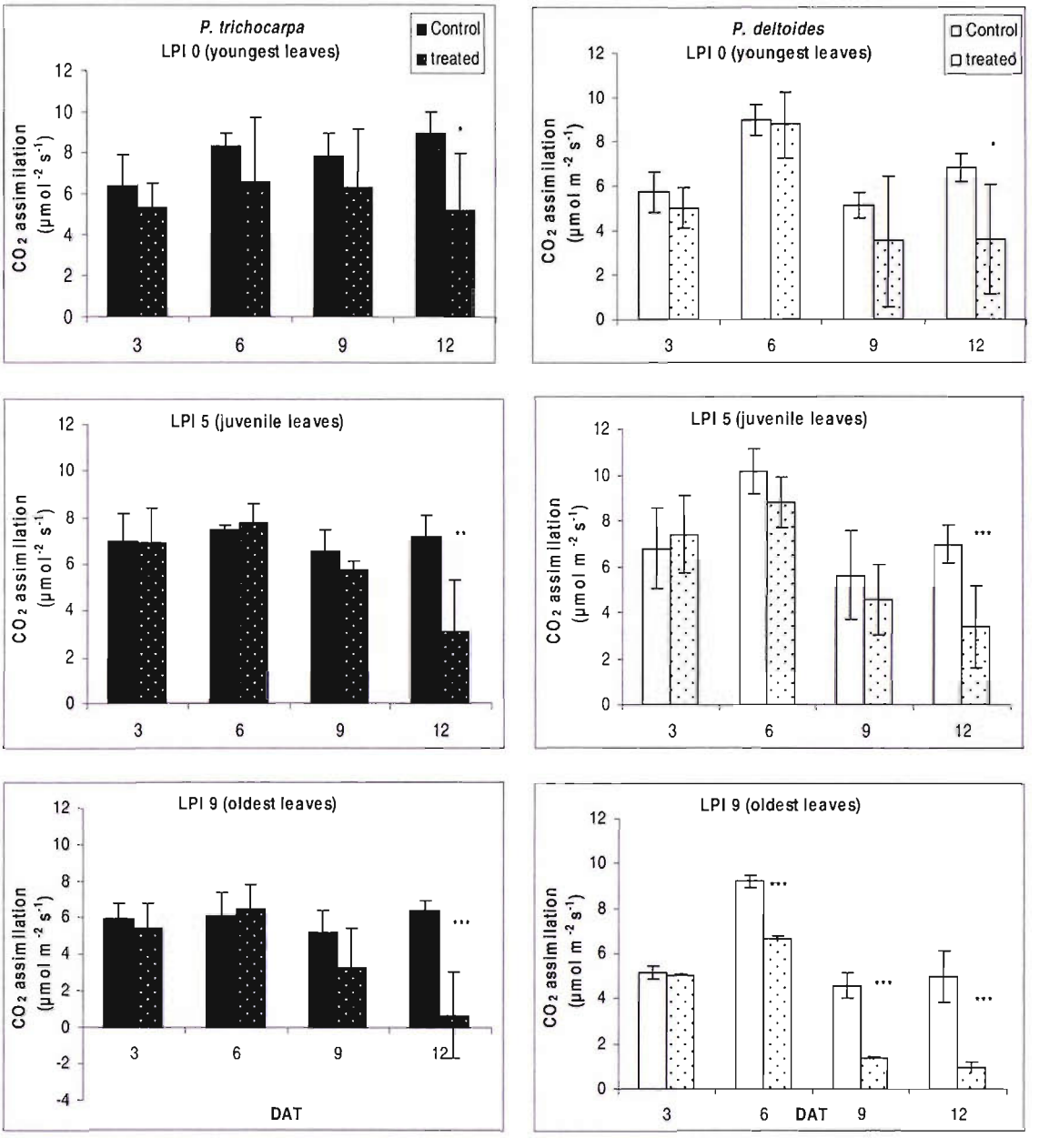


Figure 3.1 CO_2 assimilation of *P.trichocarpa* (solid bars) and *P. deltoides* (open bars) leaves of different age (contrasting LPI) at different days during the experiment. DAT = Days after starting the drought treatment. Soil water content at day 12 was 28% for the control and 15% for the treatment Results are the mean values of 5 leaves at each stage. *,**, and ***, Significant at the <0.05, <0.01, and <0.001 levels of probability. The bars indicate standard deviation.

3.4.2 Stomatal conductance

Stomatal conductance (g_s) was different for each genotype. In *P. trichocarpa* the values range from 200 to 400 mmol $m^{-2} s^{-1}$ in the control, and from 100 to 300 mmol $m^{-2} s^{-1}$ in the treated plants. In the drought treatment g_s values in LPI 9 leaves decreased to 80 mmol $m^{-2} s^{-1}$ at the end of the experiment, while g_s in leaves of LPI 0 were the first to show a response to the treatment (day 6) (Figure 3.2). In general g_s are lower and less affected by the treatment in *P. trichocarpa* than in *P. deltoides* (especially in the juvenile and oldest leaves).

Stomatal conductance in *P. deltoides* showed a marked pattern according to leaf age, with more variation in the responses. Mature leaves (LPI 9) exhibited a rapid response to water deficit (stomatal closure) decreasing rapidly to 50 (μmol $m^{-2} s^{-1}$) which was significantly different at day 3 of the treatment (Figure 3.2).

For a better representation of the photosynthetic CO_2 assimilation and stomatal conductance (g_s), Figure 3.3 shows the variation of these parameters at day 9 of the treatment. In Figure 3.3 it is possible to observe that photosynthesis shows the same variation pattern for both parental species, with regard to the same age, with less effect on photosynthesis in juvenile leaves. Larger differences occurred in mature and younger leaves, where *P. deltoides* showed a larger decrease than *P. trichocarpa*, especially in mature leaves. It is important to remark that the responses observed in young leaves in *P. trichocarpa* and in juvenile leaves in *P. deltoides* show a similar pattern.

On the other hand, stomatal conductance was more affected than photosynthesis as the decreases were more pronounced. In *P. deltoides* the effect was directly proportional to leaf age, but an opposite effect was observed in *P. trichocarpa*, where the youngest leaves were more affected than juvenile and mature leaves. The values of stomatal conductance in the youngest leaves were the most affected in this case.

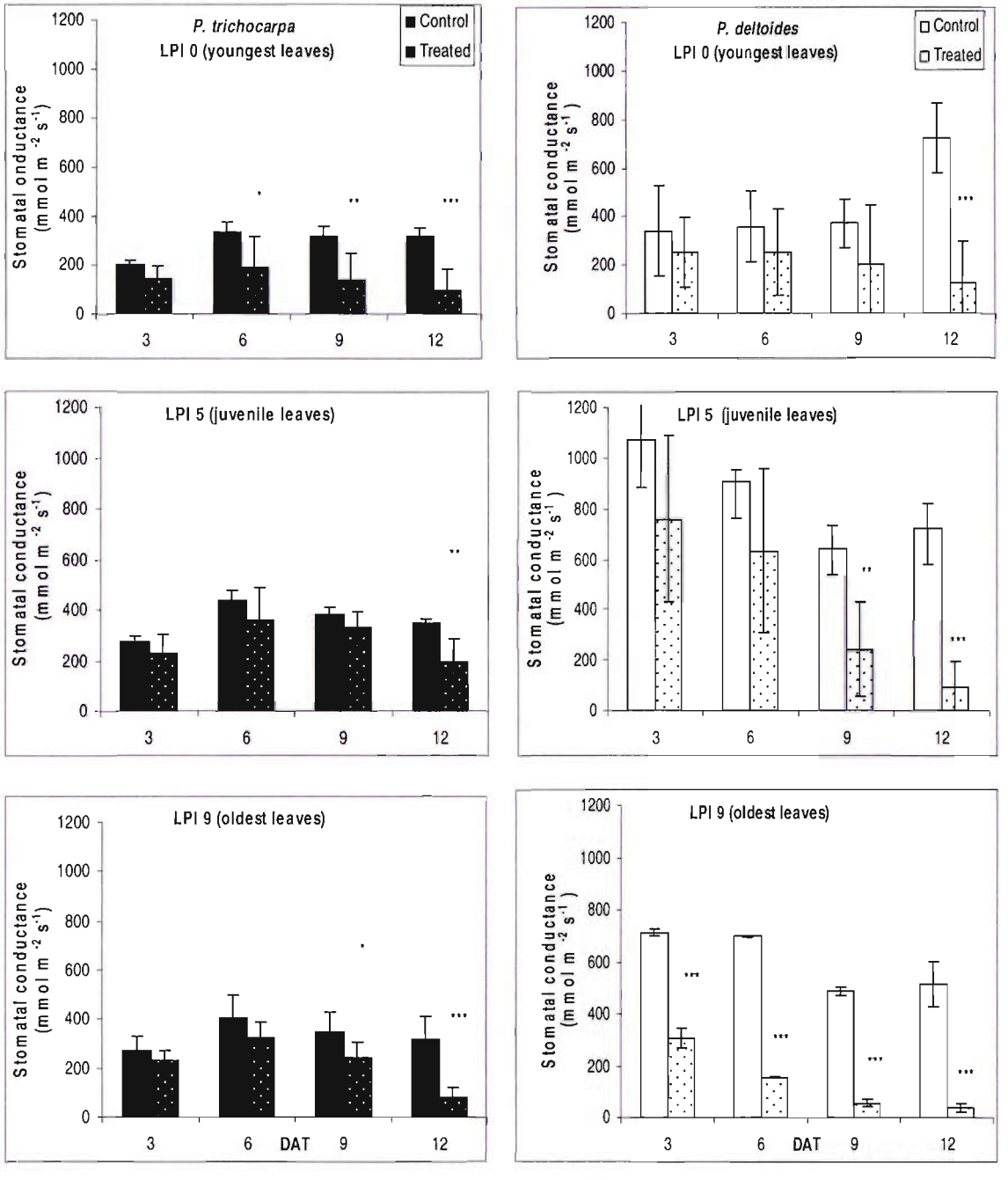


Figure 3.2 Stomatal conductance in *P. trichocarpa* (solid bars) and *P. deltoides* (open bars) leaves of different age (contrasting LPI) at different days during the experiment. DAT = Days after starting the drought treatment. Soil water content at day 12 was 28% for the control and 15% for the treatment. Results are the mean values of 5 leaves at each stage. *,**, and ***, Significant at the <0.05, <0.01, and <0.001 levels of probability. The bars indicate standard deviation.

In both species, large reduction of g_s is needed in order to cause a measurable decrease in photosynthesis. A relationship of this type can be clearly observed in LPI 0 and LPI 5 in *P. trichocarpa*, where decreases in photosynthesis of drought treated plants compared to the controls are very similar and in LPI 5 in *P. deltoides*. In these two cases, there is a large decrease in stomatal conductance, but only a slight decrease in photosynthesis. This result could means that no biochemical limitations of CO_2 assimilation occur with water content. Also, water regulation in *P. trichocarpa* occurs partially in the mature and in the youngest leaf, while in *P. deltoides* this regulation occurs in the three leaf ages (LPI 0, 5 and 9): equal to young, juvenile and mature (Figure 3.3).

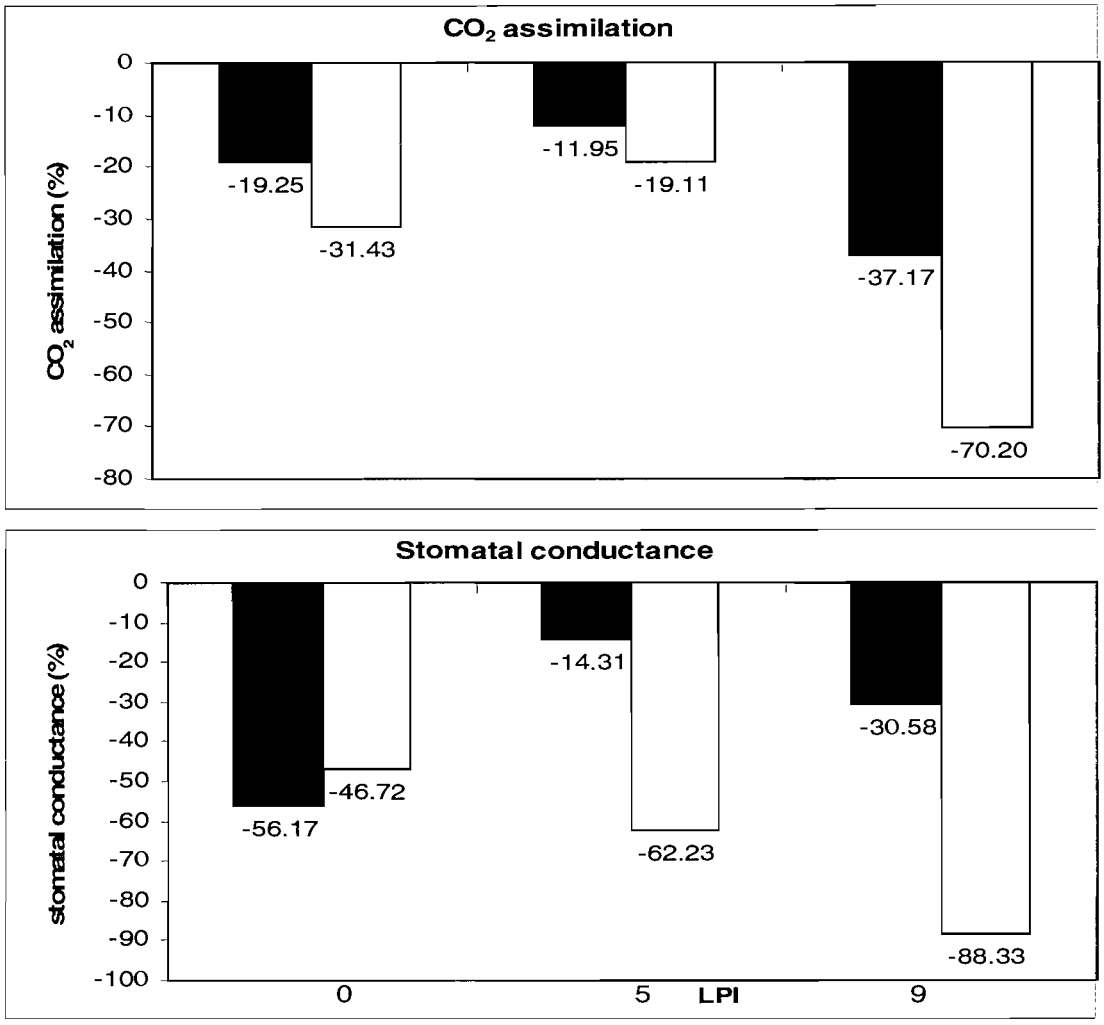


Figure 3.3 Percentage representation of CO_2 assimilation and stomatal conductance (g_s) for *P. trichocarpa* (solid bars) and *P. deltoides* (open bars) at day 9. It is shown for leaves of 3 different ages, LPI 0, 5 and 9. The statistical analysis is presented in Table 3.2 and 3.3. Day 9 (33% of water lost) can be considered as the threshold between the positive and negative effect of water deficit stress.

3.4.3 Statistical analysis

ANOVA analysis for the results of day 9 of the treatment (Figure 3.3) showed that *P. deltoides* had a larger decrease in photosynthetic CO_2 assimilation and stomatal conductance (g_s), than *P. trichocarpa*. ANOVAGLM of CO_2 assimilation showed significant effects of treatment, genotype and age (Table 3.1), no statistically significant interactions in effects were detected.

In the post-hoc analysis between genotypes, differences were shown in the youngest leaves (LPI 0) where P value was 0.011. Differences between the treatments were significant only for the oldest leaves (LPI 9) in *P. deltoides*, while in *P. trichocarpa* the treatment did not have a significant effect in all the three leaf ages.

Table 3.1 CO_2 assimilation for *P. trichocarpa* and *P. deltoides* for leaves of different age (contrasting LPI) at day 9. Values are shown in µmol m^{-2} s^{-1} for both control and droughted plants, post-hoc comparison for differences are shown. Results are the mean values of 5 leaves at each stage. *,**, and ***, Significant at the <0.05, <0.01, and <0.001 levels of probability; ns not significant. P values for significant differences between genotypes are shown where they existed.

CO_2 assimilation (µmol m^2 s^{-1})	***P. trichocarpa***		**P-value genotype**	***P. deltoides***	
	Control (mean)	**Treated (mean)**		**Control (mean)**	**Treated (mean)**
LPI 0	7.81 ± 1.144	6.30 ± 2.80 ns	0.011	5.12 ± 0.57	3.51 ± 2.418 ns
LPI 5	6.56 ± 0.901	5.78 ± 0.37 ns	ns	5.63 ±1.92	4.55 ± 1.523 ns
LPI 9	5.21 ± 1.180	3.27 ± 2.14 ns	ns	4.56 ± 0.57	1.36 ± 0.067 ***
Overall mean	6.52	5.11		5.10	3.14
Summary of GLM ANOVA	P- value				
Treatment	0.000				
Genotype	0.000				
Age	0.000				
Treatment*Genotype	0.509				
Treatment*Age	0.278				
Genotype*Age	0.219				
Treatment*Genotype *Age	8.31				

NB Genotype refers to *P. trichocarpa* and *P. deltoides* clones.

ANOVA GLM of Stomatal conductance showed significant effects of treatment, age, the interaction between treatment and genotype, and the interaction between treatment Genotype and Age (Table 3.2). Young leaves in *P. trichocarpa* are sensitive to water

deficit and respond with a decrease in g_s at day six of the treatment, while older leaves in *P. deltoides* are more sensitive. Young leaves of *P. deltoides* seem to maintain their same g_s until day twelve. In the post-hoc analysis differences were shown in the treated youngest leaves (LPI 0) and oldest leaves (LPI 9) for *P. trichocarpa*, and in the leaves LPI 5 and LPI 9 for *P. deltoides*.

Table 3.2 Stomatal conductance for *P. trichocarpa* and *P. deltoides* for leaves of different age (contrasting LPI) at day 9. Values are shown in mmol m^{-2} s^{-1} for both control and drought treated plants, post-hoc comparison for differences are showed. Results are the mean values of 5 leaves at each stage. *,**, and ***, Significant at the <0.05, <0.01, and <0.001 levels of probability; ns not significant.

Stomatal conductance (mmol m^{-2} s^{-1})	***P. trichocarpa***		***P. deltoides***	
	Control (mean)	Treated (mean)	Control (mean)	Treated (mean)
LPI 0	319.4 ± 36.0	139.9± 107.7 **	372.2 ± 100.2	198.3 ± 251.6 ns
LPI 5	385.12 ± 24.0	330 ± 63.3 ns	642.5 ± 92.8	242.62 ± 186.1 **
LPI 9	351.5 ± 77.0	244 ± 63.2 *	487.5 ± 18.0	56.88 ± 15.02 ***
Overall mean	352.0	237.9	500.7	165.9
Summary of GLM ANOVA	P-value			
Treatment	0.000			
Genotype	0.183			
Age	0.000			
Treatment*Genotype	0.000			
Treatment*Age	0.418			
Genotype*Age	0.266			
Treatment*Genotype *Age	0.025			

NB Genotype refers to *P. trichocarpa* and *P. deltoides* clones

3.4.4 Limits of drought tolerance

Populus trichocarpa and *P. deltoides* have a different response to water deficit with *P. trichocarpa* showing adverse effects of drought more quickly. In *P. trichocarpa* the signs of dehydration were clearly visible at day 9 of the treatment, when small brown spots appear on the adaxial surface, which are later manifested as small black spots on the abaxial surface (Figure 3.4 A). Leaf abscission started at day 10 and by day 11 it was severe in four of the five replicates. At day 12 all the leaves with the exception of the upper ones, exhibited signs of wilting and the lower ones falled (Table 3.3).

In contrast, signs of dehydration in *P. deltoides* were observed after 12 days of the treatment and appeared as a normal contraction of the leaves accompanied by loss of turgor (Figure 3.4 B) and leaf abscission started at day 15. At day 15 of the treatment visible signs of wilting were obvious in the basal leaves but not to the same degree as *P. trichocarpa* (Table 3.3). Control plants continued growing (3.4C).

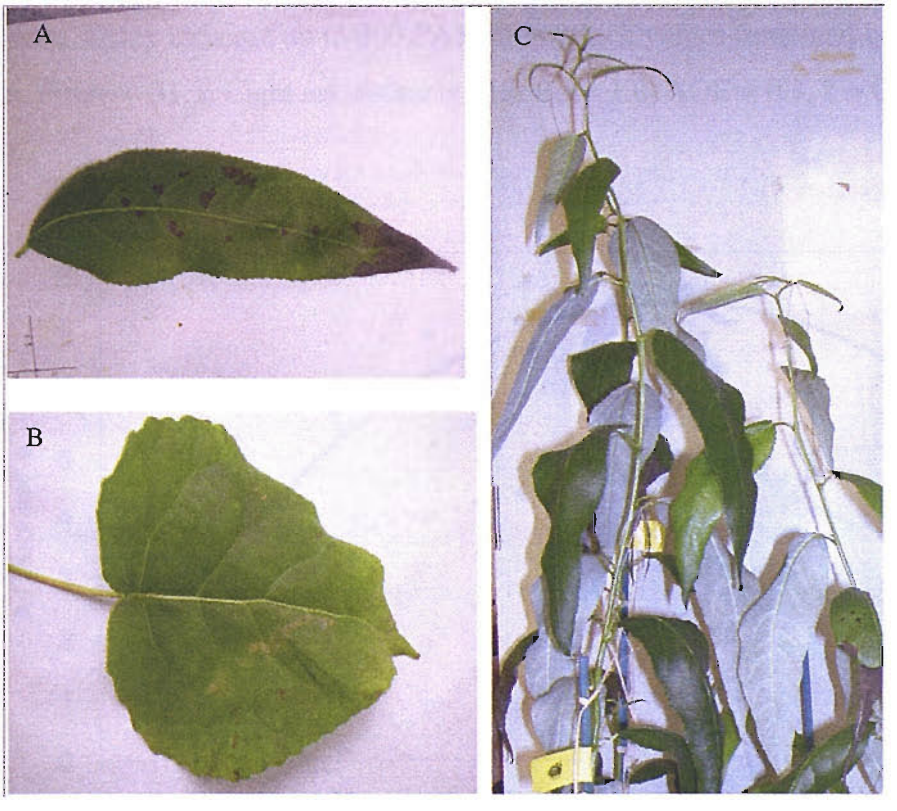


Figure 3.4 Leaf features in dry and control conditions. A) *P. trichocarpa* LPI 5 (left) at day 9 of the treatment, B) *P. deltoides* LPI 5 (right) leaves at day 12 of the treatment and C) *P. trichocarpa* control at the end of the experiment.

Table 3.3 Observable signs of drought in *P. trichocarpa* and *P. deltoides* during acute water deficit treatment. The numbers indicate the day after the treatment when the sign was observed.

Signs	***P. trichocarpa***	***P. deltoides***
Dehydration	9	12
Leaf abscission	10-11	15
Leaf shedding at day 15	Severe in 4 plants	Slight in 1 plant
Wilting	11	15+

3.4.5 Light response

The response to different light irradiances was different in the two genotypes studied. *P. trichocarpa* showed a higher photosynthetic assimilation (Amax = 6.29), than *P. deltoides* (Amax = 4.63) In *P. trichocarpa*, A (assimilation) increases at low irradiance up to 400 PAR, but decreases at higher levels of irradiance (Light com. Point = -15.4, Light sat. estimate 214, Res= 0.421, AQE = 0.0274, k = 0.993,). In *P. deltoides*, A shows a steady increase up to 800 PAR, after which values remained constant (Light com. Point = -31.3, Light sat. estimate 113, Res= 1.0, AQE = 0.3, k = 0.0058). (Figure 3.5).

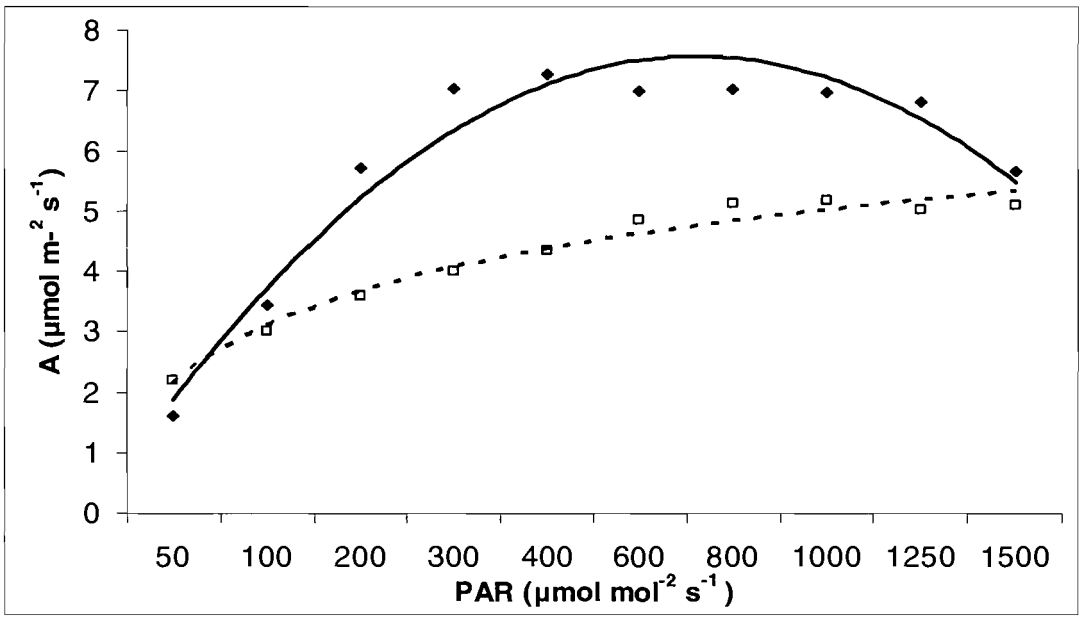


Figure 3.5 Light response in two *Populus* species: *P. trichocarpa* (♦) and *P. deltoides* (▪).The values of the mean are based on three replicates. PAR units are µmol m^{-2} s^{-1}. Trend lines are shown in each case.

3.4.6 A/C_i response

The response to increasing CO_2 concentrations in the two genotypes was different. *P. trichocarpa* exhibited a fast and higher response to lower concentrations of *Ci*. A values of 8 µmol m^{-2} s^{-1} were reached at 40 Pa of C_i in *P. trichocarpa*, while these A values are only reached at *Ci* concentration over 60 Pa in *P. deltoides* (Fig 3.6 and Fig. 3.7).

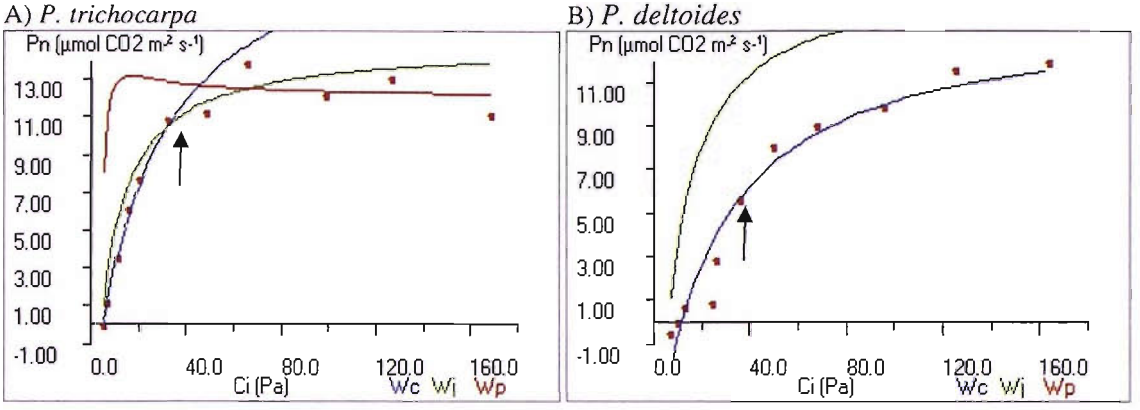


Figure 3.6 A/*Ci* response in *P. trichocarpa* (left) and *P. deltoides* (right). Graphs are presented as they are produced in Photosyn Assistant. Values reached in both cases at 40 Pa of *Ci* are indicated by the arrow. The mean values of the curve were obtained at 600 PAR (µmol m^{-2} s^{-1}) in three replicates. Trend lines for Wc= Rubisco-limited rate of carboxylation, Wi= electron transport limited rate of regeneration of RuBP, and Wp= Inorganic phosphate limited rate of regeneration of RuBP. A Pascal = Kg m^{-1} s^{2}.

The results show that a maximum response is reached in *P. trichocarpa* but this starts to decrease at 100 Pa (1000 µmol^{-1} of *Ci*). On the contrary, the response of *P. deltoides* continues steadily increasing at much higher concentrations of 120-160 Pa of *Ci* (1200 and 1600 µmol mol^{-1}). The maximum rate of CO_2 assimilation (13.78 µmol m^{-2} s^{-1}) was reached at 66 Pa (663 µmol m^{-2} mol^{-1} of *Ci*) in *P. trichocarpa* while in *P. deltoides* (11.56 and 11.91 µmol m^{-2} s^{-1}) it was reached above 120 Pa (1250-1640 µmol mol^{-1}).

3.4.7 Separation of stomatal and mesophyll limitations

3.4.7.1 Stomatal limitations

Stomatal limitation was calculated from the A/Ci curve show in Figure 3.7, using the formule $l=(A_o-A)/A_o$ given by (Farqhuar and Sharkey 1982, Hall et al. 1993). In it is possible to see the CO_2 evolution.

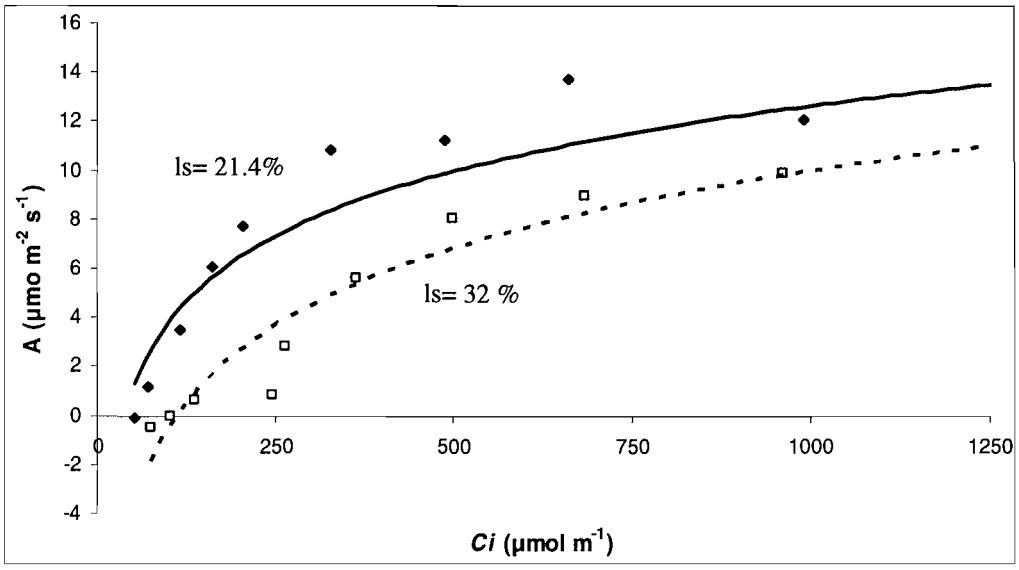


Figure 3.7 A/Ci curve for stomatal limitation (ls) calculation in two *Populus* genotypes. *P. trichocarpa* (♦) and *P. deltoides* (□). Trend lines are shown in each case.

The ANOVA analysis showed that the difference between the responses of the two species was highly significant ($P < 0.5$ and $P < 0.01$) until the values obtained at 1000, 1250 and 1600 µmol mol^{-1} where the differences are not significant (Table 3.4 and Figure 3.8).

Table 3.4 ANOVA results of the values obtained in a A/*Ci* response in two *Populus* species.

CO_2 Reference	A (µmol $m^{-2} s^{-1}$) *P. trichocarpa*	se ±	A (µmol $m^{-2} s^{-1}$) *P. deltoides*	se ±	P
50	-0.09458	0.086	-0.4958	0.089	0.12*
100	1.146	0.139	-0.04118	0.092	0.000**
199	3.48	0.577	0.64604	0.346	0.001**
299	6.096	0.980	0.85025	0.198	0.001**
400	7.69	0.926	2.81	0.348	0.001**
600	10.866	0.812	5.612	0.689	0.001**
800	11.242	0.819	8.022	0.382	0.007*
1000	13.78	0.964	8.976	0.263	0.001**
1299	12.125	0.570	9.878	0.826	0.056 ns
1600	12.98	0.733	11.566	0.934	0.268 ns
2000	11.14	0.320	11.918	1.117	0.522 ns

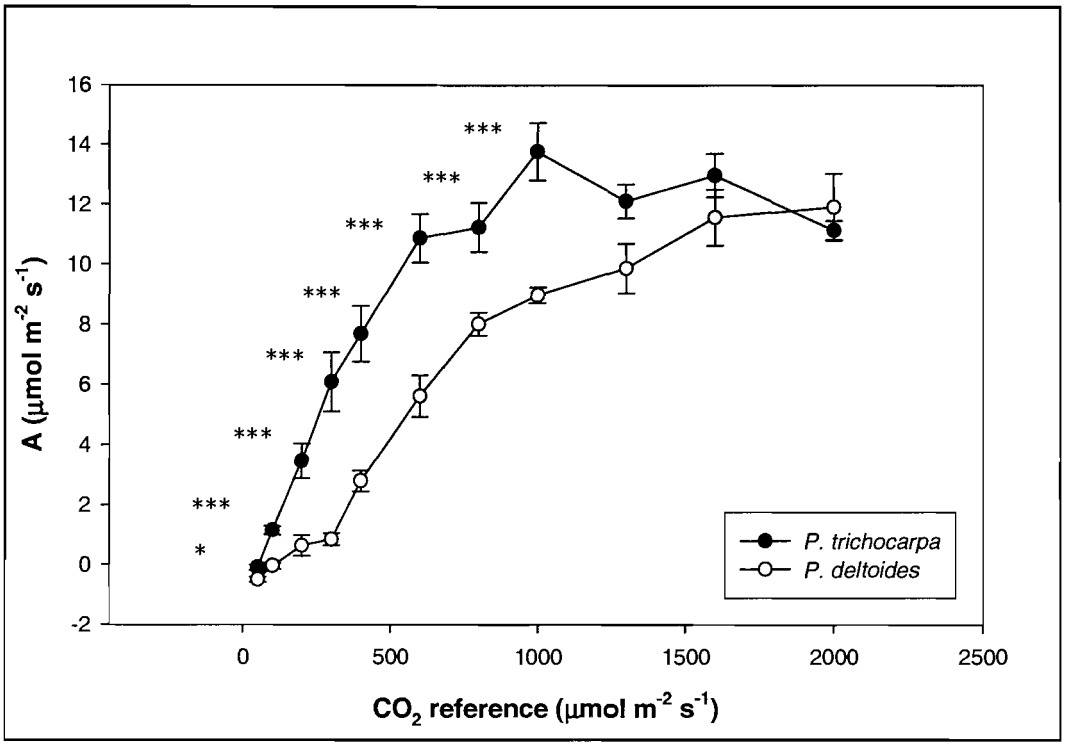


Figure 3.8 Carbon assimilation in two *Populus* species to different CO_2 concentrations. Values are the mean of 5 values with standard error. Significantly different data points analyzed by one-way ANOVA are indicated: *= P <0.5, ** = P<0.1 and *** P = <0.01 levels of probability.

3.4.7.2 Empirical and mechanistical A/*Ci* curves analysis

The response of assimilation (Amax) was determined by the internal concentration of CO_2 in the leaf (*Ci*). Curve analysis showed that Respiration (Resp), Carboxylation efficiency (CE) and CO_2 assimilation (A) were higher in *P. trichocarpa* than the values obtained for *P. deltoides* (Table 3.5). The values obtained show a high response in *P. trichocarpa* at low CO_2 concentrations, which can be observed in the initial response in the curve.

Table 3.5 Empirical A/*Ci* curve analysis for two *Populus* species

Parameter	*P. trichocarpa* ($\mu mol\ m^{-2}\ s^{-1}$)	*P. deltoides* ($\mu mol\ m^{-2}\ s^{-1}$)
Resp (Respiration)	-12.84	-3.365
CE (Carboxylation efficiency)	4.325	0.3480
CO_2 assimilation (Amax)	26.90	22.15

In the mechanistical approach, the estimated values of four parameters describing some photosynthetic characteristics of leaves are summarized in Table 3.6. The results showed a lower dark respiration (Resp) and maximum velocity of carboxylation (Vc_{max}) in *P. trichocarpa*. However, the PAR saturated rate of electron transport (J_{max}) and the rate of Triose phosphate utilisation (TPU) are higher in *P. deltoides*. The values for Rubisco activity (Wc) were higher for the latter species (Figure 3.6).

These results indicate that *P. trichocarpa* has a lower compensation point with respiration than *P. deltoides*, and a higher rate of Carboxylation efficiency that will be limited when the levels of CO_2 increase. However *P. deltoides* exhibits a higher energy input, as it values for Jmax and TPU indicates (Table 3.6).

Table 3.6 Mechanistic A/*Ci* values for two *Populus* species

Parameter	Estimated value *P. trichocarpa*	sd	Estimated value *P. deltoides*	sd
Resp	1.24	2.32E-01	4.23	8.85E-01
Vc_{max}	23.2	7.80E-02	18.5	1.54E+00
J_{max}	71.8	4.24E-02	96.8	-
TPU	4.43	9.10E-03	8.93	-

3.5 Discussion

3.5.1 Photosynthetic CO_2 assimilation

Photosynthetic carbon assimilation rates for *P. trichocarpa* and *P. deltoides* were 6-10 μmol m^{-2} s^{-1} in the control. At day 9, the differences between genotypes in response to drought were not statistically significant (Table 3.1), except in young leaves. The rates obtained in these two genotypes at the level of irradiance used in the experiment are lower than those obtained for *P. trichocarpa* (13.2 μmol m^{-2} s^{-1}) by Bassman and Zwier (1991) and Ceulemans and Impens (1980) but higher than for other clones of *P. deltoides* (5.0-7.6 μmol m^{-2} s^{-1}) reported by Dickmann (1971). However, even though this rate is higher in *P. trichocarpa* than in *P. deltoides* the differences were not significant between the controls of the two species. This could be due to the low irradiance used in this experiment, 300 μmol m^{-2} s^{-1}, which corresponds to a normal day in England in late summer. The decrease in assimilation in the droughted plants is considered a normal response when plants are exposed to stressful conditions, in this case drought (Tschaplinski and Blake 1989b). Variation in net photosynthesis has been found in several clones of *P. trichocarpa* from mesic and xeric sites and lower or higher elevations in The Cascade Mountains (Dunlap et al. 1993), and in *P. deltoides* growing in New Mexico (Rowland et al. 2001).

Photosynthetic assimilation response to water deficit was totally dependant on leaf age. The main difference between the species is a major decrease of assimilation during water deficit in *P. deltoides*, which occurs first in the older leaves at day 6. *P. trichocarpa* showed less reduction in photosynthetic assimilation than *P. deltoides*. Leaf age dependence has also been reported in *P. deltoides*, *P. trichocarpa* and some of their hybrids, showing large differences in photosynthesis with age, especially in older leaves (Bassman and Zwier 1991, Ridolfi et al. 1996, Tschaplinski and Blake 1989b), however these authors do not describe the photosynthetic and water use efficiency response of the youngest leaves in *P. trichocarpa*.

3.5.2 Stomatal conductance

The most remarkable differences between the two species of *Populus* studied here were those of stomatal conductance (g_s). *P. trichocarpa* exhibited a partial stomatal control during the experiment and at day 9 it showed for all the leaves a mean g_s value of 237.99 mmol m^{-2} s^{-1}, while *P. deltoides* was more sensitive (mean 165.93 mmol m^{-2} s^{-1}) to water deficit (Table 3.2). This behaviour in *P. trichocarpa* has been reported previously by several authors (Schulte et al. 1987, Ceulemans et al. 1988, Bassman and Zwier 1991). These results also show that after 12 days of drought, the differences between the two species were significantly different.

Our results show an age dependant relationship for both species, with a slightly different response pattern. In *P. trichocarpa* the youngest leaves were the most sensitive to water deficit showing partial stomatal closure, while in *P. deltoides* these leaves are the last to show significant effects in stomatal conductance (stomatal closure). In both species, juvenile leaves (LPI 5) have a more conservative response to water deficit, as they maintain higher values of photosynthetic CO_2 assimilation and stomatal conductance and better WUE (water use efficiency). Juvenile leaves in both clones are the least affected in growth as the photosynthetic rates were the least affected.

The results obtained confirm the age dependant pattern of response to drought which has been reported by other authors and reaffirms the lack of stomatal control in mature and juvenile leaves in *P. trichocarpa*. However in the three leaf ages it was possible to observe a partial reduction, a response that could explain the result obtained by Schulte et al. (1987) who reported that preconditioning developing leaves by water stress gives a greater stomatal conductance in foliage following rewatering. The leaf conductance of young leaves fell more rapidly and to significantly lower values than were observed in old leaves. This difference between the recently expanded leaves and older leaves could be explained by an incomplete development in the former as well as the flexibility of their cell walls, and also because of the maintenance of a higher hydraulic potential resulting from the sacrifice of branches. Cochard et al. (1996) tried to explain the abnormal stomatal behaviour in *P. trichocarpa* x *koreana* 'Peace' after adding exogenous ABA and $CaCl_2$. They showed that this exogenous application did not

produce responses to ABA (Stomatal closure) and only a slight response to darkness was obtained.

3.5.3 Limits of drought tolerance

Limits of the water deficit period could be established in 9 days for *P. trichocarpa* (33% water lost); after this point any further damage would affect plant growth. This means that lack of water for a longer period will cause leaf abscission and therefore photosynthesis will decrease as the leaf area will be considerably reduced. Even though the plants do not die, growth will be slowed to a minimum.

For *P. deltoides*, the results on day 9 show photosynthetic rates slightly different to those in the control but the stomatal conductance is lower in the two genotypes, and the leaf age differences are maintained. However, the extension of water deficit for a period of four days does not cause significant damage or leaf abscission in this species and therefore 12 days (15% soil humidity) could be chosen as the limit to drought tolerance. It would be interesting to compare biomass production at this time in the two species. An important result was that in both species, young leaves are the least affected by water deficit as they remain turgid and with a good appearance and similar g_s, but *P. trichocarpa* has higher photosynthesis which would allow young leaves to continue growing more than those of *P. deltoides*.

3.5.4 Light and CO_2 response

In the study of growth in shaded conditions, assimilation rates were higher in *P. trichocarpa* than in *P. deltoides*. The results also indicate a poor adaptation of *P. deltoides* to shade conditions, as shown by the poor growth observed. *P. trichocarpa* reached highest A values at lower light intensities than *P. deltoides*, which occurs at 400-600 PAR. *P. deltoides* showed the highest assimilation rate at higher irradiances than *P. trichocarpa*, which occurred in an steady increase up to 800-1000 PAR (Figure

3.5). These results suggest that the performance of *P. trichocarpa* is better than that of *P. deltoides* at low light intensities.

The analysis of A/C_i shows that *P. trichocarpa* has very efficient carboxylation and a low dark respiration rate which indicates an advantage for this species because at atmospheric levels this CO_2 assimilation is higher than in *P. deltoides* (Figure 3.6). This fact is in part a consequence of the lower (21.4%) stomatal limitation (Ls) or stomatal resistance that *P. trichocarpa* has (Figure 3.7). In contrast, *P. deltoides* showed a higher (32%) stomatal limitation (Ls) and requires higher input of energy efficiency for electron transport and Triose phosphate conversion which could increase the efficiency of photosynthesis in the mesophyll. These results were confirmed with the analysis of the values derived from the Empirical and Mechanistical A/*Ci* curve analysis.

The results obtained could explain that the highest rates of photosynthesis in *P. trichocarpa* in the youngest and juvenile leaves occur because of a higher carboxylation activity in comparison to its mature leaves.

3.5.5 Stomatal limitation

The results of this experiment show that there is a coordination between mesophyll and stomatal processes as mentioned by several authors (Noormets et al. 2001, Allen and Pearcy 2000). As mentioned by Fiscus et al. (1997), decreased stomatal conductance could be the result and not the cause of decreased photosynthesis, as can be observed in *P. trichocarpa.*

The lower dark respiration observed in *P. trichocarpa* is explainable by a higher Rubisco concentration at dawn, possibly because of night conductance, as reported for Snyder et al. 2003, several species of *Populus* including other clones of *P. trichocarpa.* Special attention should be given to the fact that some species of *Populus* had significant night-time conductance (g_s) and transpiration (E). It has been proved that *P. trichocarpa* night- time transpirations is much higher than in *P. tremuloides* (Snyder et al 2003). This suggests that the control of night-time g and E is more complicated than initially predicted based on habitat or photosynthetic pathway (C_3, C_4 or CAM). Night-

time stomatal opening appears to be a potentially widespread phenomenon in plants, which leads to questions about the relative costs and/or benefits of nighttime water loss. The benefit would be improved nutrient acquisition and a recovery of hydraulic conductance from partial xylem cavitation events that occur during more stressful daytime conditions (Brown and Hettersley 1989, Snyder et al. 2003). However there is still not a complete explanation for this nightime conductance, even though it has several implications for C_3 and C_4 plants (Rawsthorne et al. 1988).

3.6 Conclusions

These studies showed three main results. The first is the ability of the youngest leaves of *P. trichocarpa* to maintain higher photosynthetic rates than those of *P. deltoides* during the period of study, while the differences in juvenile and mature leaves were not significant. Photosynthetic rates of *P. deltoides* were only strongly affected after 9 days of severe drought, while *P. trichocarpa* showed a partial stomatal response to water deficit which was more accentuated in the youngest leaves. *P. deltoides* showed better stomatal control during soil drying, which could be an indication of a better adaptation to drought.

The most relevant findings in this experiment shed light on the type of response to drought in *Populus*. Certainly the gas exchange process was affected in an unexpected way. Firstly, the responses show a leaf age pattern in both species that show different responses to drought. Photosynthetic CO_2 assimilation and stomatal conductance in *Populus deltoides* will vary in an acropetal direction, while in *P. trichocarpa* this response will show a partial basipetal response. Secondly, the responses found in young leaves of *P. trichocarpa* and juvenile leaves of *P. deltoides* could reflect differences in maturity, as the common denominator is reduction in stomatal conductance but high photosynthesis. This could be explained by a complete development of structures and processes related with the biochemical photosynthetic apparatus and not only because of modification in gas exchange. The particular stomatal responses measured in *P. trichocarpa* seem to indicate a response to water potential and loss of turgor in the youngest leaves, which decreases with age.

Finally, necrosis in mature leaves and leaf abscission were the visible signs of drought in *P. trichocarpa* that occur mainly in mature leaves, reflecting the adaptation of the parental species to their native habitat, the riparian communities. The decrease in photosynthetic rate as the leaf matures, gives an extra source of metabolites to the plant to continue living during periods of reduced water supply, that occur during the different seasons. *P. deltoides* in contrast, shows a different pattern in its adaptation to more mesic habitats where the water supply will be reduced for longer periods and the strategy to take up water is observed in the root growth type.

An important consideration is that this study was conducted in a relatively low light intensity (lamp emission 400 PAR), and that different results could be found if this study was done in higher light environments. Understanding of how these species respond to light and CO_2 provides insight into the biochemistry of photosynthesis in the clones studied and might explain the differences found between them.

Undoubtedly the genotypes of *P. trichocarpa* and *P. deltoides* studied here, showed contrasting responses to light and CO_2 that are complementary. Thus *P. trichocarpa* needs less light and less CO_2 to reach saturation of A levels, while *P. deltoides* reach saturation levels at lower values than expected, but needs more CO_2 to reach its maximum assimilation (A_{max}). This experiment also gave information about the changes that the genotypes studied can have in different environmental conditions such as light and elevated CO_2 levels. The last statement is supported by the fact that the irradiance used in the CO_2 response was conservative (600 μmol m^{-2} s^{-1}) and that the plants used were already adapted to shade.

The differences found between the two species could be explained in part by their leaf structure and physiology. To support these findings, a complete analysis of the leaf structure in the parents was needed and the information produced by this will help to clarify the results obtained so far (Topic in Chapter 5).

Chapter 4

Stomatal function in the grandparents of Family 331: *P. trichocarpa* and *P. deltoides*

4.1 Introduction

Stomata are one of the most important structures that allowed the adaptation of aquatic plants to land and have been recorded from as long as 400 million years ago (Edwards et al. 1998). They have a key role in the process of gas exchange, by which plants take in CO_2 and lose water vapour through transpiration.

When plants absorb CO_2, they lose water, resulting in leaf cooling that enables the plant to maintain suitable temperatures for photosynthesis. To avoid leaf dehydration, the loss is controlled by the cuticle and stomata. Measurements of the stomatal aperture or the resistance to CO_2 and water-vapour (H_2O) transfer between the atmosphere and the internal tissue of the leaf are important for biomass production. Measurements of CO_2 taken up and H_2O lost are often expressed as water-use efficiency (WUE), defined as the instantaneous water use mass of CO_2 assimilated (or dry weight gained) per unit mass of water transpired (Beadle et al. 1993).

Water relations have been widely studied in *Populus*, in which high stomatal conductance compared to many other species gives high rates of net photosynthesis and therefore higher biomass production. However, in *Populus* higher leaf photosynthetic rates do not necessarily give higher biomass (Scarascia-Mugnozza, et al. 1986). Several genotypes of *P. trichocarpa* have been shown to produce a total dry weight up to 18-18.4 $Mgha^{-1}$ $year^{-1}$ with Pn of 14.1-15.7 $\mu mol\ m^{-2}\ s^{-1}$ while hybrid clones of *P. trichocarpa* x *P. deltoides* have yields surpassing the best of the parents, and producing 27.5 $Mgha^{-1}$ $year^{-1}$ with Pn 13.9 $\mu mol\ m^{-2}\ s^{-1}$. These Pn rates are closer to those from *P. deltoides* than for *P. trichocarpa* (Schulte et al. 1987), however it depends of the leaf age.

Scarascia-Mugnozza, et al. (1986) present data over 4 years available for five of the six *Populus* clones. High productivites seemed to be associated with high rates of net photosynthesis, high leaf conductances, and high water potential. However the maximum rate of net photosynthesis for the fastest-growing hybrid, was intermediate between the best and the worst of the *P. trichocarpa* clones, proving that superior growth need not be linked to high CO_2 exchange rates, but may be derived from a

higher leaf area index, a longer leaf area duration or from a different photosynthate-allocation pattern (Dickmann 1979). Ceulemans and Impens (1983) observed a significant relationship between net CO_2 exchange and shoot/root growth of 18 young poplar clones. The reduction of stomatal conductance in *P. trichocarpa* and related clones was by as much as 80% of maximum values, and their decreases in photosynthesis resulted from changes in the residual conductance to CO_2 and not from a decrease in stomatal conductance. In *P. deltoides* the reduction of leaf conductance and net photosynthesis rate was 40% (Regehr et al. 1975).

A great part of the success in productivity in the hybrid *P. trichocarpa* x *P. deltoides* is their contrasting stomatal response to water deficit. While *P. deltoides* stomata present responses which are as expected of most typical angiosperms (Ticha 1982), *P. trichocarpa* stomata have abnormal behaviour, showing no response to water deficit.

Extensive work on stomata in clones of *Populus trichocarpa* by Schulte and Hinckley (1987), showed that water vapour conductance in the leaf does not decrease with loss of turgor, and also that stomatal response increases after a period of stress preconditioning. The reason given for this is that stomatal activity appears to be more highly correlated with the water relations of the epidermis than with those of the bulk leaf, especially under conditions associated with rapid changes in tissue potential such as changes in relative humidity. Their results reflect that the osmotic potentials of *P. trichocarpa* guard cells are lower than those of *P. deltoides* and hybrid clone guard cells. In complete plants subjected to stress, apertures were reduced as the epidermal cells decreased their water potential, linking this response to relationships between the epidermal and guard cell, as was also reported in wilty mutant potato. They concluded that the lack of closure of *P. trichocarpa* stomata seemed to result from physical characteristics of the guard cell walls or from the physical relationship between guard cells and other epidermal cells. A lower osmotic potential would mean that a positive turgor pressure is maintained at lower soil water potentials. An epidermal layer having an especially low hydraulic conductivity to the mesophyll tissues would have lower water potentials relative to an epidermis that is closely linked to the leaf mesophyll. They also proved that ABA application to epidermal strips caused different degrees of stomatal closing in leaves of different ages, showing that stomata in younger leaves

close partially with ABA application. They found ABA in concentrations of 0.5-1.0 μg/g fresh weight (Schulte and Hinckley, 1987).

The abnormal behaviour in *P. trichocarpa* stomata has been reported in another hybrid, *P. koreana* x *P. trichocarpa* 'Peace', which has similar behaviour in stomatal regulation, responses to ABA and light (Ridolfi et al. 1996, Tang and Liang 2000), suggesting that this abnormal response could be more widespread in the genus *Populus*, especially in members of the Tacahamaca section, and therefore related to the natural selective adaptation of the section or it could have been inherited from *P. trichocarpa.*

The results obtained by Ridolfi et al. (1996) proved the complete insensitivity to ABA of mature leaves of intact 'Peace' plants agreeing with the results obtained for *P. trichocarpa* by Schulte et al. (1987). They also mentioned the partial stomatal closure at night in well watered poplars, as reported by Reich (1984), Schulte et al. (1987), Ceulemans et al. (1995, 1998) and Furukawa et al. (1990).

The study of stomatal function in these genotypes is valuable because of its strong relationship with the gas exchange process and therefore with high biomass production. The relationship between stomatal function and high productivity may be especially important in temperate forest systems. Even though poplars hybrids have superior biomass production than their parents, this cannot be sustained in all the progeny when these hybrids are back-crossed to produce the F_2 (as Family 331). An understanding of the way in which stomatal function is related to high productivity could be of real value in the selection of parameters for use in breeding programs focussed to extend the culture of *Populus* in conditions of intermittent water supply.

This study aims to give a complete picture of the dynamics of stomatal function in the two grandparental genotypes *P. trichocarpa* and *P. deltoides* in normal conditions taking into consideration the developmental changes in the leaves and the fluctuation of these changes during a normal day during the growing season. The knowledge of the normal patterns occurring in the plants of these genotypes will give a complete picture of the changes in stomatal behaviour that are part of the developmental and maturation process in the plants. This work will also provide a good starting point for comparison when water stress treatments are applied to the the plants. Part of the disagreement in

the results regarding the explanation of the lack of stomatal response in *P. trichocarpa* results from the variation in leaf age of leaves, climatological conditions and duration of the experiments.

The hypotheses here are 1) That stomatal function in *P. deltoides* and *P. trichocarpa* is in complete agreement with ontogenetic changes during leaf development and therefore leaf age. 2) That stomata in *P. trichocarpa* are totally insensitive to ABA and that the partial response reported in young leaves is related to epidermal cell properties in contrast to *P. deltoides* that responds to water deficit with mechanical and hormonal mechanisms.

4.2 Aims

This chapter is devoted to the understanding of dynamics in the stomatal responses of the grandparental genotypes of Family 331 and its relations with epidermal cell characteristics.

The first aim is to characterize the contrasting responses in the grandparental genotypes by examining:

- The dynamics of stomatal conductance during the day in conditions of natural sun irradiance in the grandparental genotypes.

- The patterns in stomatal conductance, solute content and cell extensibility through a profile in mature plants of the grandparents.

- The extent of the response to hormone stress (ABA) according to leaf age and its implications in photosynthesis.

- The dynamics of the response of conductance in juvenile and mature leaves to ABA and dehydration using thermal-imaging analysis

The second aim is to study stomatal conductance in all the grandparents, parents and hybrid Family 331 in order to find possible markers in the *Populus* genome as a starting point in the investigation of the genetic basis of drought tolerance and biomass production. The particulars objectives are:

- To study stomatal conductance in 180 genotypes of Family 331 growing under field conditions.

- To study stomatal conductance in selected extreme genotypes, and to use this to map QTL.

4.3 Methods

4.3.1 Stomatal function

In order to characterize the stomatal properties and responses to chemical and physical stress in the two grandparental genotypes, four approaches were used. The first was to study the dynamics of stomatal conductance at contrasting times (midday and midnight). This was carried out with the use of the porometer 1600 (Licor).

The second was to find the response patterns of four leaf properties related with stomatal function: stomatal conductance, cell sap osmolality, cell wall extensibility (plasticity and cell elasticity). These properties were determined by the use of the porometer 1600, osmometer and Instron. The third approach was to investigate the responses to stress hormone and acute dehydration of leaf temperature and relative conductance using thermal-imaging. The fourth approach in this chapter was to investigate stomatal conductance in all the members of hybrid Family 331 in field conditions and in a selected sample of genotypes and to use the results for QTL mapping.

4.3.2 QTL mapping in planting at Headley, UK

QTL mapping was under the responsibility of Dr. Anne Rae, using the procedure described in Chapter 2. Stomatal conductance was scored for three replicates in the parents, F_1 and 225 F_2 individuals. Two-way ANOVA showed that there was significant variation between the blocks, genotypes and a significant block x genotype interaction. The variation between genotypes suggests that QTL for this trait (g_s) may be segregating in this pedigree. Although there was a significant interaction between genotypes and blocks, the variance within the blocks shows homogeneity. From the plants growing at Chilworth, UK., stomatal conductance was scored at three different times on the grandparents, F_1s and forty F_2 genotypes selected as the highest and lowest in stomatal conductance and in biomass. ANOVA showed there to be a significant difference between the time scores, so QTL were mapped separately.

4.4 Results

4.4.1 Diurnal stomatal conductance in the grandparents

Diurnal response

Diurnal stomatal conductance measurements in the grandparents were made over a period of 19 hours, from 4.00 to 23.00. The results are shown in Figure 4.1.

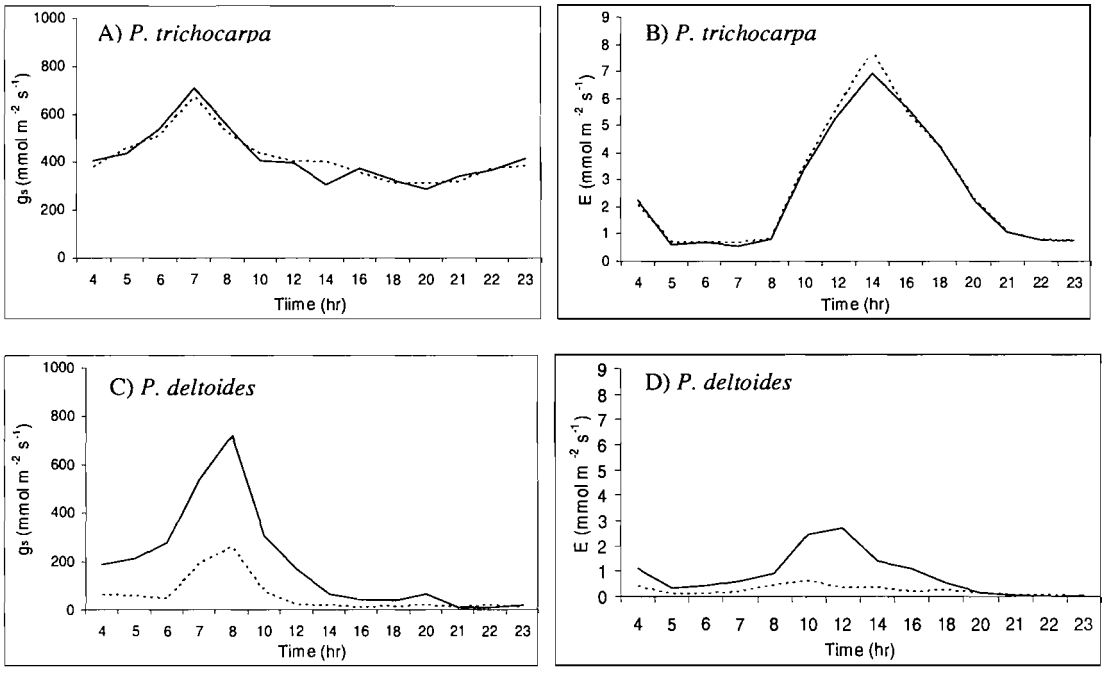


Figure 4.1 Diurnal leaf conductance in mature leaves of *P. trichocarpa* (93-968) and *P. deltoides* (ILL-129) at the Chilworth field site, under two treatments, well irrigated (–) and droughted (....). A) Stomatal conductance in *P. trichocarpa*, B) Transpiration in *P. trichocarpa*, C) Stomatal conductance in *P. deltoides* and D) Transpiration in *P. deltoides*. Values are means for two replicates.

Stomatal conductance of *P. trichocarpa* showed the same response to the two treatments (Figure 4.1A). There is no significant difference between well irrigated and droughted plants (withholding of water for one week). Leaf conductance in *P. trichocarpa* ranged from 300 to 600 mmol m^{-2} s^{-1} in both treatments. Maximum conductance occurs between 6.00 and 10.00 and remains almost constant from 10.00 until 23.00 (Figure 4.1A). Figure 4.1B shows that maximum transpiration occurs between 10.00 and 18.00. A low and constant transpiration rate occurred during the first hours in the morning and the last hour at night, even though conductance is high at 300-400 µmol m^{-2} s^{-1}.

Leaf conductance and transpiration in *P. deltoides* showed lower values than in *P. trichocarpa* with some similarities in diurnal pattern. The maximum values for conductance are reached between 6.00 and 10.00 (Figure 4.1C) and transpiration reaches maximum values between 9.00 and 13.00 (Figure 4.1D) with rates less than half those reached by *P. trichocarpa* (Figure 4.1A). The values for both genotypes are presented in Table A4.1.

Stomatal conductance at Midday and Midnight

Stomatal conductance measured on plants of both grandparents growing at Boldrewood in ambient conditions (Figure 4.2 A and B) showed a similar pattern in conductance at midday, but a very contrasting pattern at night, when in *P. deltoides* (Figure 4.2 B) but not *P. trichocarpa* (Figure 4.2A), all conductance in mature leaves (6-15) reached values near 0 mmol m^{-2} s^{-1}. In both species the conductance of young leaves (1-5) is higher at night than at midday, but there is a change at about leaf age 4 after which midday conductance is greater than the night time values. *P. trichocarpa* generally shows higher conductance that *P. deltoides*. After leaf age 4 for midnight measurements and leaf 6 for didday values, the stomatal conductance of *P. trichocarpa* drops with leaf age (Figure 4.2A).

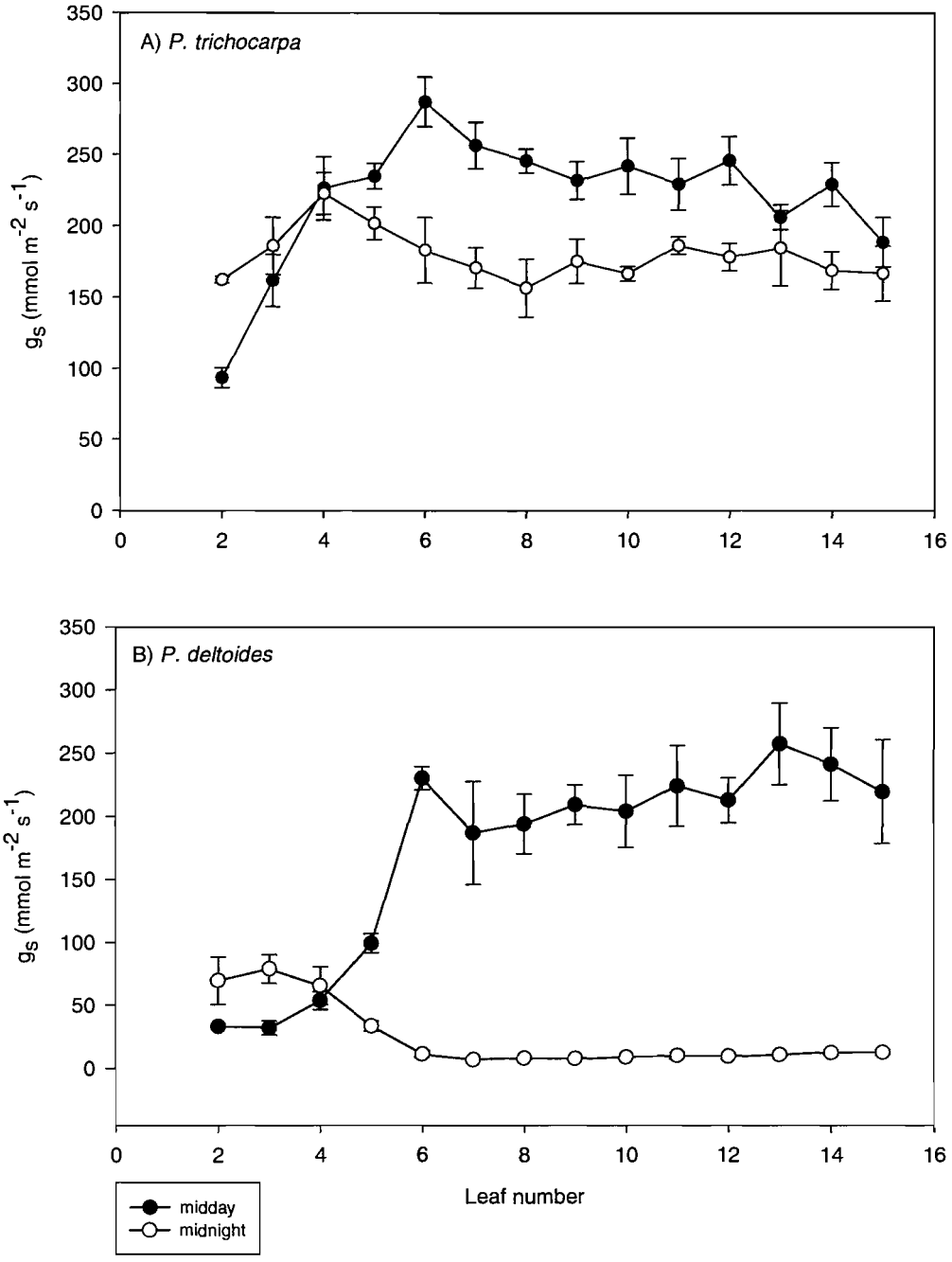


Figure 4.2 Midday and midnight conductance of A) *P. trichocarpa* and B) *P. deltoides* in field conditions at Boldrewood. Values represent the mean of three replicates. Standard error bars are shown.

4.4.2 Stomatal conductance, osmolality and cell extensibility in the Family 331 grandparents

Profile measurements of four parameters in mature plants of the grandparents growing in greenhouse conditions are shown in Figure 4.3. The values are summarized in Table A4.2.

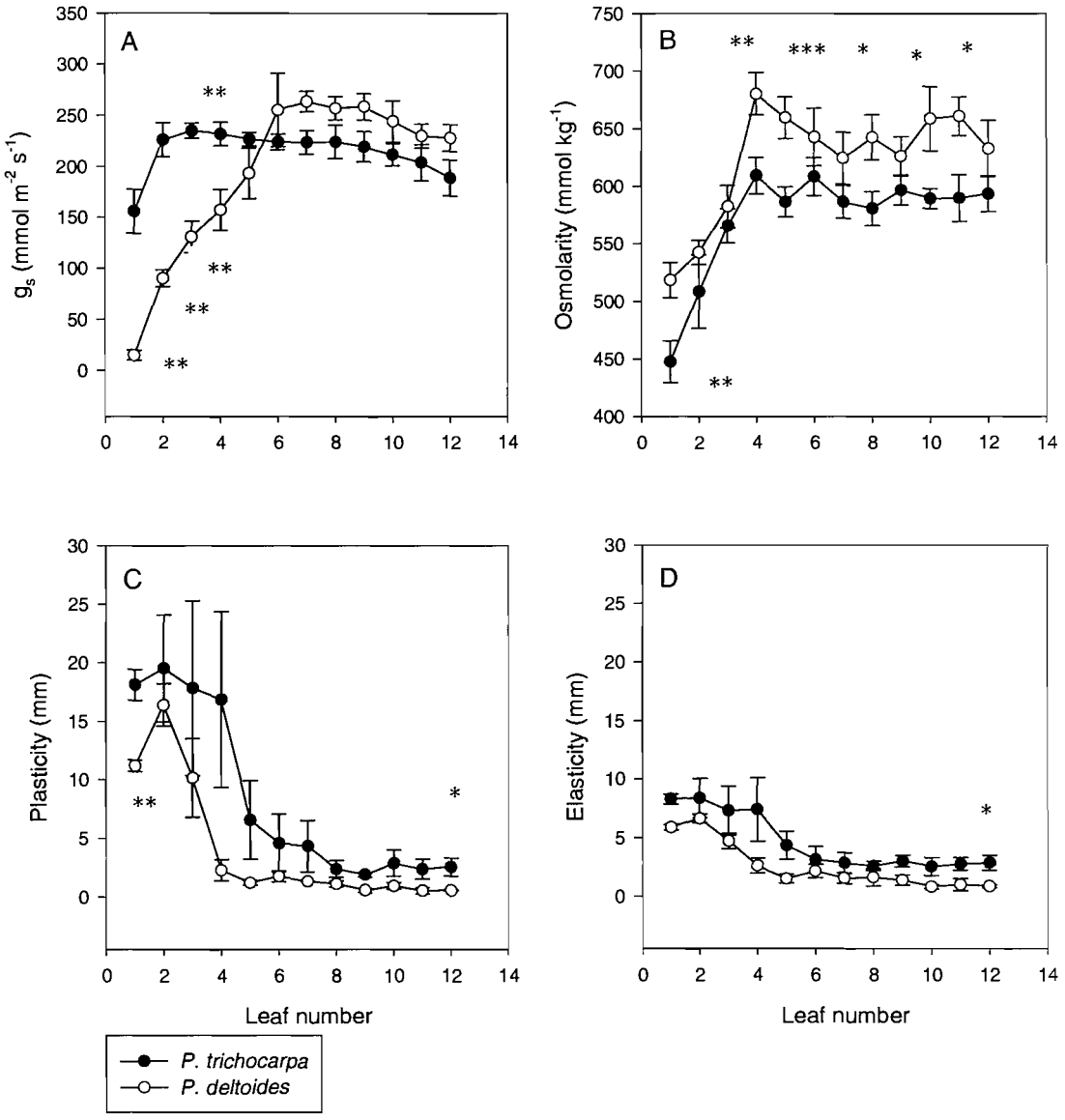


Figure 4.3 Grandparents profile for four leaf parameters in greenhouse conditions. *P. trichocarpa* (•) and *P. deltoides* (o). (A) Stomatal conductance, B) cell sap osmolarity, C) Cell wall plasticity, D) Cell wall elasticity. Values represent means of 4 or 3 replicates with standard error bars. Significant differences are indicated by *, **, and ***.

Of the four parameters shown in Figure 4.3 the largest contrast between the two grandparents is shown in the stomatal conductance values (Figure 4.3A). Conductance in *P. trichocarpa* was very similar for all leaf ages with the exception of leaf number 1, corresponding to the first expanded leaf. In contrast *P. deltoides* showed a steady increase of conductance with leaf age and maturity up to leaf age 9 and thereafter the values remained higher than those of *P. trichocarpa.*

Sap osmolality values present the same pattern in both species, steadily increasing until leaf number 4 then remaining almost constant after the leaves reach maturity. Values for *P. trichocarpa* are lower in all the profile in comparison to those for *P. deltoides* and are significantly different at all ages, with the exception of leaf number 3.

In an additional work, guard cell protoplasts of *P. trichocarpa* and *P. deltoides* were isolated using osmotic solution (data not shown). Osmotic potential values in the solution for obtaining protoplast in young leaves was similar in the two genotypes (-1.41), however values were -1.69 Mpa for mature leaves of *P. deltoides* and - 2.125 for guard cell protoplasts of *P. trichocarpa.*

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The cell wall properties evaluated in this case (plasticity and elasticity), show similar trends in both grandparents (Figure 4.3 C, D). In the case of plasticity, both species present a sigmoidal pattern where the values in young leaves (1-4) differ considerably from those of mature leaves (5-12), with the values in the first 4 leaves, almost double those obtained in cell wall elasticity in all the leaves in the profile. Elasticity values are not similar in young and mature leaves, but values in *P. trichocarpa* were higher in all cases. A general trend observed here is that these two properties reduce with leaf age. A better example can be observed in Figure 4.4, where significant differences were found in the tensil maxima in the two genotypes in mature leaves, but not in young and juvenile ones.

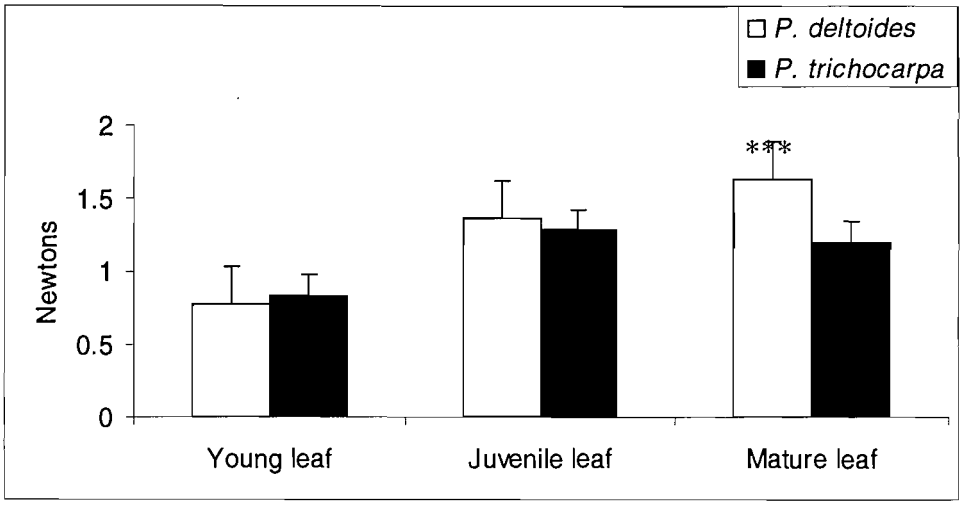


Figure 4.4 Tensil maxima in young, juvenile and mature leaves of *P. trichocarpa* and *P. deltoides*. Bars indicate standard error. Significant differences at $p<0.05$ are indicated.

4.4.3 Responses to ABA

ABA was applied as described in Chapter 2 (2.6.2). Its application to young leaves of *P. deltoides* (Figure 4.5 B) caused an immediate decrease in stomatal conductance at concentrations of 10^{-3} and 10^{-6} M, with values remaining almost constant after 60 minutes. In contrast, in *P. trichocarpa* there is only a slight decrease in conductance at the 10^{-3} M concentration that could be interpreted as a partial response to ABA, but no response was observed at the lowest concentration (Figure 4.5 A). The response observed in this case is very similar to that observed in the control of the same genotype, as in both cases the response starts after 20 minutes. Stomatal conductance ranged between 0.15 and 0.2 mol m^{-2} s^{-1} for *P. trichocarpa* and between 0.05 and 0.15 mol m^{-2} s^{-1} for *P. deltoides*.

On the other hand, photosynthetic rate in both genotypes (data not shown) continued increasing for up to two hours after ABA application, with the exception of leaves in 10^{-3}M concentration where it remained constant. In general, photosynthetic rate ranged from 4 to 7.5 μmol m^{-2} s^{-1} and it was only in the highest concentration in *P. detloides* where it remained at values of 2 μmol m^{-2} s^{-1} during the 2 hours of the experiment.

In mature leaves, the stomatal conductance responses were more defined and constant throughout the experiment. For *P. trichocarpa* (Figure 4.5 C) no response to ABA at any concentration occurred during the 180 minutes of the experiment and conductance was also very constant (0.2-0.25 mol m^{-2} s^{-1}). In contrast, the stomatal conductance of *P. deltoides* (Figure 4.5 D) was very responsive and at the time of the first reading (20 min), stomatal conductance was greatly reduced from the initial value of 0.025-0.035 mol m^{-2} s^{-1} to 0.05 in 10^{-6} M ABA and to 0.03 in 10^{-3} M ABA respectively.

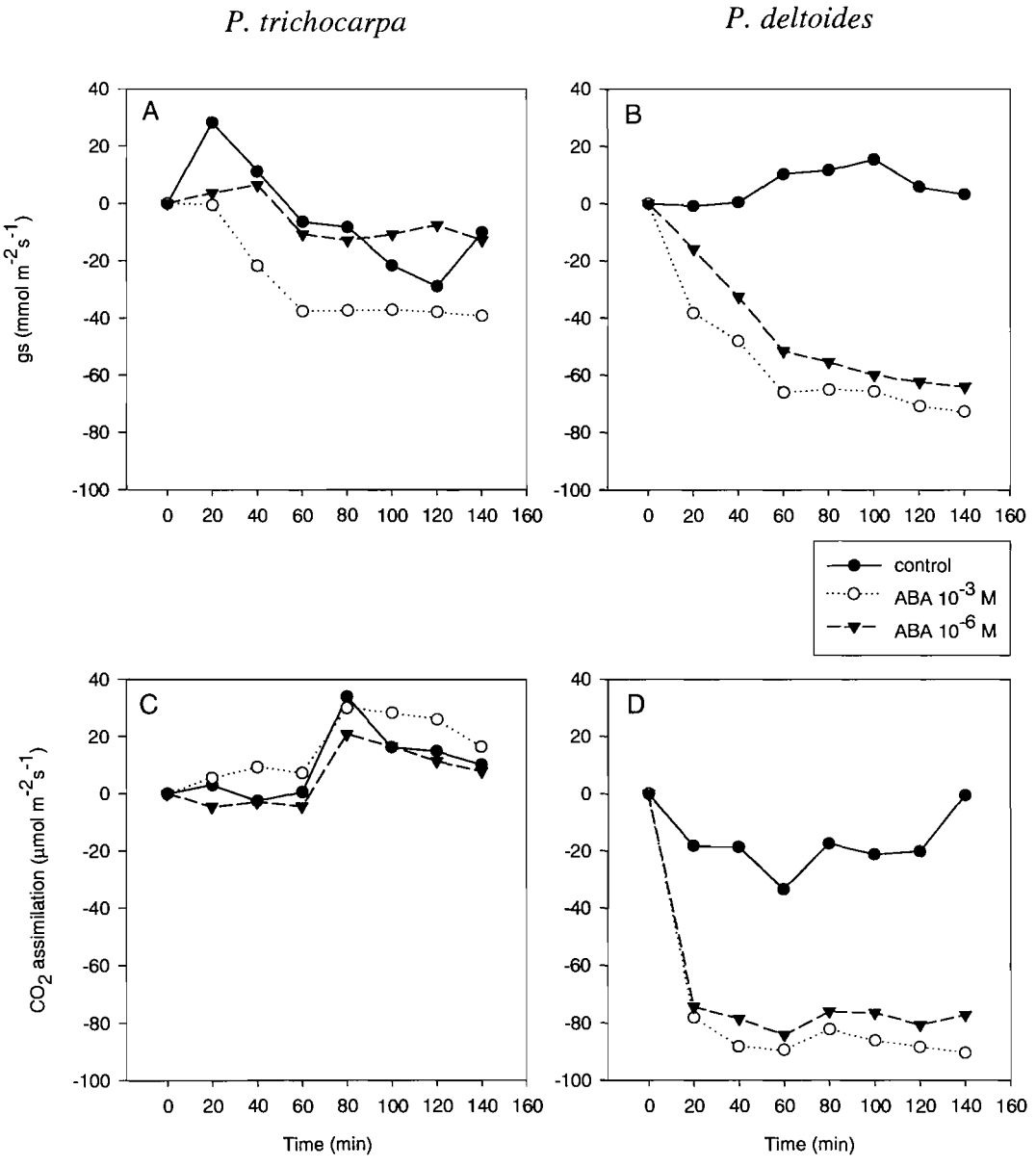


Figure 4.5 Stomatal conductance responses to ABA application in young and mature leaves of *P. trichocarpa* and *P. deltoides* expressed as percent of difference with regard to their start point. A) *P. trichocarpa* young leaf, B) *P. deltoides* young leaf, C) *P. trichocarpa* mature leaf and *P. deltoides* mature leaf. Each curve represents an individual response recorded with a Licor 6400.

It is important to say that in young leaves the relationship between stomatal conductance and photosynthesis shows an inverse pattern, while in mature leaves, there is a clear and direct relationship between conductance and photosynthesis, especially after 60 minutes of treatment. Mature leaves photosynthesis of *P. trichocarpa* declines, even though g_s values are steady at 0.2 mol^{-2} m^{-1}, probably because depletion of substrates.

4.4.4 Thermal imaging in the grandparents and parents of Family 331

Responses of leaf conductance and temperature to exogenous ABA application and dehydration were measured (as described in Chapter 2, 2.7) using thermal imaging in three groups: 1) young leaves of the grandparents, 2) mature leaves of the grandparents, and 3) F_2 parents (G 246 and G 242). In all cases, conditions were the same and videos were recorded for 2 or 3 hours. Analysis of the images allowed the calculation of g_i as an index of relative conductance and leaf temperature which illustrates the response pattern to the treatments.

There was a very marked difference in the effects of ABA on leaf temperature in the two analyses which is illustrated by the colour changes shown in Figure 4.6. In the case of young leaves, the responses of *P. deltoides* to ABA application were an immediate increase in g_i, and after 20 minutes of application, a decrease (see point 25 min in Figure 4.7). In contrast, no clear response to the dehydration treatment was observed as the g_i did not change during the experiment. Only a very slight decrease was observed at the end of the experiment that could be explained by a high sensitivity to detachment or to a loss of water conductance in the petiole when it was cut. In the case of *P. trichocarpa*, the ABA treatment and the dehydration response showed the same trend, an almost immediate increase in g_i followed by a decrease to the initial levels without any further change. Leaf temperatures ranged from 26.5°C to 29.5°C, and an oscillatory pattern was observed in all cases. The lowest temperature corresponded to the *P. deltoides* and *P. trichocarpa* controls, while the highest temperature was reached by excised leaves of *P. deltoides*. Relative conductance values reached are higher than in mature leaves which explains the loss of turgor in the leaves.

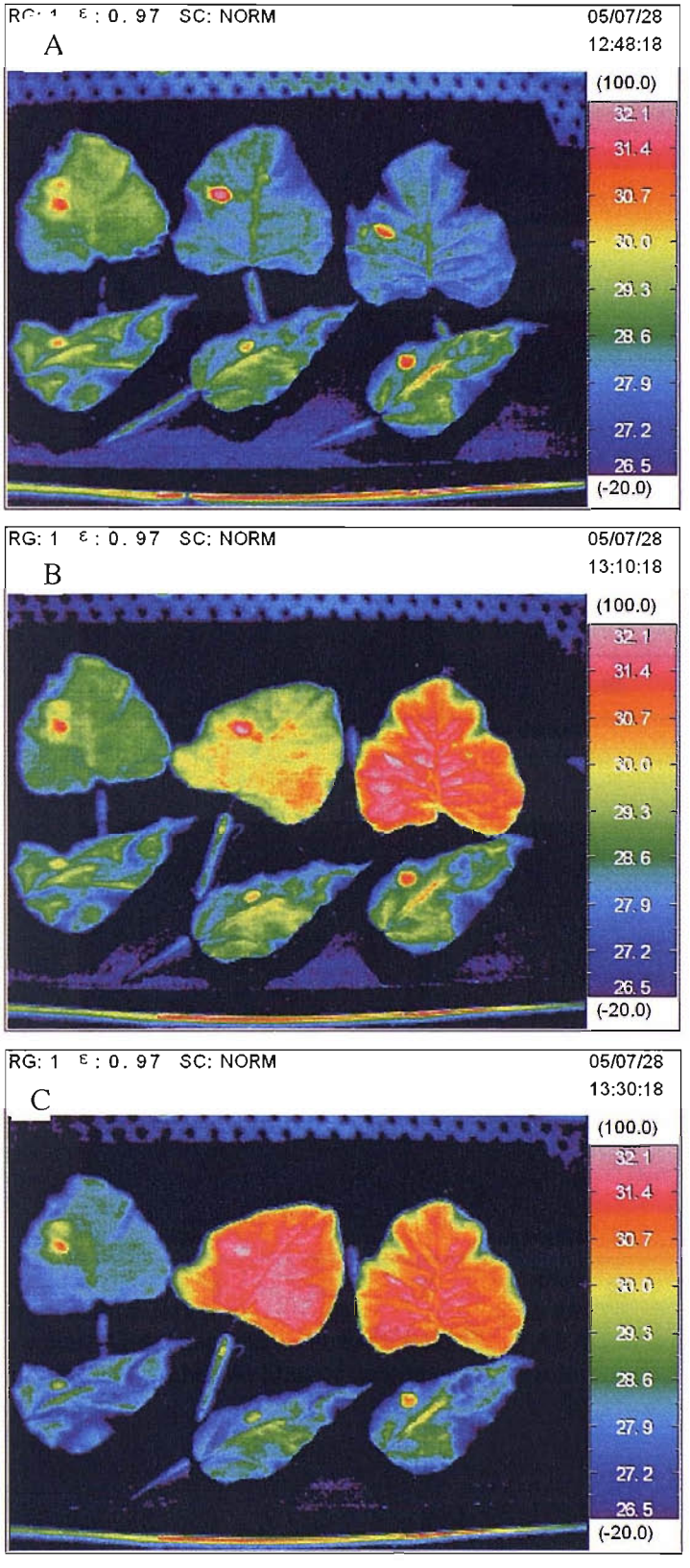


Figure 4.6 Thermal images of mature leaves of *P. deltoides* (top row) and *P. trichocarpa* (bottom row) mature leaves in response to ABA and dehydration. Treatments: control (left) with petiole in water, ABA treatment (centre) with petiole in ABA 10^{-4} M, Dehydration treatment with petiole excised (right). A) Start of treatment, B) 12 minutes after treatment and C) 33 min after treatment.

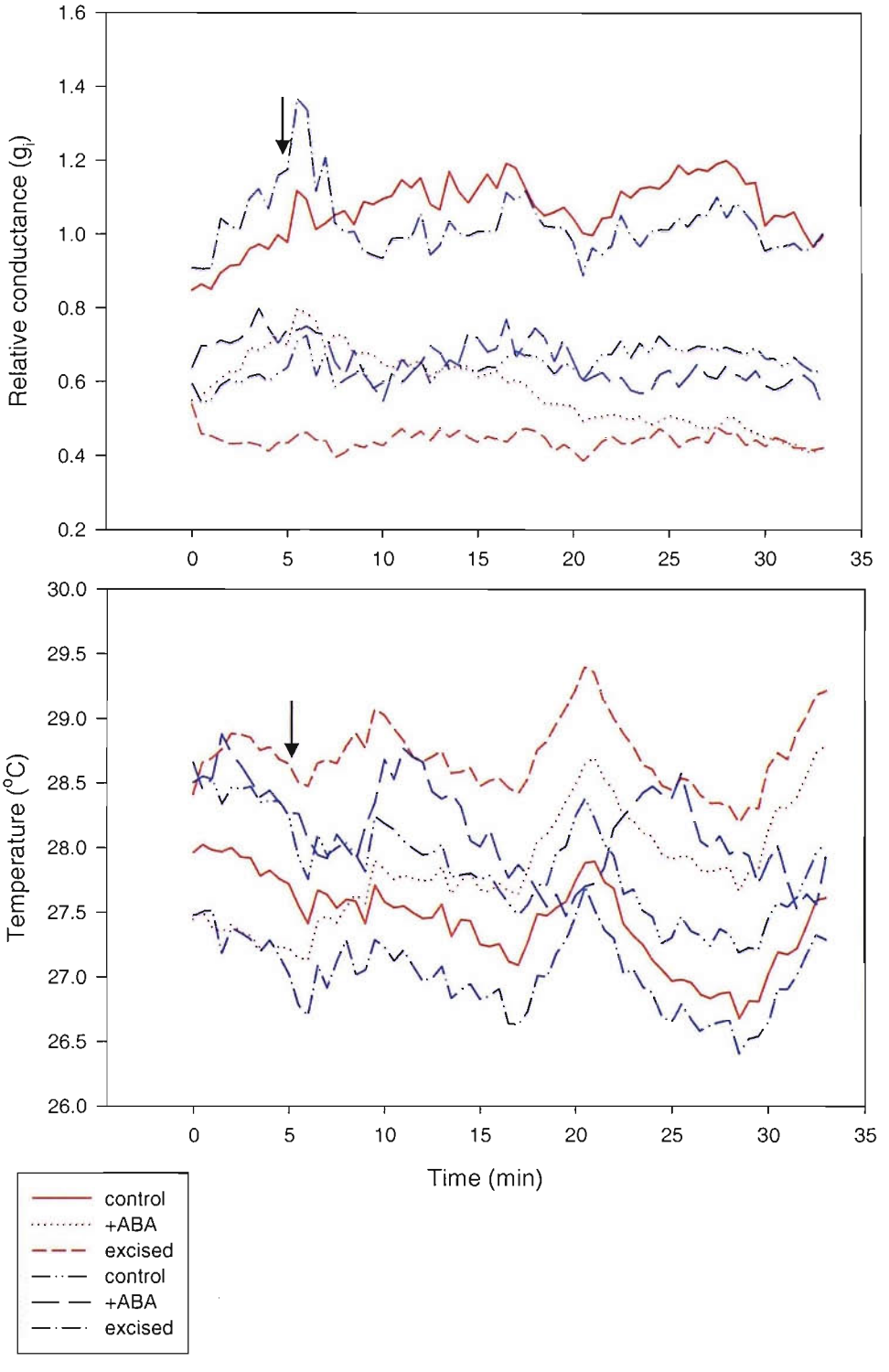


Figure 4.7 Thermal imaging in young leaves of *P. deltoides* (red) and *P. trichocarpa* (blue) in control conditions. A) Relative leaf conductance (g_i) and B) Leaf temperature. Values correspond to one leaf. Treatments started at 5 min in all cases as indicated by the arrow. Graphs are referred to a 1 h period.

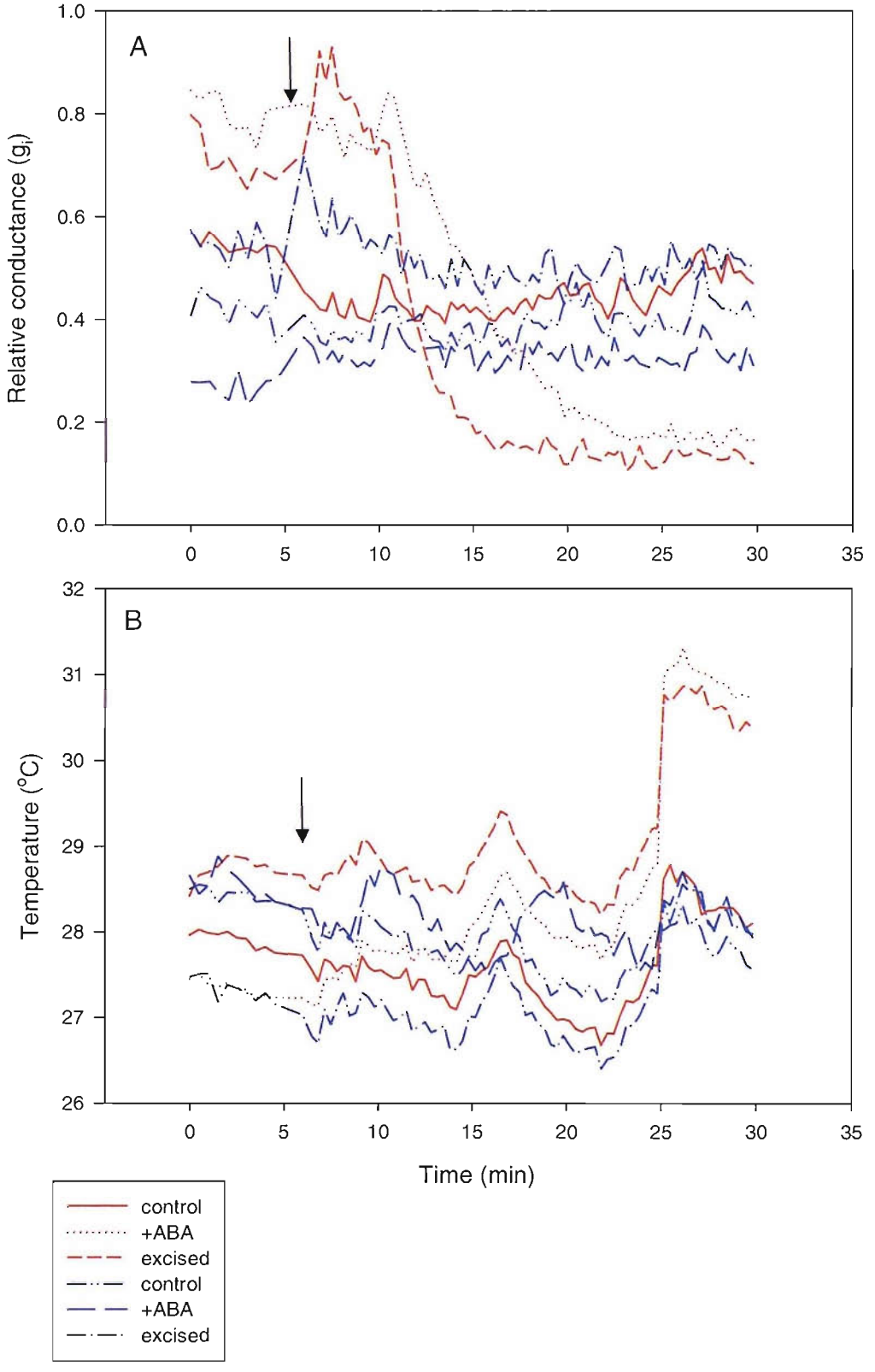


Figure 4.8 Thermal imaging in mature leaves of *P. deltoides* (red) and *P. trichocarpa* (blue) in control conditions. A) Relative leaf conductance (g_i) and B) Leaf temperature. Values correspond to one leaf. Treatments started at 5 min in all cases as indicated. Graphs are referred to a 1 h period.

Mature leaves of *P. deltoides* (Figure 4.8) have a strong response to the treatments. An increase in relative conductance was observed during the first 3 minutes of the dehydration treatment and 5 minutes after ABA application. From 15 to 18 minutes after dehydration and ABA treatments, the minimum relative conductance (g_i approx. 0.17 – 0.2) was observed in both cases and remained constant for the rest of the recording which lasted up to three hours. However, in the case of *P. trichocarpa*, the responses were completely different. There was no response to ABA at all, while dehydration caused an increase in relative conductance observable within 6 minutes of excision. Leaf conductance reached in this case the initial conductance and was maintained until the end of the experiment, however after an hour, the leaf was completely dehydrated. Leaf temperature was in the same range as that of the young leaves, however after 20 minutes temperature raised abruptly by 2°C in the case of ABA and excision in *P. deltoides* indicating complete stomatal closure to avoid the loss of water vapour.

In the case of the parents, G 246 and G 242, the responses were basically the same as in mature leaves of the grandparents but more pronounced (Figure 4.9). G 242, a male parent from generation F_1, showed a moderate and sustained increase in g_i in response to ABA that was maintained for nearly nine minutes before it steadily decreased. It also had a strong response in g_i to excision, that then decreased to the same value as the starting point. These responses are reflected in leaf temperature, as the temperature reached by excised leaves of G 242 was in the order of 31°C, while the temperature reached after 20 min of ABA treatment is slightly higher than that at the starting point. However, the value reached is in the order of 28°C, 3 degrees less than in the excised treatment.

An opposite trend was observed in G 246, the female parent. The response to ABA is not observable until 20 minutes after ABA application and it was observed as a steady decrease with the lower point ($g_i = 0.3$) reached five minutes later. In the dehydration treatment, there was a fast response to excision, observable by the fast increase in g_i, a plateau for almost 8 minutes and a steady decrease for 17 minutes until reaching the lowest g_i value near 0.25 at 25 minutes after the treatment. Leaf temperature in this case shows a similar pattern to the ABA and dehydration treatment, reaching the highest temperatures (29.5 to 30 °C) with the lowest value for ABA treatment. Temperature

oscillation was the same as in G 242, and ranged between 25 and 31°C, being the widest range in the three groups of leaves studied (a difference of 5°C).

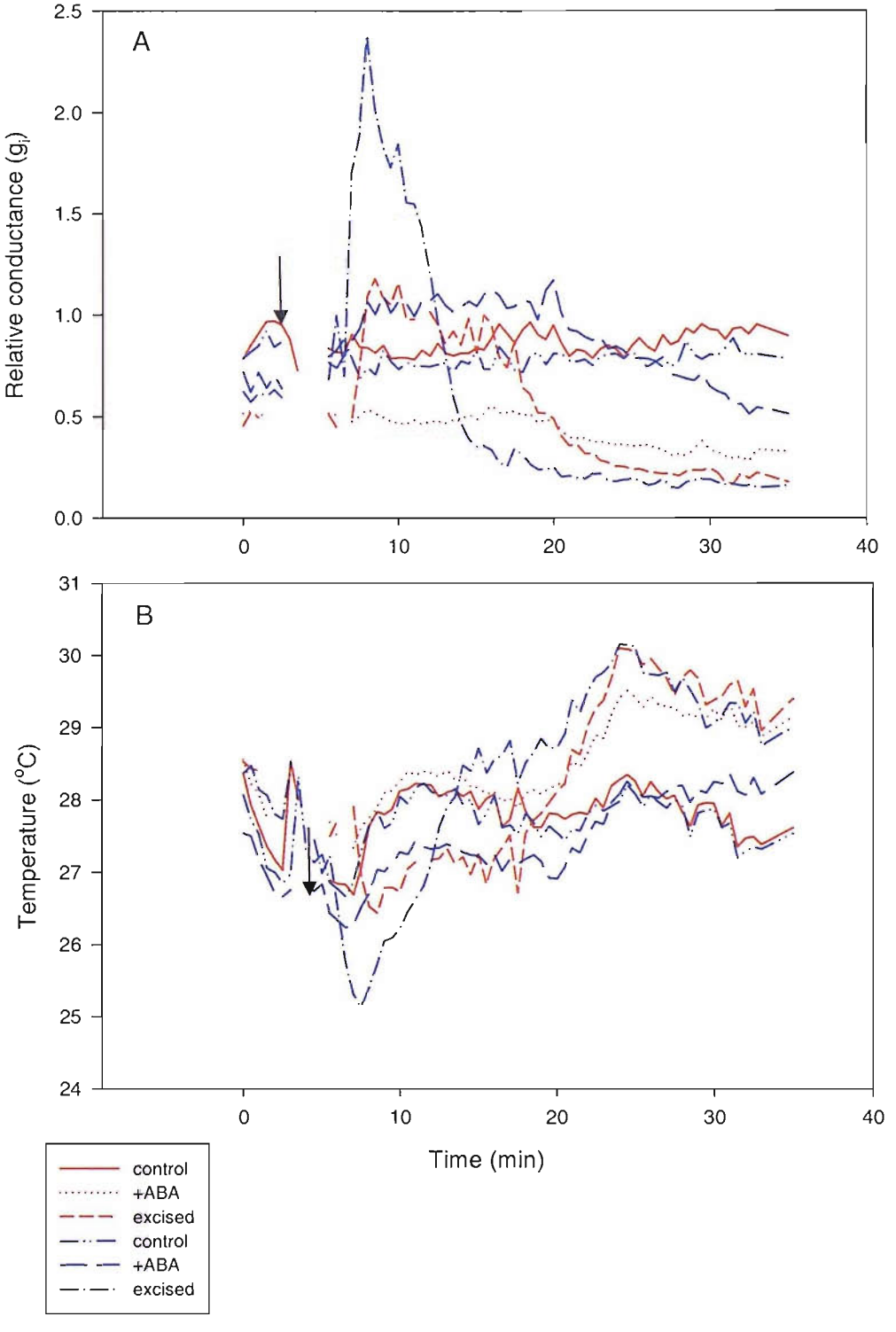


Figure 4.9 Thermal imaging in mature leaves of G 246 (red) and G 242 (blue) in control conditions. A) Relative leaf conductance (g_i) and B) Leaf temperature. Values correspond to one leaf. Treatments started at 5 min in all the cases. Graphs are referred to a 1 h period. Leaf conductance ranges from 0.25 to 2.5, while temperature ranges between 25 and 30.5 ºC. The response to dehydration treatment is remarkable in G 242.

4.4.5 Stomatal conductance in field planting at Headley Experimental site in the UK

Frequency distribution of the F_2 population stomatal conductance does not have a normal distribution (Andersson-Darling 19.78, $P < 0.005$) illustrated in Figure 4.10, but values are skewed to the right. However, after $\log_{10}$ transformation, the data was normal (Andersson-Darling 0.312, $P > 0.005$).

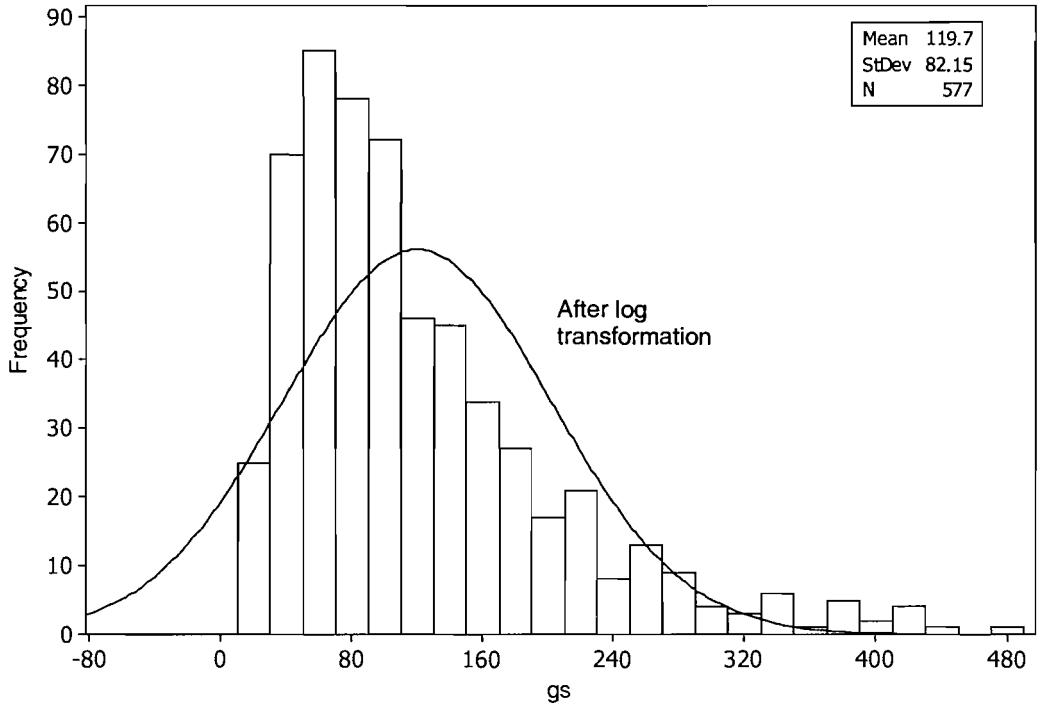


Figure 4.10 Frequency distribution of stomatal conductance in Family 331.

In general, g_s in the F_2 progeny was much lower than in the F_1 parents G 242 and G 246 (Figure 4.11). The parents, especially G 246 (the female parent) kept the higher conductance in all three blocks sampled, followed by the male parent G 242 which showed a reduction of 21.6% compared to the female parent. In the case of the grandparents the decreases relative to G 246 are larger, but the trends in the female and male are the same as in the grandparents. Figure 4.11 shows the stomatal conductance in the F_2 grandparents and parents, as well as the mean stomatal conductance for the F_2 population. The highest mean conductance value was for G 246, the mean conductance in the F_2 population decreased (-63.2%). One-way ANOVA on Log_{10} transformed data to comply with homogeneity of variance (Bartlett's Test 1.10, $P > 0.05$) shows that

there are no significant differences between the genotypes (F=2.31, d.f. = 3, P > 0.05). Even with the inclusion of 225 F_2 genotypes means in the ANOVA, causing an unbalance in the data, the result indicates the same (F= 1.85, d.f. = 216, P > 0.05).

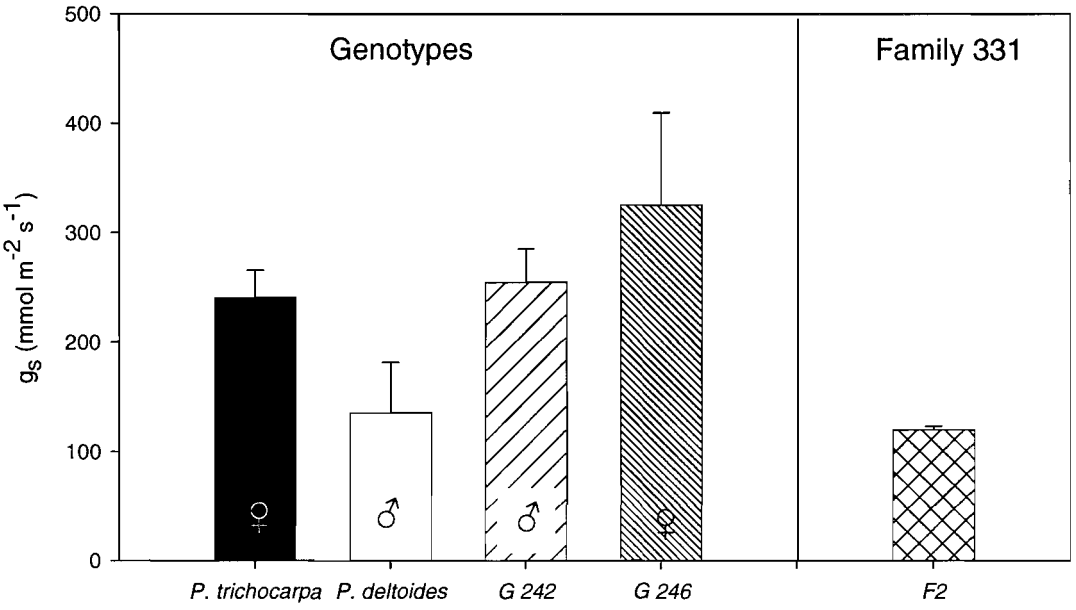


Figure 4.11 Stomatal conductance in different species and genotypes of *Populus* in field conditions. Bars show the means and se of three replicates for each one of the relatives in Family 331. *P. trichocarpa* (female grandparent), *P.deltoides* (male grandparent), G 242 (male parent), G 246 (female parent) and values progeny F_2 (577 values). No significant differences were found at P>0.05.

Thirty four F_2 genotypes were selected with to high or low conductance. The group with high conductance included those with values above 200 mmol m^{-2} s^{-1}, while the group with low conductance had values of up to 50 mmol m^{-2} s^{-1}. To these two groups grandparents and parents were added.

4.4.5.1 QTL mapping for stomatal conductance

QTL mapping was carried out for all genotypes for all blocks simultaneously. Three QTL mapped for stomatal conductance at Headley. These were in LG I, IV and VI (Table 4.1). From these QTL, the one in LG I corresponded to a position that overlaps with other QTL mapped in other experiments in this research work. The position is coincident with leaf area, leaf length, leaf wide, and stomatal number. This QTL was collocated in the physical map of *P. trichocarpa*, close to a candidate gene in a position just 2 cM from the QTL. XTH28 is considered the best candidate gene in this case (see Chapter 7 for more detail).

Table 4.1 Leaf conductance QTL mapped in a Family 331 planted at Headley, UK.

Linkage Group	Position cM	CI	$\%V_A$
I	136	4 - 172	6.3
IV	25	6 - 84	7.4
VI	71	28 - 84	5.1

The two QTL remaining, found in LG IV and VI, do not have common or similar positions to other QTL mapped in this work. It is important to say that these QTL were mapped when blocks were taken separately not on an average over blocks. Mapping of the average values over the blocks failed to identify any QTL. The variances were relatively small (Table 4.1), but similar to most of the QTL mapped in this research (Figure 7.2).

4.4.5.2 Stomatal conductance in selected genotypes growing in glasshouse conditions

QTL in selected genotypes at Chilworth

The 34 selected genotypes (as indicated in 4.4.5), gave g_s values between 285 and 700 mmol m^{-2} s^{-1} when grown at Chilworth. A single QTL was found to map to linkage group XI at time 2. No other QTL were mapped for the other times. The position was at 24 cM with a confidence interval of 15-49.5 and 29% V_A.

This QTL was collocated in the physical map of *P. trichocarpa* near to a candidate gene at position 27.3. It is import to say that the 29% of variance in this case indicates a locus that could be responsible for the control and regulation of this trait.

QTL in selected genotypes under drought and irrigated treatments

The selected genotypes were scored under drought and non-drought conditions. ANOVA showed there to be no significant variation between genotypes in either the drought or non-drought treated plants. A significant difference was observed between the drought and non-drought conditions. A single QTL mapped for stomatal conductance in each treatment with a high percent of variation was mapped in this case (Table 4.2). This implies that there are few genes which account for a large proportion of the coding for this trait.

Table 4.2 QTL mapped in selected genotypes for irrigated and droughted treatments. In each case there were three replicates

Treatment	Linkage Group	Position cM	CI	$\%V_A$
Irrigated	I	50	25 - 134	38.4
Drought	XVIII	68	35 - 78	41.5

The two QTL mapped in this case have common positions to other QTL mapped in Chapter 7. In addition they also collocated to two candidate genes in the same position as in LG XVIII or in the interval as in LG I (Figure 7.2).

4.5 Discussion

P. trichocarpa and *P. deltoides* showed distinctly different pattern of stomatal dynamics. The most relevant finding is that the daily pattern of conductance in *P. trichocarpa* never dropped to values less than 200 mmol m^{-2} s^{-1} and no differences between droughted and well irrigated plants were found.

Because of the earlier measurements of g_s in *P. trichocarpa*, this response was expected, however it was surprising that night conductance values remained high. High conductances have been reported in other poplar genotypes and species in dark conditions or at night. In different *Populus* hybrids like Columbia River, 'Beaupre', 'Unal' T x N, D x (T x N) and 'Robusta', conductance is 2 – 5.5 mM s^{-1} (Ceulemans et al. 1988). These hybrids also showed hysteresis, as at 22.00, they still showed high conductance. *P. balsamifera*, another *Populus* species belonging to the same section as *P. trichocarpa,* has been observed to show high conductance at night. This diurnal pattern of g_s could confer advantage in growth over other species as a consequence of allowing a longer period of high gas exchange (Blake et al. 1984 in Ceulemans et al. 1988) , particularly if there was a mechanism for night time CO_2 fixation or storage. The high rate of transpiration at midday indicates a close relationship with the atmosphere (vapour water deficit) as temperature increases and guarantees the supply of nutrients through the xylem. The high productivity of poplars is particularly expressed in places with a constant supply of water (Scarascia-Mugnozza et al. 1986).

The response in *P. deltoides* reveals a stomatal control sensitive to water deficit, as significant differences were found between droughted and well irrigated plants, and lower rates of transpiration than in *P. trichocarpa* were observed. These results indicate a high sensisitivy to water deficit. Similar results were mentioned by Scarascia-Mugnozza (1986). The diurnal patterns also indicate that maximum conductance is reached during the early hours of the morning, one hour later than in *P. trichocarpa.* The diurnal pattern of g_s also showed that during that first three hours, the conductance values can reach the same values as in *P. trichocarpa*, showing its good performance in the first hours of the morning. Suggestions that the stomata have more oscillations

during the afternoon, when they remain open for longer periods in *P. deltoides*, are recognized as the stomata "hunting" for favourable apertures (Ceulemans et al. 1988).

The conductance profile in the two genotypes studied confirmed the variations in the diurnal patterns, especially in mature leaves. In the case of young leaves, conductance was always slightly higher at night, indicating that at this time leaves have more turgor than at midday, a fact that could be related to the processes of leaf growth and the stiffening of cell walls with leaf age, a normal change in the leaves as indicated by Van Volkeburgh and Taylor (1996). On the other hand, values of midday conductance in both genotypes showed no significant differences between the two species.

Relating conductance to other leaf properties such as cell sap osmolality and cell plasticity and elasticity, shows the existence of two groups of leaves defined by age: young leaves (leaves 1-5) and mature leaves (6-12). This is similar to the anatomical changes during leaf ontogeny in *P. deltoides* found by Isebrand et al. (1973), who showed that maturity of the leaf in *P. deltoides* occurs at LPI 6.0 signifying a transition with respect to structure and function. In both grandparents, mature leaves show a decrease in conductance with age, in accordance with the loss of stomatal function in ageing hybrid poplar leaves (Reich 2006).

The lowest values of conductance in leaves 1 and 2 of *P. deltoides* are explained by the lack of maturity in the stomata on the abaxial side of the leaf, which is reached at LPI 3. Maturity in stomata on the adaxial side is not reached until leaf LPI 6, when highest values are measured, implying an increase in size of stomata and therefore an increase in gas exchange area. The pattern of conductance in *P. trichocarpa* also indicates that the stomata in leaf 2 are completely developed, including the intercellular spaces, in comparison to leaves of the same level of insertion in *P. deltoides*. Even though leaf size has not reached its maximum, stomatal density will be higher than in mature leaves, with larger stomata. It could also be that young leaves in *P. trichocarpa* have more water content because their solute content is lower than in *P. deltoides*. The faster development of the shoot of *P. trichocarpa* compared to *P. deltoides* is noticeable. This difference in development rate at time of maturity has also been highlighted previously (Van Volkenburgh and Taylor, 1998).

Response to stress hormone ABA and dehydration.

P. deltoides exhibited a fast response to ABA observable at 20 minutes after ABA application in young and mature leaves. This type of response has been reported in previous work (Scarascia-Mugnozza et al. 1986). In contrast, a complete lack of response in mature leaves of *P. trichocarpa* was observed even after 3 hours of treatment application. This result agrees with that reported in the same species (Schulte and Hinckley 1987) and in hybrids such as *P. koreana* x *trichocarpa* 'Peace' (Ridolfi et al. 1996). On the other hand, the slight decrease in stomatal conductance in young leaves of *P. trichocarpa* has been interpreted as a partial response to ABA or as a response to acclimatization after drought treatment (Dunlap et al. 1993, Schulte et al. 1987). In this case, the partial response in young leaves is explained by a reduction in turgor in the epidermal cells related to their cell extensibility, or by possible damage of the vascular system in the petiole when cut, as this partial response also occurred in the control after 60 minutes.

One important finding in this experiment was the fact that in *P. trichocarpa*, photosynthesis rates (data not shown) continued increasing even though stomatal conductance values remained constant or slightly decreased, indicating a lack of relationship between stomatal conductance and photosynthesis. This response could be explained by the existence of a favourable *Ci* pressure in the intercellular spaces and by ontogenetic differences between young and mature leaves (Catsky and Ticha 1982, Ticha 1982). Higher rates of photosynthesis in younger leaves have been reported for *P. deltoides* in conditions of enhanced UV-B radiation (Bassman et al. 2001), and this has been linked to higher WUE.

The use of thermal-imaging shows in detail the dynamics of the response to ABA and dehydration through the measurement of leaf temperature. The biggest change that occured was when leaves of *P. deltoides* reached their minimum relative conductance. It also showed that in *P. trichocarpa* the extreme dehydration treatment did not cause any change in the temperature or conductance, even when after an hour, the leaf showed physical evidence of dehydration or damage. This showed that even in extreme turgor pressure, no changes in the stomatal or epidermal cells occurred, suggesting that this characteristic is related to the epidermis and turgor pressure in the epidermal cells.

The response in the youngest leaves of *Populus trichocarpa* can be explained as a response to turgor in the excised leaf, which clearly shows an increase immediately after cutting followed by a steady decrease to normal values. In the case of *P. deltoides* a similar response was observed and it can be concluded that in this case the response to turgor is independant of the response to ABA. This hypothesis was confirmed in the results shown by the parents G 242 and G 246, where high increases in the response to dehydration (G 242) and ABA (G 246) suggest the transmission of alleles responsible for these responses.

Stomatal conductance in the Family 331 was low (reduction of 63.2% compared to the best of the parents), suggesting 1) that in general most of the genotypes have a low conductance, or 2) that the population has sensitive stomata as in the grandparent *P. deltoides*, which also showed a large decrease in stomatal conductance relative to the F_1 parent G 246 (-58.2%). The high conductance in the female G 246 indicates a maternal influence in the stomatal function. Ceulemans et al. (1988) found a maternal effect in several clones of *P. deltoides* and *P. trichocarpa* that seems to lead to lower ratios of stomatal conductance and frequency in black cottonwood and higher ratios with eastern cottonwood clones. The low conductance values probably contribute to the small number of QTL mapped in the population when conductance was measured in detached leaves. Conductance was higher in the selected genotypes grown in greenhouse conditions where measurements of g_s were made in situ. As a result of the stomatal conductance measurements and the mapping of QTL, six QTL were obtained in total, with three of them having high % of variance indicating that there is a higher probability that this part of the genome has an important control in this property.

4.6 Conclusions

The study of stomatal function in the two original grandparental species of Family 331 using the different approaches in this chapter gave a complete picture of the behaviour patterns of the stomata. It showed clear differences between both species expressed in ontogenetic and developmental changes in the leaves.

The results presented here prove the hypothesis postulated in this research work that the responses in both genotypes are in complete agreement with: 1) the ontogenic changes, 2) developmental processes and 3) the cell division and expansion mechanisms of each genotype. The data presented here do not support, indeed conflict with, the earliest suggestion that acclimation occurs in the responses of these genotypes when they are exposed to intermittent water supply. They support the hypothesis expressed by Ridolfi et al. (1996) in this regard.

They also prove that *P. trichocarpa* is insensitive to ABA even when it has been found in high concentrations in leaf tissue (see Chapter 5). Similar results were found by Ridolfi et al. (1996) in the cultivar 'Peace'. However, the response in young leaves is linked with the loss of turgor in epidermal and guard cells. They also prove a high ABA sensitivity in young leaves of *P. deltoides* that increases as the leaf matures and allows the leaves to remain well hydrated. The strategy to conserve water potential in *P. trichocarpa* is completely different to *P. deltoides* with the former showing acropetal branch sacrificing in order to maintain high water potentials in the younger leaves where, as was observed, higher rates of photosynthesis occur.

The stomatal conductance rates measured in the Family 331 were low (Figure 4.11) and therefore the prospects for increasing biomass with member of the Family 331 could be described as limited. The QTL mapping results presented here give a clear indication of some regions of the genome which may participate in stomatal regulation (indirectly in biomass production) and thus be of interest in breeding for increased productivity. Until now there has been no candidate genes proposed for the regulation of gas exchange rates, therefore any indicator of its regulation in the genome would be of value.

Chapter 5

Characterization of leaf morphology and growth in the grandparents of Family 331: *P. trichocarpa* (93-968) and *P. deltoides* (ILL-129)

5.1 Introduction

Leaves are important structures in plants, capturing light and CO_2 and their basic molecular and biophysical processes are highly consistent (Press 1999). Leaf arrangement and leaf morphology vary at microscopic and macroscopic levels. It is considered that the variation reflects different trade-offs such that plants maintain an equilibrium between the performance of primary leaf functions (photosynthesis) and those needed for other purposes such as mechanical support, prevention of water loss, regulation of energy exchange and defence (Parkhurst and Loucks 1972, Gates 1980, Givnish 1986, Niklas 1999, Press 1999).

Photosynthesis in terrestrial plants may be limited by stomatal behaviour and leaf biochemical capacity (Geber and Dawson 1997), as they face the trade-off between maximizing carbon fixation and minimizing water loss. Any increase or decrease in carbon uptake means an associated increase or decrease in the uptake of water, through control of the stomatal aperture.

Stomata are found in the epidermal cell layer over the aerial surfaces of plants (Bird and Gray 2003). Stomatal frequency can be described by their area density (stomatal density: number per unit area) or by the fraction of epidermal cells that they represent (stomatal index: the ratio of stomatal to epidermal cells). Individual stomatal aperture can be affected by changes in light or humidity (Buckley et al. 1997, Gutschick 1999), and also by plant nutrition, leaf age, water stress and other physiological factors (Catsky and Ticha 1982, Von Caemmerer and Farquhar 1981).

Stomatal density is an important ecophysiological parameter that affects gas exchange, stomatal conductance and the efficiency of water use. However stomata are not the only leaf structures that participate in the maintenance of the best photosynthetic CO_2 assimilation rate; trichomes, cuticle composition and mesophyll structure also have a particular role in this process (Press 1999, Beerling and Kelly 1996, Gutschick 1999, Bird and Gray 2003). The diversity of all these structures is demonstrably under genetic control (Geber and Dawson 1990, Gutschick 1999, Jarvis and Davies 1998, Haefner et al. 1997).

If plants show long-term anatomical leaf adjustments to altered atmospheric CO_2 concentrations, the instantaneous leaf water use efficiency might be enhanced by the altered stomatal density and/or dimensions (Idso et al. 1987, Woodward 1987, Woodward and Bazzaz 1988, Ceulemans and Mousseau 1994, Ceulemans et al. 1995). This statement has been proved by the analysis of axial Silurian and basal Devonian fossils which form the basis for speculation on the selective pressures (e.g. the generation of a transpiration stream, H_2O and nutrient acquisition, temperature control) that led to the evolution of stomata (Edwards et al. 1998).

Populus is a genus of fast growing tree species that apart from their small genome, efficient transformation and ease of propagation (Sheppard et al. 2000, Taylor 2002), exhibit a wide site-specific as well as interclonal variation in the stomatal characteristics of dimension, density and index (Ceulemans et al. 1995). In several experiments held in Open Top Chambers, a decrease in stomatal density was associated with a fall in the stomatal index, (Ceulemans et al. 1995, Ferris et al. 2002). Leaf stomatal and epidermal cell development, as well as leaf growth in response to free-air CO_2 enrichment (FACE) have also been widely studied (Ferris et al. 2001, Taylor et al. 2001b, Taylor et al. 2003), including others as the ones held by Norby et al. 1999, Pritchard et al. 1999). All of them suggest that there was also a change in stomatal initiation.

Even though it is not known how stomatal density is controlled during leaf growth, it is likely to be genetically regulated. Ferris et al. (2002) provided preliminary evidence that leaf stomatal and cell traits are amenable to QTL analysis and concluded that these traits are robust enough to be developed for accelerated tree breeding programs and could assist in the finding of traits related to high biomass productivity.

This study characterizes the leaf morphology of *P. trichocarpa* and *P. deltoides* (as a control) growing in two contrasting environmental conditions in Europe, aiming to find morphological characteristics that explain the contrasting stomatal responses reported for both species and that can be used as traits to assist in the selection of drought tolerant

clones in *Populus*, specifically in the *P. trichocarpa* x *deltoides* F_2 hybrid pedigree Family 331.

The interest is in the epidermal/cell-guard cell relationship and other leaf structures that can participate in the perception of environmental conditions and signalling them to the plant. The hypothesis tested is that growing the grandparental genotypes with such contrasting leaf morphology in different environmental conditions would make it possible to identify the most significant traits that would segregate in the progeny when these are growing in the same conditions. It is predicted that these differences will be expressed to the greatest extent when plants are grown in extreme environmental conditions which will favour the extremes of the genetic variation. The roles of water deficit combined with solar irradiance and temperature in two contrasting environments are seen as major factors that would allow expression of the differences through epidermis-guard cell communication in the two mentioned genotypes.

5.2 Aims

The particular aims of this research are to study the morphology and structure of leaves of the two parental species growing in UK and to observe the changes when the same parents are growing in more extreme environmental conditions.

It is expected that a deep knowledge of the morphology and structure in such a contrasting grandparental genotypes would be a valuable tool in the selection of traits to be searched in the F_2 progeny, when they are grown in the same conditions.

5.3 Results

5.3.1 Leaf Area and Specific Leaf Area

Leaves of the two species exhibit differences in shape and leaf area inherent to the genetics of each species, which have been described in the taxonomic description (Chapter 1). The most remarkable feature at a macroscopic level is the presence of thick waxy witish cuticle covering the abaxial side of the leaves of *P. trichocarpa.*

Three features were observed in *P. trichocarpa*, which do not appear in *P. deltoides* when observed with transmission light microscopy x160. Firstly, the cuticle on the abaxial surface is a thick continuous layer that shows the guard cells apparently elevated on this layer. Secondly, the presence of short trichomes on the adaxial side of leaves of *P. trichocarpa*, which are very scarce or absent on the abaxial side and thirdly a larger stomatal size. *P. deltoides* shows a thinner and transparent cuticle in both surfaces, with no trichomes and a smaller stomatal size.

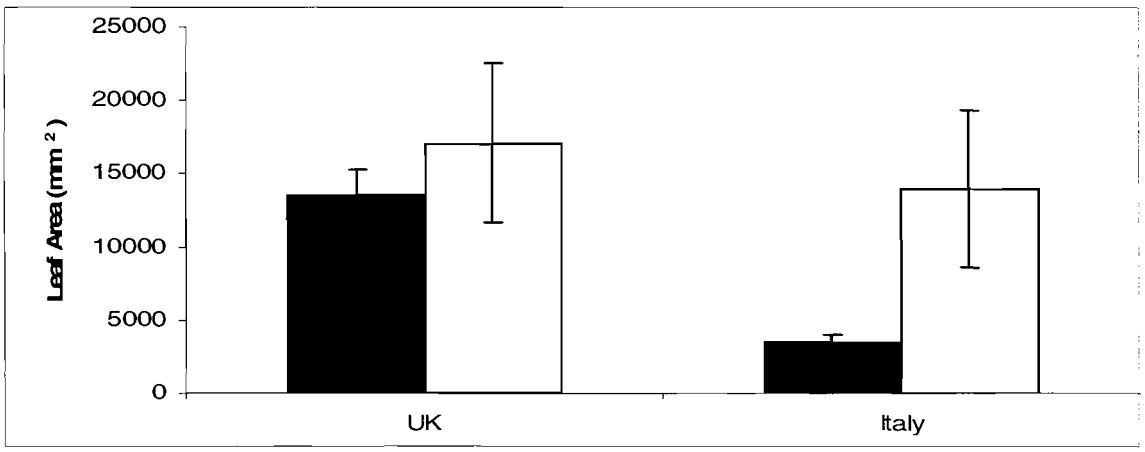


Figure 5.1 Mean individual final leaf area in mature leaves of *P. trichocarpa* (solid bars) and *P. deltoides* (open bars) growing in two contrasting environmental conditions in England (UK) and Italy. Data points are means ± se of 3 leaves for each species at each location. The result of two ways ANOVA were significant at $P< 0.05$.

Individual leaf areas were larger in *P. deltoides* than *P. trichocarpa* at both study sites. *P. trichocarpa* leaves in Italy showed a considerable reduction in their leaf area (74.82%)

compared with those growing in the U.K. (Table 5.1, Figure 5.1), and the leaves had a waxy appearance with slightly revolute margins and with slight brown spotting on the epidermis of the abaxial side. *P. deltoides* leaves also showed a reduction in their area (18.23 %) in Italy compared with England. The differences in leaf area were significant at P <0.05, but there were no interactions in the effects of species and site (Figure 5.1, Table 5.1).

Table 5.1 Mean Leaf Area in *P. trichocarpa* (Clone 93-968) and *P. deltoides* (Clone ILL-129) growing in two contrasting environmental conditions. Means from 3 replicates, s.d. and % of variation are shown. Percentage of difference in the values was calculated with the formula % difference= ((Ita-UK)/UK)*100.

Number	Species	Area (mm^2)	s.d. ±	%	SLA ($mm^2 g^{-1}$)	%	s.d. ±
UK	*P. trichocarpa*	13630.28	1744.22		16514.44		4155.62
UK	*P. deltoides*	17124.39	5433.79		13632.14		637.46
Italy	*P. trichocarpa*	3431.673	660.52	-74.82	8838.77	-46.47	95.65
Italy	*P. deltoides*	14001.41	5359.39	-18.23	8577.86	-37.07	728.97
Summary of 2 Ways ANOVA							
Species		0.015	*		0.238	ns	
Site		0.019	*		0.001	**	
Species x Site		0.157	ns		0.140	ns	

Both parental species showed the same response in SLA. SLA is higher for both species at Headley, UK (Table 5.1, Figure 5.2). *Populus trichocarpa* showed a significantly decreased SLA at the site in Italy (-47.47 %), while *P. deltoides* showed a slightly smaller decrease (-37.07 %). However between species, differences were not as marked at the same site and

there were only small and not statistically significant differences between the two species at both of the sites, but significant differences between sites.

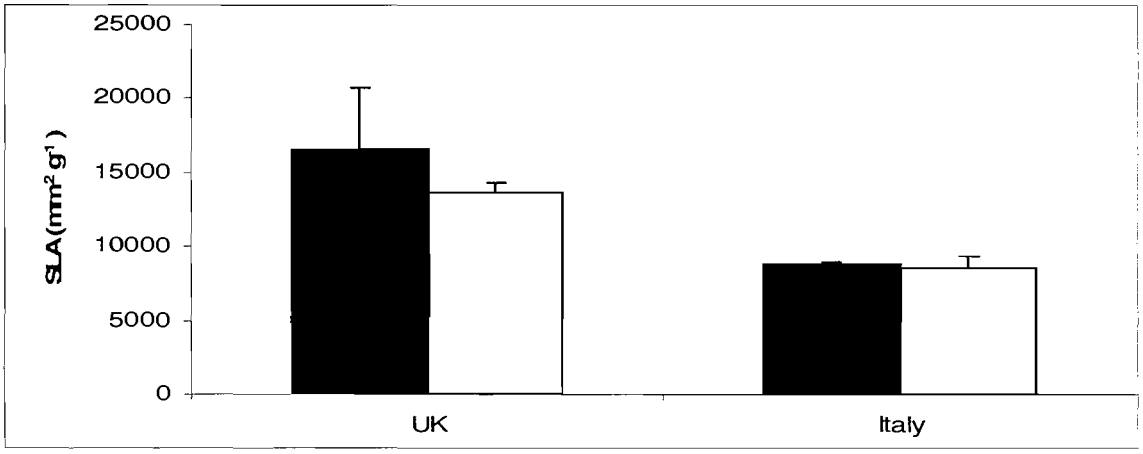


Figure 5.2 SLA (Specific Leaf Area) of *P. trichocarpa* (solid bars) and *P. deltoides* (open bars) at the two study sites. *P. trichocarpa* showed the highest decrease in comparison with *P. deltoides*. Data points are means ± SE of 3 leaves for each species at each location. The result of two ways ANOVA were significant at $P< 0.01$ for species but there were no significant differences for sites or between sites and species interaction.

5.3.2 Transverse sections and SEM

5.3.2.1 Transverse section

The following observations were made on transverse sections viewed with a transmission microscope.

a) Young leaves

Mesophyll of *P. trichocarpa* is well structured when the leaf is young (Figure 5.3). At this developmental stage, cuticle, epidermis, palisade and spongy parenchyma are easily observable. It is important to note that on the abaxial side the spongy parenchyma has extremely large cells with an amorphous shape. Epidermal cells on the adaxial side seem to continue the dividing process, while on the abaxial surface this process is less evident and also guard cells are observable. Large numbers of chloroplasts are observable in the middle of the mesophyll.

In *P. deltoides* the mesophyll is thinner than in *P. trichocarpa* and its cell differentiation still appears incomplete. The nuclei in the epidermis are very noticeable. Epidermal cells are narrower but longer and they are very symmetrical on the two leaf surfaces. Palisade parenchyma and spongy parenchyma is observable, but vascular bundles are still in the differentiation process. Guard cells are still not differentiated on any of the surfaces. A thin cuticle is observable on both sides of the leaf. In Figure 5.3, two images of transverse sections are shown. They show the structures in each of the leaves and the differences between them.

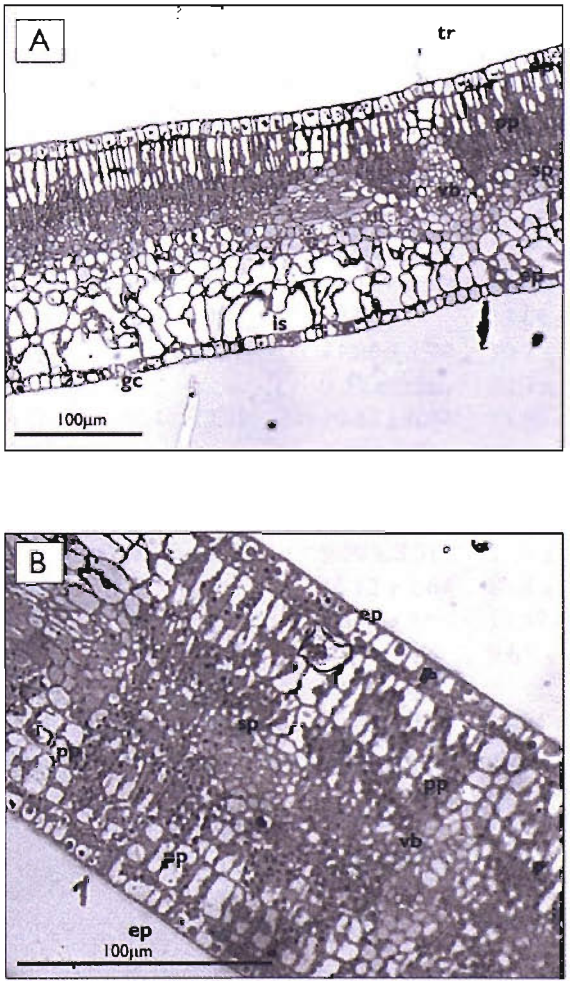


Figure 5.3 Transverse sections of young leaves (LPI 3) of A) *P. trichocarpa* (x20) and B) *P. deltoides* (x40) growing at a field site in Italy. Leaf age was calculated as LPI 3. Legend: ep= epidermal cell; gc= guard cell; pp= palisade parenchyma; sp= spongy parenchyma; Tri= trichome; and vb= vascular bundle.

b) Mature leaves

In a mature leaf, the same structures are observed, with the difference that there are more intercellular spaces between the palisade parenchyma on the adaxial side and the spongy parenchyma on the abaxial side of the leaf seems stretched leaving only thin junctions between amorphous cells and extremely big intercellular spaces. Leaf thickness is almost double in the mature leaf (300 μm) in comparison to the young leaf (175 μm) (Figure 5.4 and 5.5).

Table 5.2 summarises the means of the structures observable in the transverse sections of mature leaves in the grandparents growing in UK and in Italy.

Table 5.2 Leaf structure dimensions for the two study locations. The results are the means of 3-6 values in μm including standard deviation when possible.

	UK		Italy	
Structure	*P. trichocarpa*	*P. deltoides*	*P. trichocarpa*	*P. deltoides*
Mesophyll width	288 ± 3.47	201.60 ±5.59	284.75 ± 2.9	240 ± 4.24
Adaxial epidermal cell width	29.63 ± 9.6	12.56 ± 5.20	22.7 ± 4.27	17 ± 2.75
Abaxial epidermal cell width	36.37 ± 12.92	13.143 ± 0.8	10.4 ± 1.4	11.23 ± 1.23
Adaxial epidermal cell height	20.44 ± 4.7	14.345	13.09 ± 0.3	15.41 ± 2.3
Abaxial epidermal cell height	15.89 ± 1.9	11.26 ± 1.24	10.7 ± 2.54	8.63 ± 0.25
Adaxial guard cell width		9.38 ± 0.56	10.3 ± 1.26	8.95 ± 0.29
Abaxial guard cell width	18.44 ± 2.79	10.43 ± 0.64	13.59 ± 1.72	7.40 ± 2.27
Adaxial guard cell height		9.31 ± 0.21	10.25 ± 0.57	8.68 ± 3.8
Abaxial guard cell height	11.45 ± 0.37	10.23 ± 0.5	10.55 ± 0.48	7.56 ± 1.19
Adaxial subsidiary cell width		12.23 ± 1.76	13.17 ± 0.742	7.92 ± 5.75
Abaxial subsidiary cell width	18.14 ± 6.89	9.79 ± 3.51	9.24 ± 6.41	10.80 ± 0.13
Adaxial subsidiary cell height		16.12 ± 1.64	8.68 ± 1.22	13.5 ± 0.04
Abaxial subsidiary cell height	14.36 ± 6.2	12.66 ± 3.6	10.57 ± 0.24	13.44 ± 0.52
Adaxial palisade cell width	13.02 ± 0.48	9.99 ± 0.74	9.61 ± 1.08	10.93 ± 2.46
Abaxial palisade cell width	absent	8.4 ± 0.46		
Adaxial palisade cell height	46.32 ± 6.83	26.98 ± 0.54	44.64 ± 2.9	34.3
Abaxial palisade cell height	absent	39.43 ± 1.67		
Adaxial cuticle width	1.74 ± 0.32	1.5	2.43 ± 0.233	1.412 ± 0.24
Abaxial cuticle width	1.58 ± 0.16	1.46 ± 0.35	2.46 ± 0.175	2.48 ± 0.7
Adaxial ledge exterior		3.41 ± 0.93		2.64 ± 0.147
Adaxial ledge interior		1.57 ± 0.428		1.134 ± 0.18
Abaxial ledge l exterior			2.82± 0.743	5.08 ± 0.44
Abaxial ledge interior			1.12 ± 0.73	1.89 ± 0.61

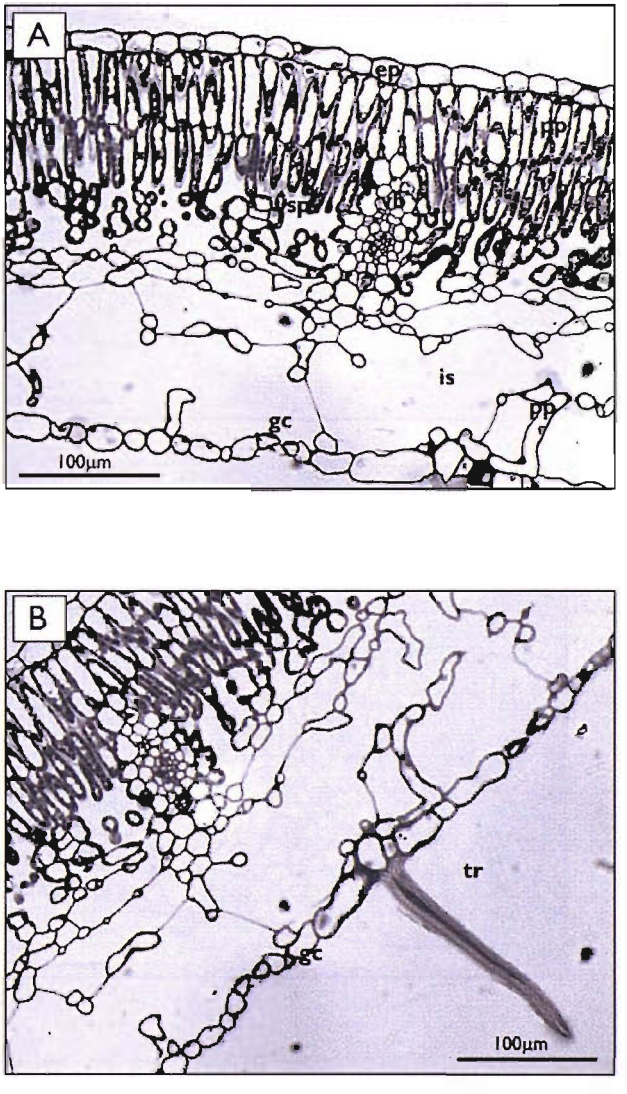


Figure 5.4 Transverse sections of mature leaves of *P. trichocarpa* growing at the field site in UK. A) Mesophyll view showing a well defined palisade parenchyma and a scarce spongy parenchyma – sp- in the middle. The abaxial side of the leaf contains large intercellular – is - spaces. B) The same slide showing a detail of trichome. Labels as in Figure 5.4. Images x20.

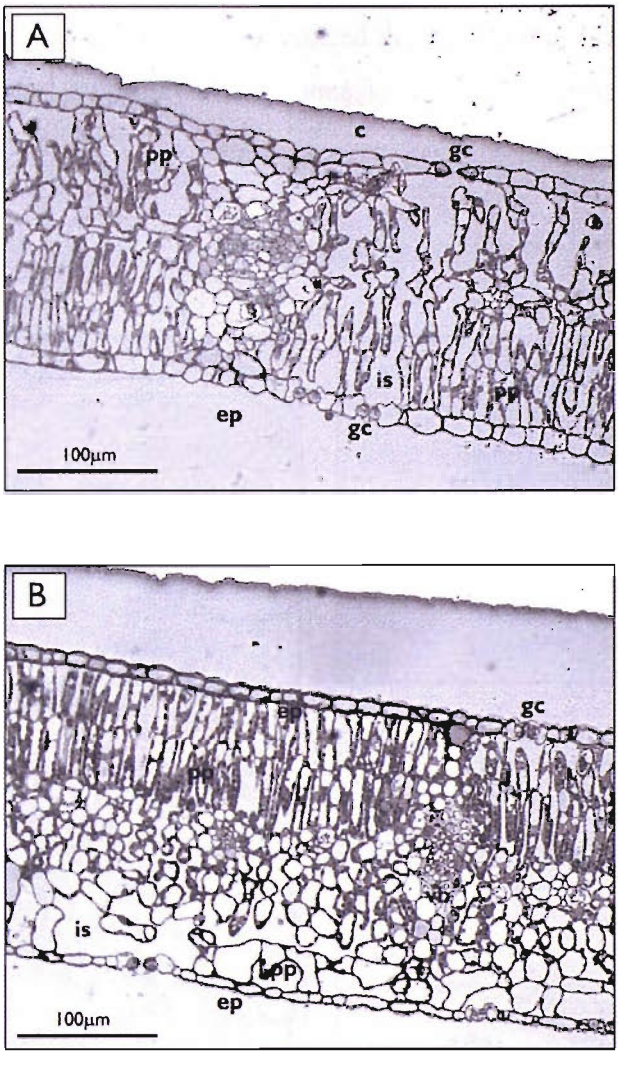


Figure 5.5 Transverse sections of mature leaves of *P. deltoides*. A) Mesophyll from a plant growing in the UK showing a more open arrangement as the leaves are more turgid. B) Mesophyll from a plant growing in Italy showing an increase in the mesophyll thickness, a more compact arrangement of palisade parenchyma – pp - and spongy parenchyma – sp- is observed. A light deformation in palisade parenchyma on the abaxial side is also observed. Labels as in Figure 5.4. Images x20.

Guard cells in *P. trichocarpa* and *P. deltoides* are shown in Figure 5.6. This shows changes in size in the plants growing in Italy compared to those growing in UK. *P. trichocarpa* guard cells were extremely reduced in size, while *P. deltoides* guard cells showed little change. A summary of the dimensions of all the structures observable is presented in Table 5.2.

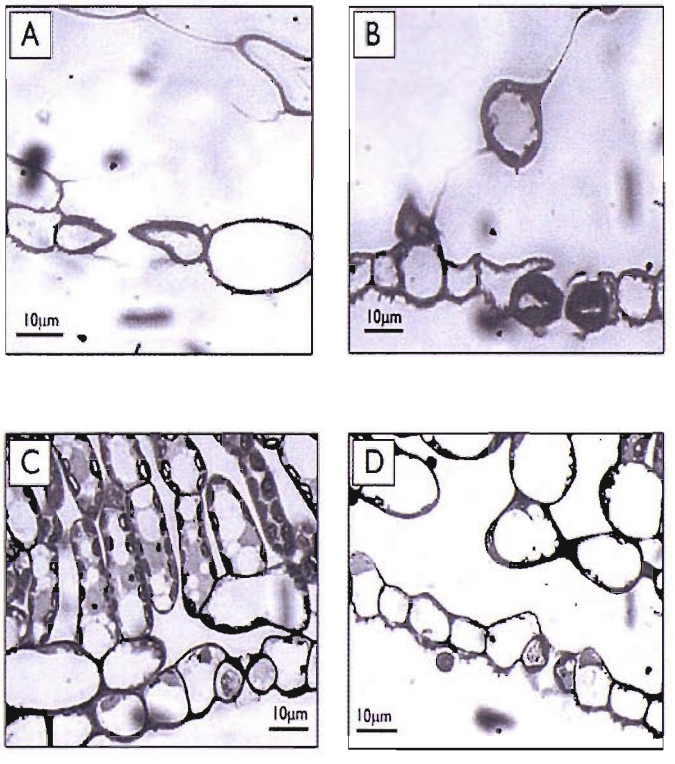


Figure 5.6 Guard cell structure views from transverse sections, at different magnifications. A) *P. trichocarpa* guard cell of a plant growing in Headley Experimental Station UK x100. B) *P. trichocarpa* guard cell showing a remarkable thickening in the guard cell x40 of a plant cultivated in Cavallermaggiore Field Station in Italy. C) *P. deltoides* guard cell growing in UK and D) *P. deltoides* in Italy. Guard cell dimensions in *P. deltoides* are about half that those found in *P. trichocarpa*. All Images x100.

5.3.2.2 SEM

Scanning Electronic Microscopy (SEM) images showed a large difference in cell shape and arrangement between the two species. Stomatal number and wax concentration in *P. deltoides* were increased in Italy (Figure 5.7 C, D) compared to the plants growing in UK (Figure 5.7 A, B). This response was observed on both leaf surfaces.

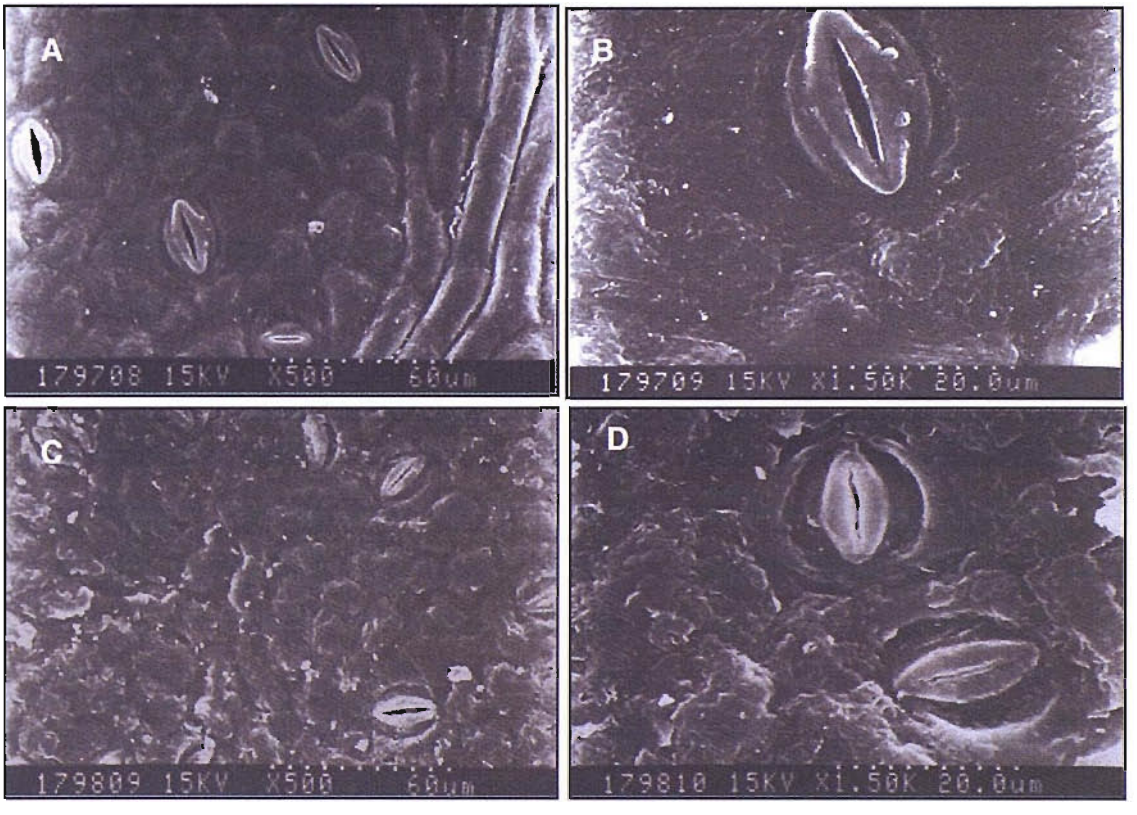


Figure 5.7 SEM of adaxial leaf epidermis in *P. deltoides*. Magnification (x 500 and x 1.50K) is shown on each photo. A and B plants growing in UK; C and D plants growing in Italy. An increase in number of stomata and wax deposition was observed in the plants growing in Italy, compared to those growing in the UK, together with a decrease in the size of stomata.

The adaxial side of leaves of *P. trichocarpa* growing in Italy showed an increase in stomata and wax deposition (Figure 5.8 C, D), compared to plants growing in UK (Figure 5.8 A, B). A reduction in stomatal size and pore size was also observed. Cells on the adaxial side showed a puzzle shape at both locations.

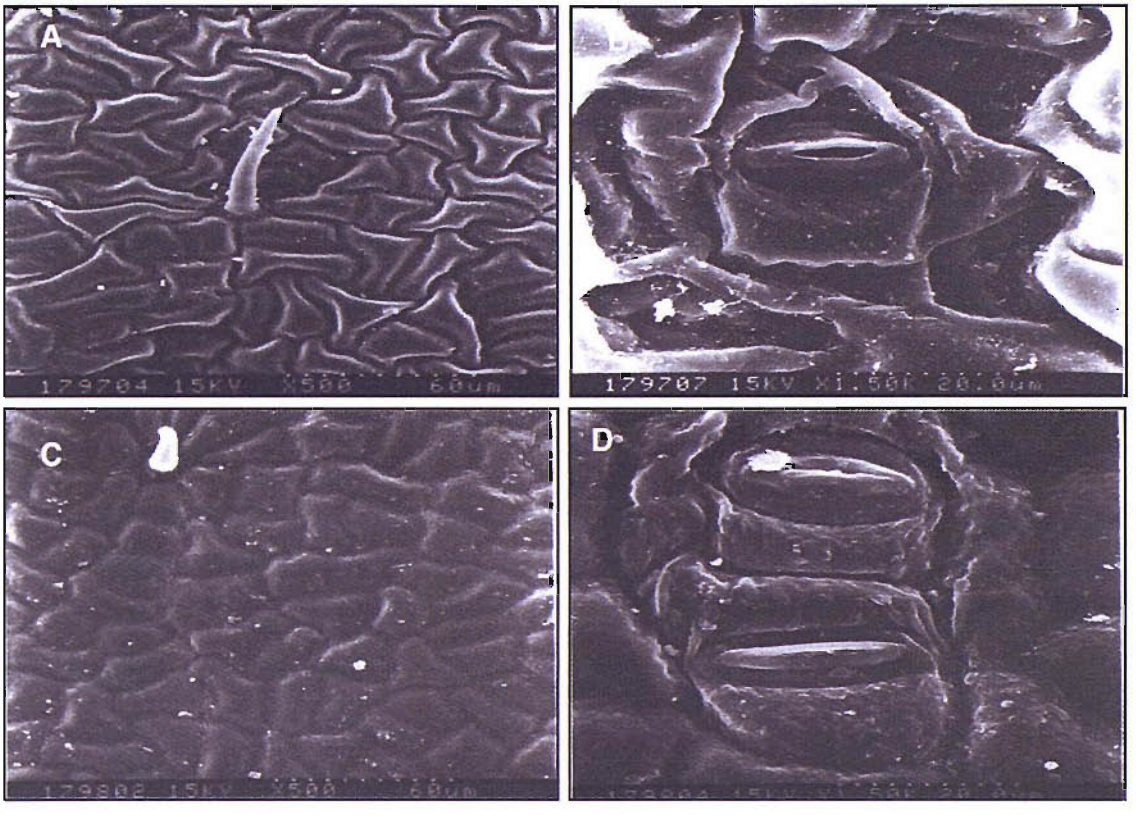


Figure 5.8 SEM of adaxial leaf epidermis in *P. trichocarpa.* Magnification (x 500 and x 1.50K) is shown on each photograph. Plants growing in UK (A,B) and Italy (C,D). An increase in number of stomata and wax deposition was observed in Italy compared to the plants growing in the UK, together with a decrease in the size of stomata.

The abaxial surface of leaves of *P. trichocarpa* showed a complex wax deposition pattern with more wax deposition in the leaves of the plants growing in Italy (Figure 5.9 C,D) than those in UK (Figure 5.9 A,B). The epidermal cells are large and have a puzzle shape. This arrangement was not observed in *P. deltoides,* which has a polyhedral cell shape and a smaller cell size.

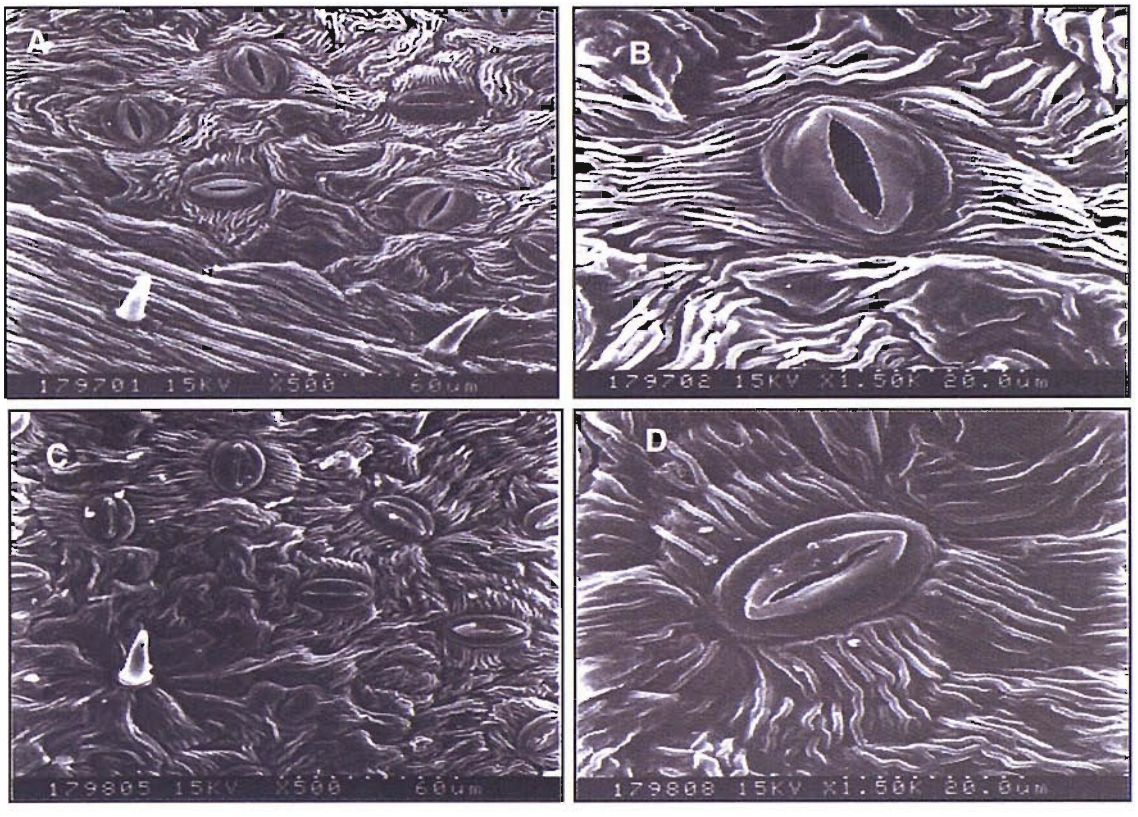


Figure 5.9 SEM of abaxial leaf epidermis in *P. trichocarpa*. A, B), plants growing in the UK; C, D) plants growing in Italy. Magnification as indicated (x 500 and x 1.50K) is shown on each photograph. An increase in number of stomata and wax deposition was observed in Italy with compared to the plants growing in the UK, together with a decrease in the size of stomata.

5.3.3 Stomatal density and stomatal index

In both genotypes, *P. trichocarpa* (93-968) and *P. deltoides* (ILL-129), the stomatal density showed an increase in the plants growing in the experimental field in Italy compared to the UK (Fig 5.10). Stomatal density increased to 164.51 % (adaxial) and 78.76% (abaxial) in *P. trichocarpa* and 67.89 % (adaxial) and 24.63 % (abaxial) in *P. deltoides*, in the leaves of plants growing in Italy (Table 5.3).

Stomatal number in *P. deltoides* does not vary significantly at the two sites (Figure 5.11). The values on the adaxial side in both genotypes maintain the same tendency to increase, even though those in *P. deltoides* are much higher. The differences between the two

genotypes are greater on the adaxial side, where the stomatal density of *P. trichocarpa* is very low.

For the abaxial side, the increase in stomatal density was also much higher in *P. trichocarpa* than in *P. deltoides* (78.76% and 24.63% respectively). The effect of contrasting environment on stomatal density was more pronounced in *P. trichocarpa.*

Table 5.3 Stomatal density expressed as stomatal number per mm^2 and as percentage change in Italy with regard to the UK. ANOVA for the 6 replicates in the two field sites are included.

Species	No. stomata/mm^2	s.d. ±	% change in Italy
UK			
Adaxial			
P. trichocarpa	16.50	0.78	
P. deltoides	163.51	1.16	
Abaxial			
P. trichocarpa	169.51	1.67	
P. deltoides	304.52	1.84	
Italy			
Adaxial			
P. trichocarpa	43.619	0.87	+ 164.33
P. deltoides	274.52	1.16	+ 67.89
Abaxial			
P. trichocarpa	303.02	3.39	+ 78.76
P. deltoides	379.53	2.98	+ 24.63

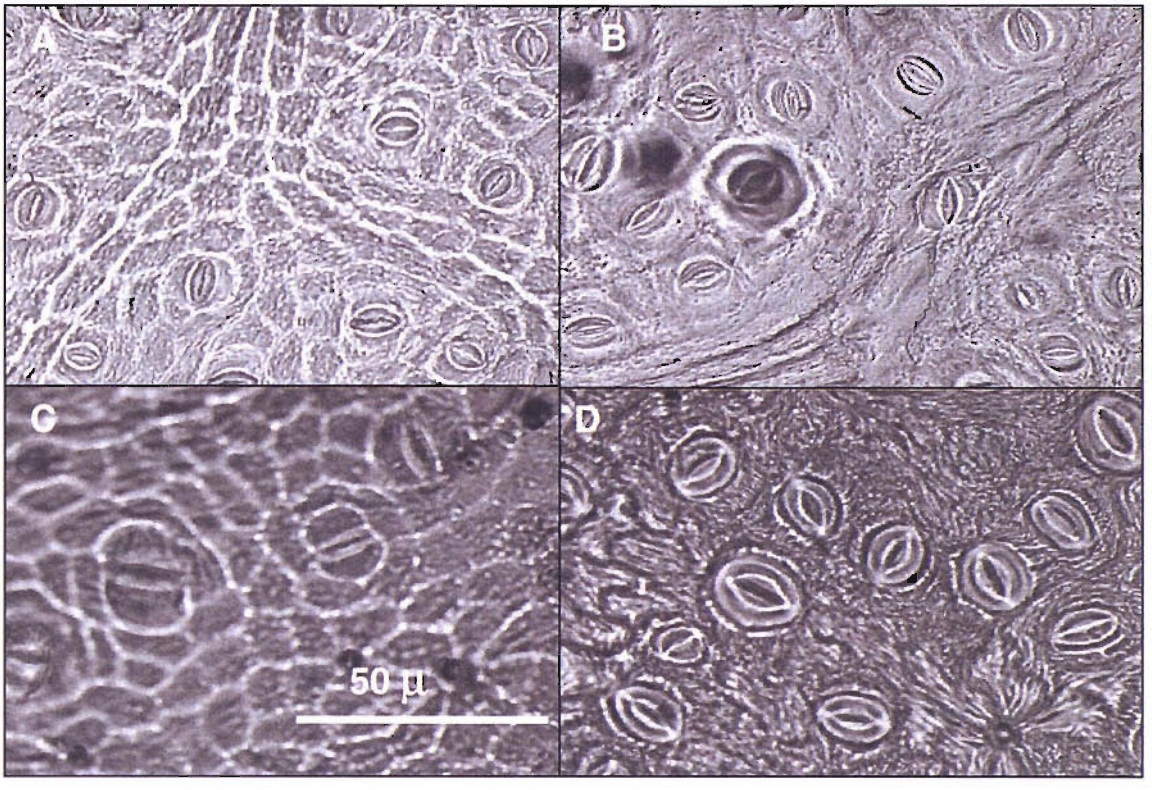


Figure 5.10 Microscopic view of leaf surfaces of *P. trichocarpa* and *P. deltoides* from Italy. A,B) *P. deltoides* adaxial and abaxial side. C,D) *P. trichocarpa* adaxial and abaxial side respectively. Images were taken from epidermal strips at x400 magnification using a Nikon microscope. Photographs were taken using MetaMorph Imaging System (Westchester, Philadelphia U.S.A.).

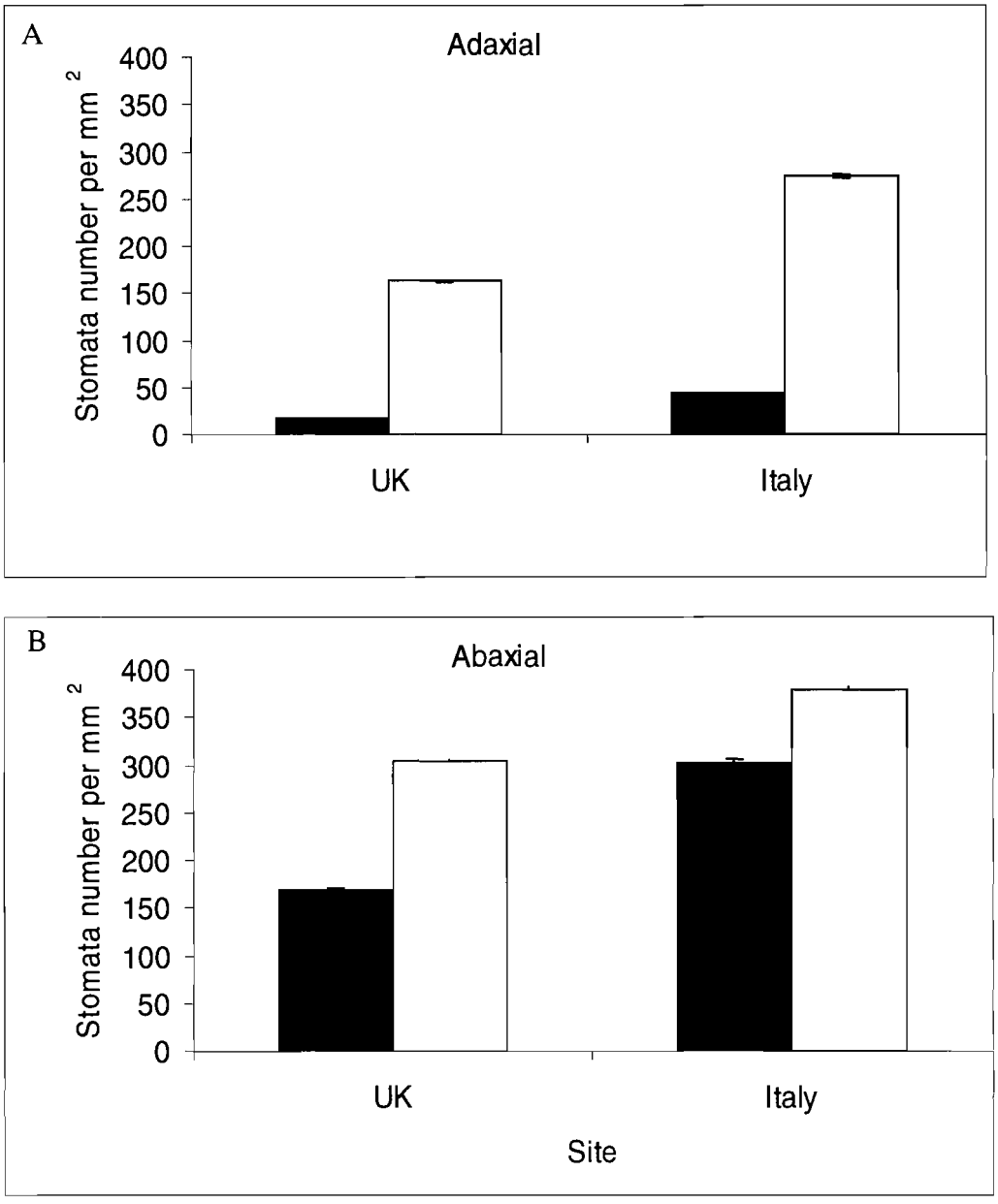


Figure 5.11 Stomatal density in A) Adaxial and Abaxial sides of the leaf for *P. trichocarpa* (solid bars) and *P. deltoides* (open bars) at the two study sites U.K. and Italy. Standard deviation bars are shown.

The values for stomatal density in UK are slightly higher that those reported for the same species growing in open chambers at the same experimental site in 1999 (Ferris et al. 2002), but they are similar to those reported for clones from xeric habitats by Dunlap and Stettler (2001).

5.3.4 Stomatal pore length and width

Stomatal pore length in *P. deltoides* is very similar in adaxial surfaces in the UK and Italy, with reductions of only 2.5%), but it was considerable reduced in the abaxial side in Italy in comparison to the (Figure 5.12). *P. trichocarpa* has not stomata in the adaxial side. The pore length mean for *P. trichocarpa* and *P. deltoides* on the abaxial side decreased by 32.64 and 15.63 % respectively. Pore width in *P. deltoides* adaxial surface was reduced by near 21%, while in *P. trichocarpa* and *P. deltoides* decreased by 53.60 and 44.62 % respectively when they are compared between UK and Italy (Table 5.4). These changes could indicate a decrease in gas exchange in Italy compared to England. In both genotypes, changes are less marked in the adaxial side.

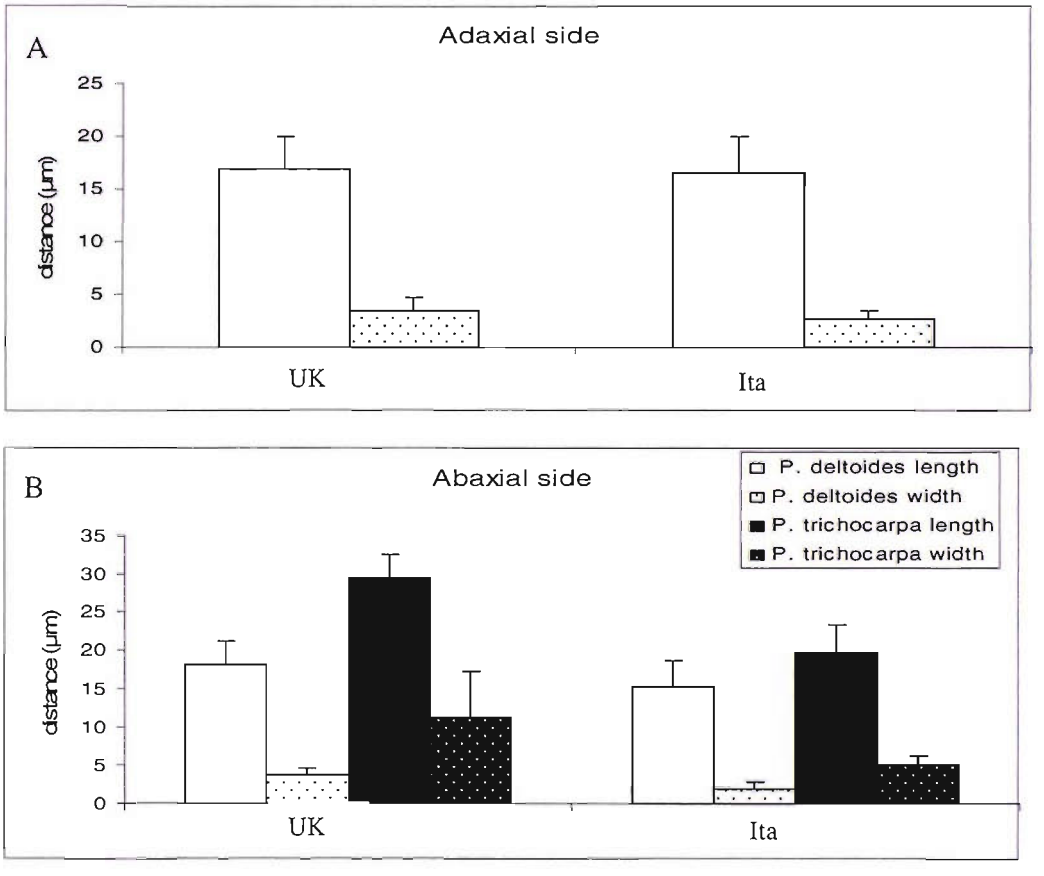


Figure 5.12 Stomatal pore length and width in A) adaxial side and B) abaxial side. A) Stomatal presence on the adaxial side only occurred in *P. deltoides*. B) Stomatal presence in the two genotypes *P. trichocarpa* (solid bars and solid stippled bars) and *P. deltoides* (open bars and open and stippled bars) stomatal pore length and width. Distances are given in µm with bars of s.d. indicated. Pore length is indicated by open and solid bars, width is indicated by open and solid stippled bars as indicated in the legend.

Table 5.4 Stomatal pore length and width in *P. trichocarpa* and *P. deltoides*. The values are means of 5-10 stomata for each of the 6 replicates.

Species	Pore length (μm)	s.d. ±	%	Pore width (μm)	s.d. ±	% change in Italy
UK						
Adaxial						
P. trichocarpa						
P. deltoides	16.97	3.06		3.40	1.30	
Abaxial						
P. trichocarpa	29.50	3.22		11.36	5.98	
P. deltoides	18.23	1.76		3.72	0.93	
Italy						
Adaxial						
P. trichocarpa						
P. deltoides	16.57	3.37	-2.35	2.69	0.75	- 20.88
Abaxial						
P. trichocarpa	19.87	3.60	-32.64	5.27	1.11	- 53.60
P. deltoides	15.38	2.06	-15.63	2.06	0.75	- 44.62

5.3.5 Trichomes

A noticeable increase in trichome number was observed in *P. trichocarpa* grown in Italy compared to plants of the same clone growing in the UK (Table 5.5). Trichome density was used to calculate Trichome index which was increased in Italy in a similar proportion on the two sides of the leaf (Figure 5.13). Trichome density was higher on the adaxial side of the leaf, where usually they are present. However, trichomes rarely can be observed in the abaxial side under normal conditions. These trichomes have a single stem, a transparent testa and a fluorescent base with a very defined and symmetrical pattern of epidermal cells around them (Figure 5.4 B and Figure 5.8 A,C, and Figure 5.10). Even though some small points that could be trichomes were observed in *P. deltoides*, these were not well-defined as in *P. trichocarpa*.

Table 5.5 Trichome density (TD) and Trichome index (TI) of *Populus trichocarpa* on the adaxial and abaxial side of the leaf, from plants growing in two contrasting environmental conditions. The increase in percentage in Italy compared to UK is shown.

Site	TD adaxial	TI adaxial	sd	% change	TD abaxial	TI abaxial	sd	% change
UK	1.98	2.97	0.133507		0.54	0.45	0.483498	
Ita	6.39	5.14	0.867283	73.05	3.06	1.62	0.099357	72.22

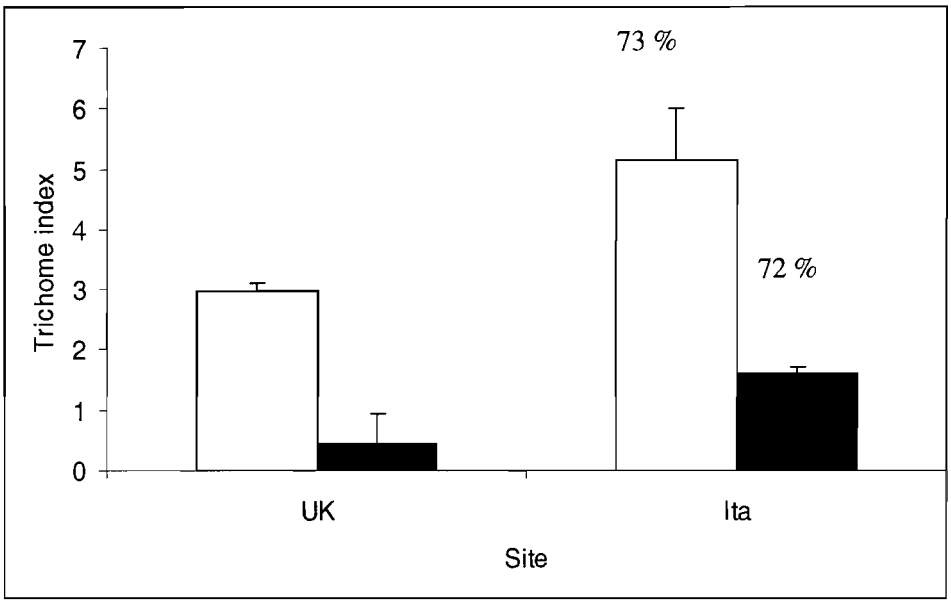


Figure 5.13 Trichome Index on adaxial (open bars) and abaxial (solid bars) sides of the leaf in the UK and in Italy. Trichome index in Italy increased on the two sides of the leaf in similar percentages. Standard deviation is indicated.

5.3.6 ABA content in *P. trichocarpa* and *P. deltoides*

Content of ABA was determined in µg per gram of dry weight (gDWT) in mature leaves in three different locations: Glasshouse, Headley experimental station in UK and Cavallermaggiore in Italy. In Table 5.6 the results for the three sites show that ABA is present in all the replicates for both parental species.

Table 5.6 Abscisic acid concentration in mature leaves of *P. trichocarpa* and *P. deltoides*. ABA was determined in µg per gDWT.

Site	*P. trichocarpa*	*P. deltoides*
Greenhouse	1.15	2.18
Greenhouse	1.5	1.59
UK	1.19	1.75
UK	1.38	1.9
UK	1.19	1.63
Italy	5.35	2.7
Italy	3.25	3.16
Italy	3.61	2.4
Fvalue	5.71	0.57
Pvalue	0.029*	0.458 ns
UK vs. Italy Two –Way ANOVA		
Site	0.001	**
Genotype	0.276	ns
G x L	0.030	*

A single factor ANOVA of the values obtained for *P. trichocarpa* showed that there are significant differences between the plants growing at the three locations, but no significant differences occurred in *P. deltoides* between the different locations (Table 5.6).

ABA production in each parental clone seems to be the same, however in the case of *P. trichocarpa* production of ABA is influenced by the site. A two ways ANOVA was used to examine the interactions between genotype and site (environment). This analysis also showed that genotype had no significant effect, but that there was a significant between site effect ($p = 0.01$), and site-genotype interaction ($p < 0.05$) (Table 5.6).

These results indicate that the environment influences ABA production in leaves of *P. trichocarpa* and *P. deltoides* in a differential manner. Figure 5.14 shows the concentrations of ABA in both genotypes at the two field sites. The ABA concentration in both genotypes increased in Italy but *P. trichocarpa* shows a higher increase and also a higher standard variation.

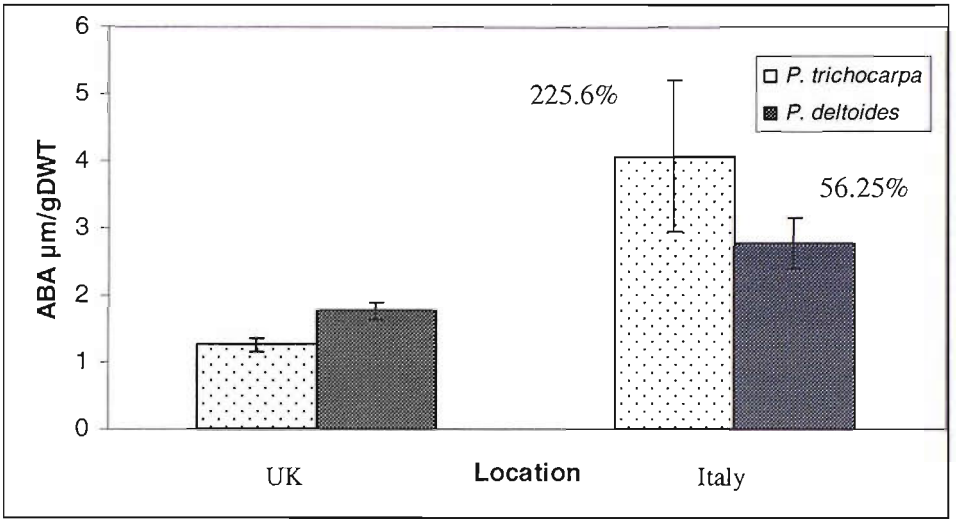


Figure 5.14 ABA concentrations in leaves of the parental species growing in two field sites. ABA values (µg/gDWT) are means of three replicates. Percentage increase is Italy in indicated. Standard deviation bars are shown.

5.4 Discussion

5.4.1 Leaf Area and SLA

Leaf area and Specific Leaf Area (an indirect estimate of leaf thickness), were reduced in the plants of both species growing in Italy especially in *P. trichocarpa*. It is remarkable that SLA does not decrease in *P. trichocarpa*, in the same proportion as leaf area, a fact that suggest a leaf thickening in response to the environmental conditions in Italy. *P. deltoides* showed small decreases of leaf area and SLA in Italy, and leaf thickness is affected less than in *P. trichocarpa*. This increase in weight could reflect the increase of cuticle thickness and the metabolites accumulated in the leaf. A leaf thickening has also been observed in other *P. trichocarpa* clones in xeric regions (Dunlap et al. 1993, 1994, 1995).

5.4.2 Transverse sections

One of the most distinctive features in leaf structure found is the dorsoventrality in leaf blade, where the spongy parenchyma on the abaxial surface of *P. trichocarpa*, a difference from *P. deltoides*, is replaced by lacunar spaces. Parenchyma and intercellular spaces are better defined in young leaves than in mature leaves where the spaces are more extendend and more amorphous. This tissue resembles what is known as aerenchyma comprising a high proportion of gas filled lacunae that is found in aquatic plants and which functions to provide the plant with an alternative strategy for obtaining O_2. Spaces form either by cell separation at the middle lamella during development (schizogeny) or by cell death and dissolution (lysigeny). Lysigenous aerenchyma provides not only an internal pathway for O_2 transfer, but also simultaneously reduces the number of O_2 consuming cells, a feature that would prevent them from functioning in O_2 transfer.

Intercelullar spaces are a feature in spongy mesophyll related to stomatal frecuency, they increase with ontogeny and changes dialy. Its presence determines the velocity of gas exchange (Bolhar-Nordenkampf and Draxler 1984).

Aerenchyma formation is inducible by flooding in maize. In the coastal grass and in other native species, both mono and dicot that occupy wetland habitats (Drew et al. 2000) and its presence in the riparian genotype of *P. trichocarpa* is not surprising.

The differences in cell shape between the two species suggest that their cell division and extension may be different. This could explain the results in cell expansion of *P. trichocarpa* and *P. deltoides* obtained by Street et al. (2006). They proved that *P. deltoides* has a very active cell division and a large leaf area, while *P. trichocarpa* produces fewer and larger cells and has a smaller leaf area. Our microscopical analysis showed that *P. trichocarpa* cells have a distinctive tubular cuticular wax arrangement that seems in principle different to that in *P. deltoides*. The study of the waxes in *Salix* and *Populus* cuticles shows that its production is species specific and that their role in drought tolerance, pest and pathogen resistance may be a useful selectable trait in a breeding programme (Cameron et al. 2002).

5.4.3 Epidermal traits

The epidermal and stomatal traits were further separated by leaf side since leaves of *P. trichocarpa* are dorsiventrally distinct (Van Volkenburgh and Taylor 1996).
The distinct increase in stomatal density on the abaxial leaf surfaces of both species in Italy, agrees with the response obtained in other clones of *Populus*, which is considered a response to acclimatization to a xeric habitat (Rogers et al. 1989, Dunlap et al. 1993, 1994, 1995). In regions where the temperatures are 26-32°C and 24-26°C, stomatal size also decreased, agreeing with our results of a decrease in pore length and width in the plants growing in warmer conditions than in England. However, stomatal pore length in *P. deltoides* was hardly affected in contrast to stomatal width, a more variable trait in response to environment. The number of stomata per mm^2 in *P. trichocarpa* (303) increased distinctly in Italy compared with a xeric place in Yakima (206) in the Cascade Mountains (Dunlap and Stettler 2001).

Broad-sense heritabilities averaged 72% in the epidermal and stomatal traits in *P. trichocarpa* indicating moderately strong genetic control in most of them (Weber et al. 1985, Dunlap and Stettler 2001). The existence of a xeric *P. trichocarpa*, with thinner leaves greener beneath, with smaller, densely packed stomata, and higher photosynthetic rates in the two surfaces (Dunlap and Stettler 2001) shows the existence of clonal variation that probably reflects adaptation to the environment. As in this study, Dunlap and Stettler reported smaller stomata and stomatal pore length and aperture in *P. trichocarpa* in drier places. In contrast *P. deltoides* is very responsive to water deficit, decreasing size and stomatal pore length and width when it was submitted to water deficit in Italy.

5.4.4 Trichomes

The role of the trichomes found in *P. trichocarpa* and the reason for the increase in their number in Italy is still not clear. However, it is expected that as in *Arabidopsis*, they could play a role in the promotion of new cells which could later become stomata. The arrangement, development and maturity of the trichomes on the epidermis seem to have a defined pattern and it is particularly significant that they start from large, undefined interveinal cells. The trichome density increases on both sides but is greater on the adaxial side. The base of the trichome is particularly responsive to fluorescence in the epidermis and in the stomata, being stronger under UV or blue light, but the meaning of this is still not clear and more work is needed. The higher trichome density on the adaxial side of the leaf could be explained as differential responses by the adaxial and abaxial surfaces to maintain an energy balance in the leaf of *P. trichocarpa*. The trichome would help in water acquisition from the air and at the same time promote the number of stomata to cool the leaf.

The morphological traits we have mentioned can be indicators of water stress including leaf area, specific leaf weight, and chlorophyll content. They also help to predict changes in photosynthesis, water vapor exchange and water potential (Baldocchi et al. 1987, Iacobelli and McCaughey 1993, Rowland et al. 2001).

Physiological traits important for plant responses to stress are often quantitative rather than qualitative, suggesting that they are determined by several gene products. Many complex traits, such as a large leaf area and stomatal index, are unlikely to be entirely described by the inheritance of a single gene (Bradshaw and Stettler 1995, Wu et al. 1997, Ferris et al. 2002). We consider that the traits studied represent strong features to indicate acclimatization to hotter and drier environments. In particular wax and trichomes are features easy to observe and which need more complete study. Cell shape and stomatal density can also serve as good indicators of adaptation to xeric environments. These traits could be traced in the progeny to assist in the search for new signals for clones best adapted to drought (Davies et al. 2002). The study of epidermal cell number and cell area (see chapter 6), will provide more elements that can support our research.

5.4.5 ABA presence

The work on ABA content shows the ability to produce ABA in the two genotypes studied. *P. deltoides* ABA concentrations do not vary significantly between the glasshouse and the experimental field conditions as the ANOVA analysis showed, while the opposite occurs in *P. trichocarpa.* In this last genotype in well irrigated plants the concentration of ABA is slightly lower than in *P. deltoides*, but in drier environments ABA production increases considerably, which could be interpreted in two ways: Firstly *P. trichocarpa* could be more affected by the stress caused by a dry environment resulting in ABA concentrations higher than in *P. deltoides*, or secondly the difference in concentrations reflects the high stomatal sensitivity to drought in *P. deltoides* and the delay or lack of stomatal response in *P. trichocarpa.*

As the ABA quantification was determined in mature leaves, it would be very interesting to know the results for recently developed and juvenile leaves. We know (from Chapter 1) that juvenile and mature leaves maintain the same photosynthetic and stomatal conductance rates in well irrigated plants in control conditions, but we do not know how this process varies in field conditions. The knowledge of how the gas exchange process occurs in

mature plants at the two locations would therefore be interesting and would show the role of ABA in stomatal regulation in these contrasting environmental conditions in the field. Also, gas exchange variation linked with leaf morphogenesis in controlled and field conditions would be important.

5.5 Conclusions

This study showed the distinctive characteristics in the leaves of each species and also showed how these features were modified when they grow in a harsher environment (Italy).

The morphology of *P. trichocarpa* leaves was affected more by the conditions in Italy than that of *P. deltoides* leaves. This may reflect a greater effect of harsh conditions on the growth of *P. trichocarpa* leaves and a better response to those environmental conditions in *P. deltoides*.

In addition, spomgy mesophyll structure in *P. trichocarpa* is very different to that in *P. deltoides*. The main feaure of this is the presence of large cavities in the mature leaf, a fact that has been mentioned before (Volkenburgh and Taylor 1996). The presence of the cavities could be explained by the ontogeny of intercellular spaces (Bolhàr-Nordenkampf and Draxler, 1993) and the formation of aerenchyma in *P. trichocarpa* as in other species (Drew et al. 2000). The function of this would be to make available more O_2 to the plant in conditions of flooding, something that could happen in a riparian ecosystem. This hypothesis is reinforced by the fact that in our work, this aerenchyma formation is observable in the different leaf developmental stages in *P. trichocarpa*. The presence of big lacunae joined walls by stretching with age and dissolving seems to reinforce this idea. In adition, *P. maximowiczii*, another member of the Tacamahaca section in *Populus* (Reich 1989) seems to have the same structure observed in mature leaves in *P. trichocarpa*.

The variation in wax and trichome presence was one of the most easily observable changes, as in both genotypes it was registered an increase. It also seems that cell wall properties are between the factors related with stomatal conductance and therefore gas exchange, as it was showed in chapter 4.

Particularly important are the changes in stomatal density and trichome number because these parameters may provide information on adaptability. These, and the decrease in leaf area and presence of trichomes are easily observable in the plant and they could be

considered as good traits to follow in the progeny. Even though root evaluations were not made in this experiment, it is known that *P. trichocarpa* has a shallow root system which differs from that of *P. deltoides*. The increase of trichomes on the leaf in *P. trichocarpa* when grown in Italy could be linked with the proliferation of roots in order to increase water supply.

The reduction in leaf growth of *P. trichocarpa* in contrast with *P. deltoides* shows that the conditions of high solar irradiance and temperatures found in Italy, are not suitable for providing the good growth rates that have been reported. However, it would be interesting to see the response of these two species in the same conditions when the minimal dose of water is applied to them.

From the work reported in this chapter it is not possible to draw firm conclusions on the role which changes of leaf and cell structure have in drought response of poplar. However the work has identified some key morphological responses to drought which are shown by two grandparental genotypes of Family 331 (*P. trichocarpa* (93-968) and *P. deltoides* (ILL-129), linked to stomatal dynamic and ontogenic changes found in Chapter 4.

Chapter 6

Populus leaf growth in the grandparents, parents and the F_2 hybrid Family 331 growing in contrasting environmental conditions

6.1 Introduction

As previously mentioned, poplars have been accepted as a model tree for forestry. There have been intensive *Populus* breeding programs around the world which have relied upon interspecific hybridization and F_1 heterosis (hybrid vigour) to make genetic gains in stem volume growth (Bradshaw et al. 2000, Taylor 2002). Their advanced genomics offers a good platform to gain insight into biomass producer regulators.

The understanding of how much genetic variation is available in organs such as leaves that critically affect tree growth, is vital. Leaf development and area display is fundamental in light interception and therefore in carbon gain (Ferris et al. 2001). Leaf growth is also highly responsive to the environment and its responses to environmental factors are clearly observed in the cell production and cell expansion processes that determine leaf growth. The accurate knowledge of these processes and how to manipulate that variation through selection and breeding programmes (Bradshaw et al. 2000, Ferris et al. 2001, Taylor 2002, Wullschleger et al. 2002a) has a vital role in revealing the genetics of variation.

When submitted to different environmental conditions, leaves often show large increases or decreases in area, changes in shape and initiation, as well as reduction or increases in stomatal number, and changes in cell production and expansion have been recorded in tree species, especially in response to eCO_2 (elevated CO_2). Although stomatal and epidermal cell development has been widely studied (Ferris et al. 2001), little is known about the genetic basis of leaf morphological variation in forest trees and there is no information on how leaf stomatal and cell traits are genetically and developmentally correlated (Ferris et al. 2002). However, the results obtained in the studies of *Populus* growth and epidermal traits in eCO_2 by Ferris et al. (2001, 2002), have shown that leaf epidermal traits give information about the traits critically linked to growth that are suitable for further studies. Information about the traits can be obtained by QTL analysis.

In this context, the leaf, the principal plant structure responsible for capture of light and CO_2, and therefore critical in biomass production, is a good structure to use as an

indicator of the effects of different environmental factors. Leaf arrangement and leaf morphology vary at microscopic and macroscopic levels. It is considered that this variation reflects different trade-offs which allow plants to maintain an equilibrium between the performance of photosynthesis and its other functions such as mechanical support, prevention of water loss, regulation of energy exchange and defence (Parkhurst and Loucks 1972, Gates 1980, Givnish 1986, Niklas 1999, Press 1999).

Therefore, the study of leaves under different environmental conditions could show evidence of traits that are expressed in the clones as a consequence of environmental changes. This will identify the variability of the traits which are measured within the F_2 Family. These responses could also show some of the changes that occur in the epidermis; a multifunctional tissue of which development and morphogenesis is not completely understood. Trichomes, stomata, cuticle and waxes and their differentiation processes are likely to be important amongst the morphological features which respond to environment (Glover 2000).

In this study, the responses that the leaves in a second generation of a hybrid *Populus*, Family 331, have at macro- and microstructural levels when they are grown under extreme environmental conditions were studied. Also included were the growth responses of the original parental species (grandparents) which were sampled in a separate set from those used for Chapter 5 and of the parents (F_1), in order to compare the responses in all the related genotypes. With regard to changes in leaf, Ferris et al. (2002) provided preliminary evidence that leaf stomatal and cell traits were amenable to QTL analysis, and concluded that these traits are robust enough to be developed for accelerated tree breeding programs and potentially to be traits related to high biomass productivity. These traits appear to show a continuous range of variation in a population, which is more or less normally distributed (Kearsey 1998). Consequently these traits can be used as indicators of response under different environmental conditions.

The study of epidermal cells will enable us to understand the pattern and relationship between pavement cell, and stomata and trichome differentiation in *Populus*, as described in *Arabidopsis* (Glover 2000). Glover found that pattern formation occurs through the cell lineage mechanism that give place to stomatal complex formation, or

cell interactions which may reinforce any tiny initial differences to produce significantly different cells that may adopt different fates, as in the case of trichomes. The understanding of stomatal development in *Populus* would help to show the basis of fast growth and biomass productivity in this model tree.

6.2 Aims

This study aims to characterize the leaf growth of the grandparents, parents and all F_2 hybrid progeny from Family 331, when they are grown in extreme environmental conditions in two locations in Europe, where the main differences are in water supply and solar irradiance. This should allow identification of changes in morphological characteristics in an F_2 pedigree that can used as indicators of adaptation to drought.

Our particular objectives were:

- to assess the effects of contrasting environments (mainly differing in soil moisture and temperature), on leaf development in *Populus*.

- to identify those traits useful in selecting the more drought tolerant and productive genotypes at a macro- and microstructural level.

- to give information for use in further QTL analysis.

6.3 Materials and Methods

The materials and methods used in this chapter are described in Chapter 2. In order to compare growth in the original parental species or grandparents, parents (F_1) and progeny (F_2), the responses of each one of them were separated in tables and indicated in the graphs.

In this chapter, only the responses of the 26 traits evaluated are presented, while the distribution of these traits in all the 210 genotypes studied is presented in Chapter 7, together with the QTL analysis.

6.4 Results

6.4.1 Leaf traits

The grandparents of Family 331, *P. deltoides* and *P. trichocarpa*, differ in leaf and growth characteristics (Figure 6.1 A-C). In both the UK and Italy there were differences between the two grandparents of Family 331 as follows: *P. deltoides* had longer and wider petioles which also had larger diameter, larger width/length ratio, higher leaf areas, lengths, width, and dry weights than *P. trichocarpa*. However, *P. deltoides* had marginally lower SLA than *P. trichocarpa* in the UK and that difference is maintained in Italy. Leaf extension rate (LER) was greater in *P. trichocarpa* than in *P. deltoides* at both sites (Table A6.1).

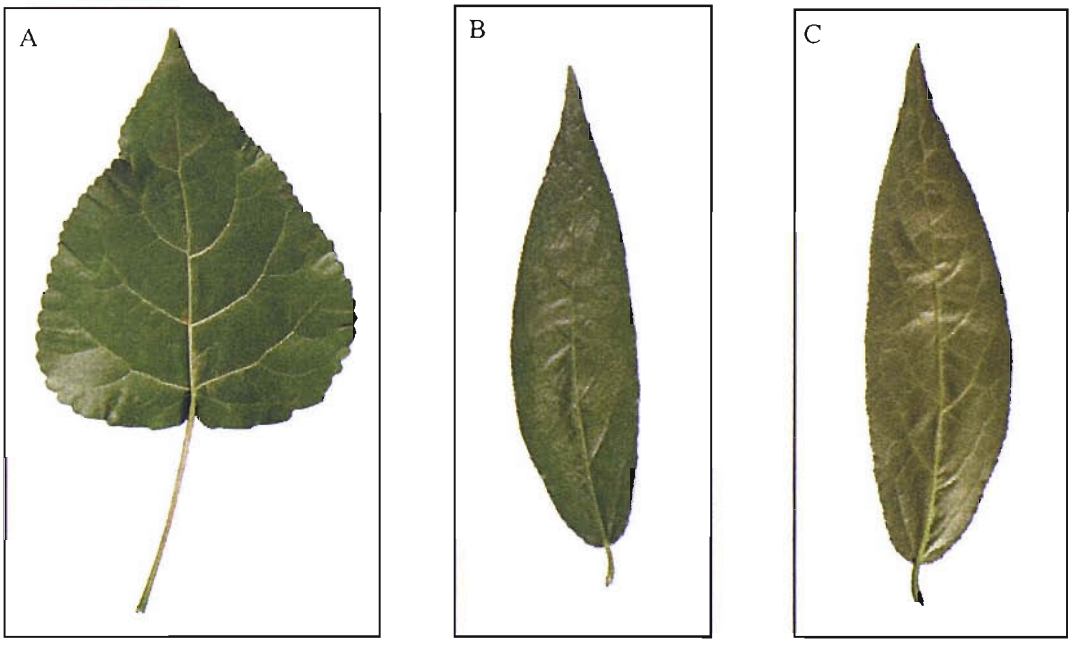


Figure 6.1 Leaf shapes of the Family 331 grandparental genotypes. A) *P. deltoides*, B) and C) *P. trichocarpa*. *P. deltoides* adaxial and abaxial surfaces are very similar. *P. trichocarpa* B) adaxial and C) abaxial surfaces are different. The adaxial side has trichomes and almost no stomata while the abaxial side has a whitish and very reflective waxy cuticule.

Means of all traits evaluated for the grandparental and F_1 clones are shown in Tables A6.1 and A6.2 in Appendix 6 respectively. The means for leaf area, leaf length, width, width/length ratio, dry weight, specific leaf area (SLA), petiole width, height and LER for all Family 331 are summarised in Table 6.1.

P. trichocarpa showed the largest decreases in Italy, except in SLA, where the decrease shown by *P. deltoides* was slightly larger. For *P. trichocarpa*, the smallest decrease in Italy was of petiole width (8 %) and the largest decrease was of leaf extension rate (LER) (78.23 %). In both grandparents, LER showed the greatest difference between the two environments and petiole height increased considerably in *P. deltoides* (52.5 % - Figure 6.2 A).

In the UK, *P. deltoides* showed higher values in leaf area, length, width, dry weight and petiole length than *P. trichocarpa*, but SLA and LER were higher in *P. trichocarpa.* The petiole traits were different for each genotype, as *P. deltoides* has a flattened petiole while in *P. trichocarpa* it is more rounded, and therefore the variation in the two dimensions measured is less significant. Petiole width and height increased in *P. deltoides*, but decreased in *P. trichocarpa* in Italy

Leaf trait values in the genotypes 242 (♂) and 246 (♀) (F_1 parental generation) grown in both sites were greater than those of the grandparents (Table A6.2). G 242 showed values slightly higher than G 246 in most of the traits measured. Similar values were observed in leaf area and SLA and in clone 242 the standard deviation is much higher than in clone 246. In the plants growing in Italy the same trend as in the grandparents was observed, as all the leaf traits had lower values. Exceptions are petiole height, dry weight and width/length ratio values in G 242 which increased in the same way as in the male parent *P. deltoides*. Width/length ratio showed little difference (with a variation of 1.3%), while dry weight increased by 12.7% and petiole height, increased considerably, by 110.5%.

The leaf traits of Clone 242 that showed a reduction in plants grown in Italy were leaf area (-34.7%), leaf extension rate (-87.50%) and SLA (-41.4%), while less affected traits were petiole length, followed by leaf width and leaf length (-6.31, -19.03, -19.63% respectively).

Nine Clone 246 leaf traits decreased in Italy compared to UK: only petiole width, showed a moderate increase (38.38%). The most negatively affected leaf traits were LER (-76.54%), leaf area (-50.9%), and SLA (-37.35%). The least affected traits were width/length ratio (-3.03%), petiole length (-13.84%) and petiole height (-18.29%).

Table 6.1 Leaf and cell traits in F_2 genotypes of Family 331 growing in two contrasting environmental conditions. Means of 26 traits are included. Values were obtained from 3 replicates including standard deviation. Percentages difference represent the differences between plants growing in Italy with regard to those growing in UK, according to the formula % variation = ((mean Italy-mean UK)/(mean UK))*100.

Trait	UK			Italy			% difference
	mean		sd ±	mean		sd ±	F_2
Leaf							
Leaf area (mm^2)	13185		4331	5968.9		2785.7	-54.73
Leaf length (mm)	168.32		27.26	113.6		25.6	-32.51
Leaf width (mm)	114.4		22.27	74.24		21.56	-35.10
Width/length ratio	0.68	**	0.08	0.65	**	0.1	-4.41
Dry weight (g)	0.97		0.37	0.79		0.42	-18.56
SLA ($mm^2\ g^{-1}$)	13814.8	***	1390.6	8242.75	**	3036.64	-40.33
Petiole width (mm)	1.69		0.31	1.53		0.33	-9.47
Petiole height (mm)	2.91		0.57	2.46		0.7	-15.46
Petiole length (mm)	61.92		14.2	44.46		14.78	-28.20
Leaf rate extension (mm)	12.98		3.4	1.94		1.32	-85.05
Adaxial Cell Traits							
Cell area (μm^2)	666.8	**	149.5	370.84	*	114.3	-44.39
Cell number (mm^{-2})	225.6	**	50.8	430.7		136.9	90.91
Stomatal density (mm^{-2})	83.69	**	32.5	124.2		48.2	48.40
Stomatal Index	5.01	***	1.8	4.13		1.6	-17.56
Trichome density (mm^{-2})	3.28		3.46	13.35	*	9.34	307.01
Trichome index	0.2		0.22	0.46	**	0.39	130.00
Abaxial Cell Traits							
Cell area (μm^2)	711.6		169.5	392	*	135	-44.91
Cell number (mm^{-2})	215.8	*	59.4	415.6		133.5	92.59
Stomatal density (mm^{-2})	186.9	*	40.9	262.55		68.7	40.48
Stomatal Index	11.3		2.3	9.7	*	5.35	-14.16
Trichome density (mm^{-2})	1.3		2.13	6.7		6.07	415.38
Trichome index	0.09		0.157	0.25	*	0.26	177.78

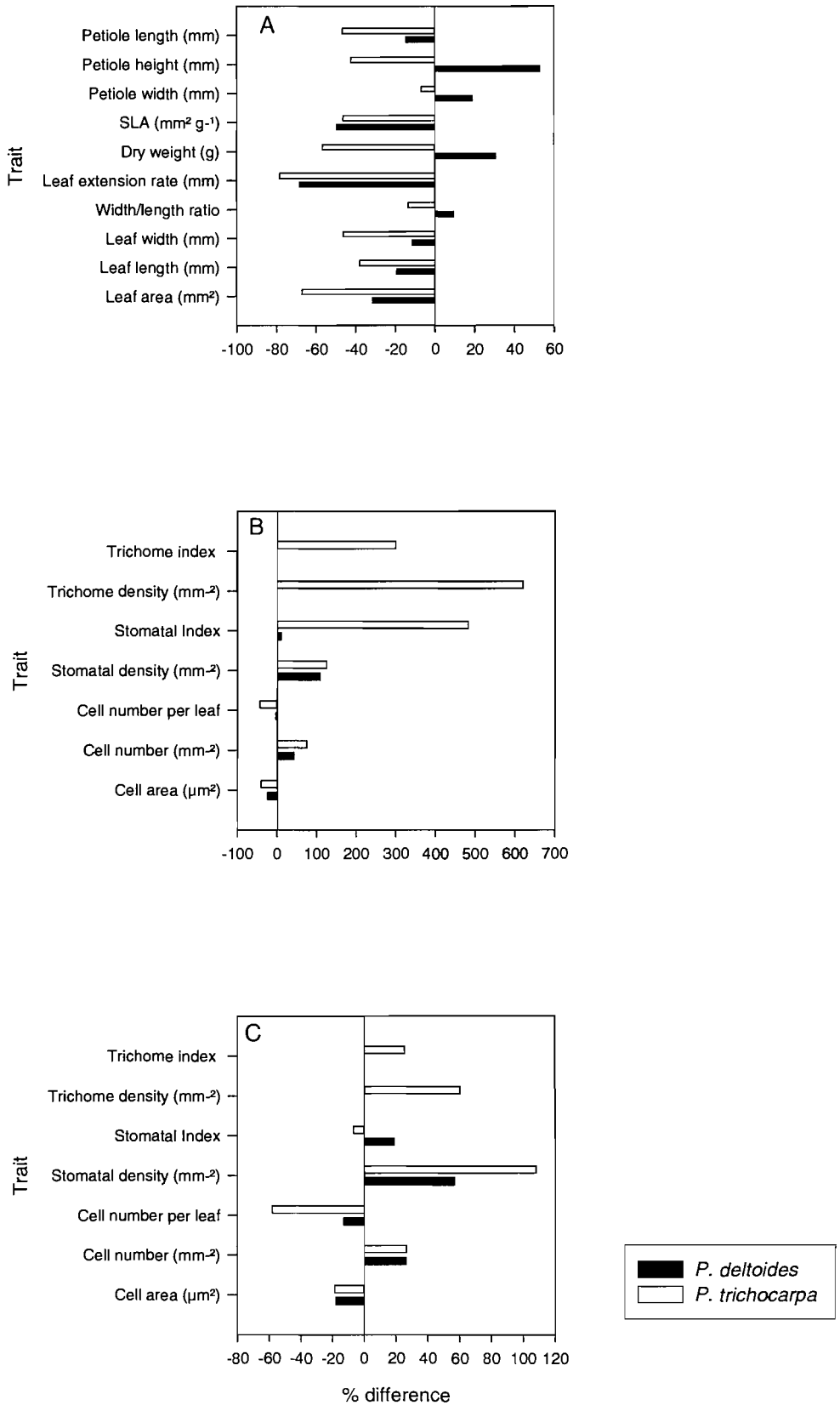


Figure 6.2 Percentage difference of traits evaluated in Italy compared to the UK for the grandparental genotypes. A) Leaf traits, B) adaxial and C) abaxial cell traits. *P. deltoides* (black bars), *P. trichocarpa* (open bars). + and - values represent an increase and decrease respectively.

The F_2 pedigree exhibited marked transgressive segregation in different traits (Table 6.3), and also leaf shape was very variable. Mean SLA for the F_2 genotypes grown in the UK was marginally smaller (13814.8 mm^2/g) than in the grandparents (14271 and 15728 mm^2/g for *P. deltoides* and *P. trichocarpa* respectively) and slightly larger than the F_1 (12921 and 12921.52 mm^2 for clones 242 and 246 respectively), while mean leaf area was almost half (13183 mm^2) that of the F_1 parents (29251.27 and 28589 mm^2 in genotypes 242 and 246 respectively) (Tables A6.1, A6.2 in Appendix 6 and Table 6.1). The ranges in mean leaf area of the F_2 were from 4000 to 30000 mm^2. In Italy, the F_2 genotypes showed the same response as the grandparental species and parental genotypes. All the leaf traits were reduced in the plants grown in Italy. Mean leaf area was 5968.9 mm^2 with a range between 2000 and 20000 mm^2. LER was by far the most affected trait (85% reduction) followed by leaf area, which decreased by 55%. Leaf width/length ratio (Figure 6.3 A) was the least affected trait (4.4% reduction in Italy) in the population together with dry weight (18.6% reduction).

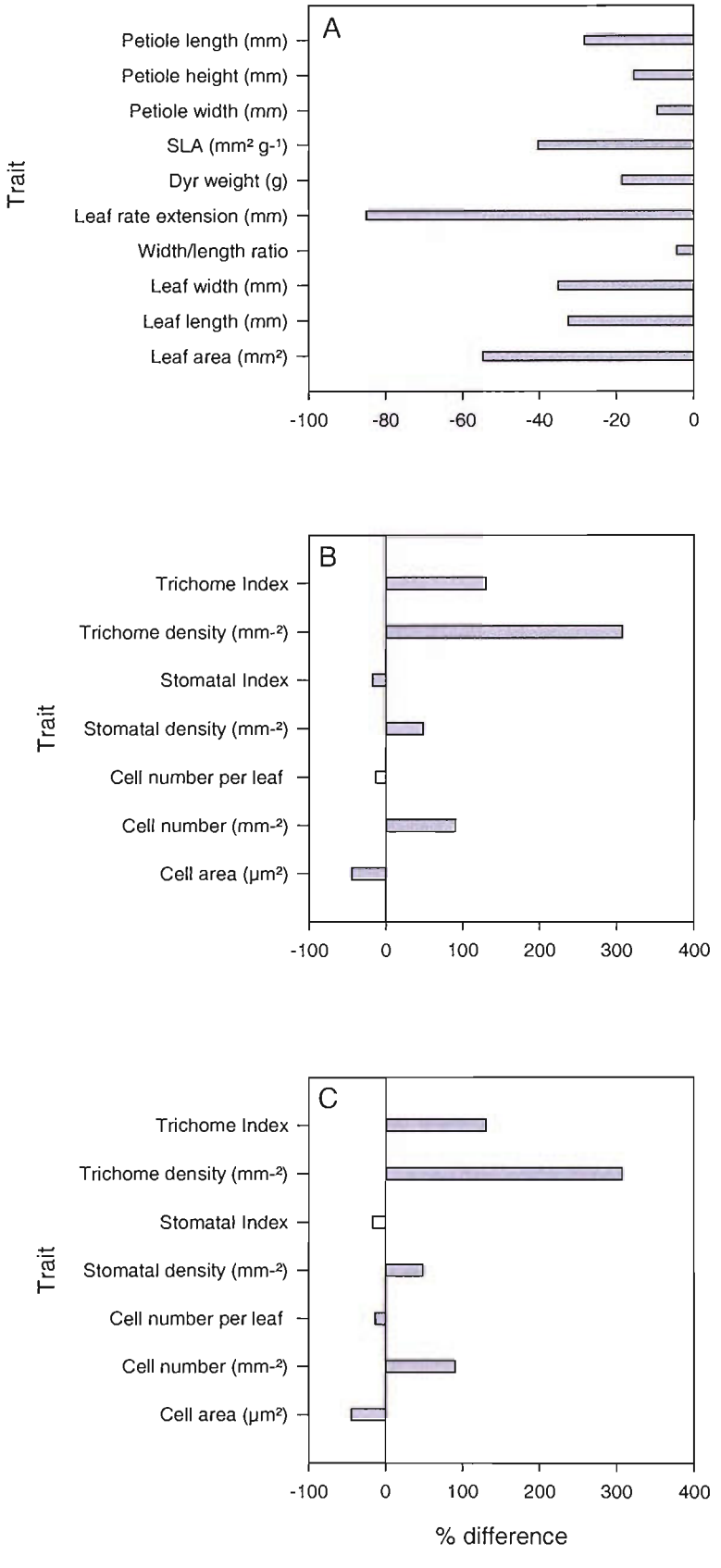


Figure 6.3 Percentage difference of traits evaluated in the Family 331. A) Leaf, B) adaxial cell and C) abaxial cell traits. + and - values represent an increase and decrease respectively.

6.4.2 Adaxial cell traits

Cell traits on the adaxial surface were affected similarly in the two grandparental species. The means of the measured traits are presented in Table A6.1 (in Appendix 6). Cell area in the UK, was almost twice as large in *P. trichocarpa* compared to *P. deltoides,* with a corresponding reduction in cell number per area. Stomatal number was significantly higher in *P. deltoides* with stomata absent or scarce in *P. trichocarpa*, where trichomes occur instead.

In *P. deltoides*, only cell area was reduced by 24.7% in the population grown in Italy, but increases occurred in stomatal index (10.7%), cell number per area (42.4%) and stomatal density (109.08%) (Figure 6.2B). Total cell number per leaf was almost constant as only a reduction of 2.3% was observed. Trichomes were not observed on either leaf side in the male grandparent (Table A6.1). On the other hand, *P. trichocarpa* showed a reduction in cell area of 40.4% in Italy compared to the UK. However, other traits measured showed higher increases than in *P. deltoides* ranging from 75% for cell number up to 620.5 % for trichome density (Table A6.2). Increases in stomatal and trichome index in *P. trichocarpa* were significantly greater but cell number per leaf was severely reduced, by 42.2%.

Cell traits in the parents (clones 242 and 246) in the UK have similar values and both clones showed a decrease in cell area and trichome traits (Table A6.2). However, stomatal traits and cell number increased. Clone 242 increased cell number by 49.68% and stomatal density and number by 72%, consequently stomatal index is increased by 43%. In Clone 246, cell number showed a small increase of 3.32% but stomatal number and density showed a higher increase (162%). Stomatal index decreased by 64.15%, being so far the most distinctive feature in this population. Trichome traits and cell area also showed a decrease in Italy Like in the grandmother (Table A6.2).

F_2 genotypes showed a decrease in cell area and stomatal index of 44.4 % and 17.6 % respectively, but considerable increases in stomatal density (48.4%), stomatal number (49.2%), cell number (90.9%), trichome index (130%), and trichome density (307%) (Table 6.1) were also observed. Trichome traits were increased significantly in this

population, as in the female grandparent (See data in Chapter 5). This feature has been linked to the expression of *TTG1* (*TRANSPARENT TESTA GLABRA 1*), *GL1* (*GLABRA 1*) and *GL2* (*GLABRA 2*) genes (see in discussion). Cell number per leaf was reduced by 13.6%.

6.4.3 Abaxial cell traits

The same seven traits were measured on the abaxial side of the leaf as on the adaxial side. The most distinctive features of the plants growing in UK were cell area and number, where they are smaller in *P. deltoides* in comparison to the large cells of *P. trichocarpa* (Table A6.1). In general, cells are larger on the abaxial side in comparison with the adaxial side in both cases. Stomatal index is also higher in *P. trichocarpa* than in *P. deltoides*, where there are few, but larger stomatal cells. The lack of trichomes in *P. deltoides* is also a distinctive feature on the abaxial side. In Italy, cell area was the only trait reduced (-17.8%) on the abaxial side in *P. deltoides*, but stomatal number, cell number, stomatal density and stomatal index were increased in *P. deltoides* by 81.13, 26.41, 56.82 and 18.75% respectively.

P. trichocarpa showed a decrease (-18.48%) in cell area similar to *P. deltoides* and also a slight decrease in stomatal index (-6.72%). However, trichome index was increased by 25.41% and cell number by 26.7%. The increases in trichome density (60.1%) and stomatal number (120%) were exclusive to this parent and the values were moderate compared with those observed on the adaxial side (Table A6.1).

Values on the abaxial side in clones 242 and 246 are very similar in the UK, with the only difference that clone 246 has a very low number of trichomes while in clone 242 they were not observed. The environmental conditions in Italy mainly affected cell area (-46.4%) and stomatal index (-25.9%) as changes in trichome traits were not observed in clone 242. Stomatal density and cell number had higher values (68.4% and 96.8% respectively). With the exception of percentage difference in trichomes, which were remarkably reduced in clone 246 as no trichomes were observed (-100%), the values for trichome density and cell area were of the order of -60%, -66.8 and 62.1-%

respectively), while stomatal index was slightly reduced (-9.0%). Stomatal number and cell number were the most contrasting positive values between the two clones. Clone 242 had a higher number of stomata and clone 246 had a higher cell number (96.8%), similar for both clones (Table A6.2). Cell number per area in clone 246 increased more than in clone 242, because of a greater reduction in the cell area of clone 246.

The F_2 population showed two main differences corresponding to cell area and stomatal index, which had observed reductions of 44.9%, and 14.2% respectively, with considerable increases in stomatal density, cell number, trichome density and trichome index (40.5, 92.6, 415.4 and 177.8%, respectively) in Table 6.1. A summary of all the percentage differences in the grandparents, and F_2 is presented in Table A6.3.

6.5 Discussion

6.5.1 Leaf traits

For the traits evaluated the values recorded in Italy were generally lower because of the higher water deficit and sun irradiance than in UK. Leaf extension rate was the most affected trait in Italy. This is consistent with the results reported by Lambers et al. (1998) in other plants. Their low relative growth rate can be attributed in part to the production of small, or slowly expanding cells. In general, the male parent *P. deltoides* was less affected than the F_1 and the F_2 showing higher resistance to stressful conditions. For example *P. deltoides* in Italy showed a decrease of about 28% in leaf area, while in *P. trichocarpa* and the F_2 the reduction was more than 50%.

The responses of the siblings show how different clones can adapt to different habitats by changing their growth rates and leaf areas (Poorter and van der Werf 1998). As expected, leaf areas were significantly reduced in the F_2, in G 246 and *P. trichocarpa*, and were less affected in *P. deltoides* and G 242, coincident with the changes in petiole length, width and depth. G 246 (female clone) seems to behave in a similar way to *P. trichocarpa* (female) and that could indicate the effect of sex in the genotype responses. This difference between sexes in the grandparents has been mentioned before (Isebrand

et al. 1998, Tschaplinski et al. 1994). Differences in allocation patterns have also been mentioned, as female clones allocate more carbon to lateral branches under water stress contrasting with *P. deltoides* (male genotype) that displays a greater degree of stress tolerance than the female.

In the two environments leaves showed a range of shape and size (Figure 6.4). Leaf shape varies with the position on the plant and also affects leaf size while size and thickness affect the efficiency of resource use. Larger, thicker petioles are demanded for broader and thicker leaves. For *P. trichocarpa* the decreases of leaf dry weight in Italy relative to the UK were more pronounced than in *P. deltoides* and G 242. Leaf dry weight was only slightly reduced in Italy for G 246 and in the F_1. This response agrees with the statement that "sun" leaves are thicker than shade leaves, for example. The costs of petiole actually make sun plants less effective than shade plants of the same size, for intercepting PAR (Gutschik 1999).

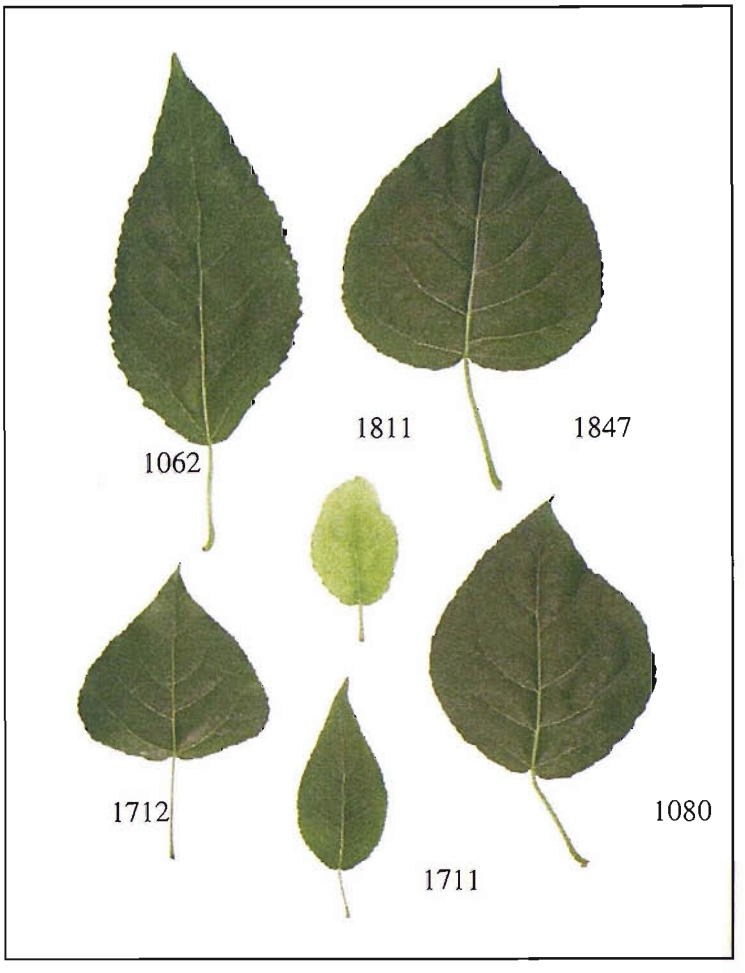


Figure 6.4 Contrasting leaf shapes in Family 331. Six genotypes of Family 331 are shown, with leaf areas differing by up to 600%.

Unidimensional traits like leaf length and width were reduced in all cases in Italy, however in the ratio width/length *P. deltoides* showed a big increase that could be interpreted as a greater reduction of length. Leaf length was one of the traits that showed a significant degree of heritability and the similarity in the ratios was a good indicator of leaf shape maintenance in varied environmental conditions. As has been reported, the shape of plant organs has been thought to be fundamentally regulated by polar cell elongation with *AN* and *ROT3* reported to be involved in this control (Kim et al. 2002).

The increases in leaf dry weight in *P. deltoides* and in G 242 and the reduction observed in the female parent and grandparent G 246 and F_2, together with the increase in diameters and decrease in petiole length, could be explained by different composition at a cellular level reflecting different needs for elasticity and for mobility of various types of molecules in the cell wall (Somerville et al. 2004). This also shows that leaf area in

the parents G 246 and G 246 was not reduced as in the other genotypes. A stronger petiole allows more movement of the blade and also enables the plant to keep the leaf blade longer, thus continuing photosynthesis in order to continue metabolite synthesis.

An increase in petiole thickness can also be interpreted as an investment for enhanced light interception, which is more valuable early and late in the day, when water vapour pressure differences are smaller and WUE is larger. Shade plants would be more damaged by photoinhibition from high PAR interception on leaves of limited electron-transport capacity. Angles that favour high light interception (normal to the sun, and perhaps actively tracking the sun) favour high efficiencies in using light (much light is intercepted at irradiances far exceeding the light-saturation point of the leaf) and in using water (Gutschik 1999).

SLA was higher in the UK, showing a more efficient use of low light, in comparison with the reduction in SLA in Italy. This reduction could be a result of a different allocation pattern and to an extreme reduction in LER. Decreases in SLA have been observed in other environmental conditions, for example under high CO_2 concentrations (Ceulemans et al. 1995). However the decrease of this trait in Italy was more pronounced than in the conditions mentioned above and is closely related to the decrease in LA and mesophyll thickness, while in the example given it could be explained by the fast increase in leaf area. Sun leaves are markedly thicker and can have additional palisade layers compared with shade leaves (Gutschick 1999). However, because of the many environmental differences between Italy and the UK, it is difficult to attribute responses to specific factors.

Palisade plus spongy mesophyll represent an investment in N and in total construction costs. Nitrogen is allocated to leaves increasingly as the opportunity for photosynthesis increases, especially with increasing PAR flux density. This is clearly adaptive, at least for plants in which fast growth is valuable, and the patterns along the gradient of microenvironments in a canopy have been so analyzed. Accompanying the increasing mass of N per leaf area in high sunlight is an increasing partitioning to carboxylation enzymes at the expense of light-capturing chlorophyll complexes, clearly another pattern (Gutschick 1999).

6.5.2 Epidermal traits

The epidermal traits evaluated corresponded to the three types of cell that form part of it: pavement cells, stomata and trichomes. In Italy relative to the UK there were increases in cell number, stomatal density and in trichome density and index, but a decrease in the rest of the traits studied.

The decrease in cell area in the grandparents, parents and F_2, was accompanied by an increase in cell number. This response seems to be a compensatory mechanism to avoid a higher decrease in leaf area. This mechanism was mentioned by Tsukaya (2003), and even though the number of cells is not necessarily reflected by leaf shape the genetic analysis of leaf development in *Arabidopsis* shows that a compensatory mechanism acts on leaf morphogenesis and an increase of cell volume might be triggered by a decrease in cell number.

Traits such as stomatal number and density, but not stomatal index increased in Italy in the two leaf surfaces. It has been reported that stomatal density is variable according to environmental conditions during leaf development. This has been observed in other cases as a response to environmental conditions, and it is accepted that light levels and exposure to the drought hormone ABA affect stomatal numbers in some species (Quarry and Jones 1977, Schoch et al. 1980, Franks and Farquhar 2001, Lake et al. 2002). Factors such as humidity (Schürmann 1959), temperature (Srivastava et al. 1995), CO_2 partial pressure (Clifford et al. 1995) also affect stomatal number. Stomatal density is also subject to genetic (endogenous) control, as illustrated by the differences between varieties of the same species (Reich 1984; Buttery et al. 1992, 1993; Ramos et al. 1992) or F_1 hybrids (Abak and Yanmaz 1985).

Because stomatal density may be affected by alterations in epidermal cell size and/or changes in the ratio of pavement cells to stomata, stomatal indices (SI) are very important (Berger and Altmann 2000, Sachs 1993). In general, adaxial stomatal index was reduced in the F_2 and in G 246, but increased in *P. deltoides* and in G 242. In *P. trichocarpa* the increase in SI on the adaxial side was about 500%, suggesting that there was a cell fate modification, promoting the trichome differentiation. However on the abaxial side, stomatal index only increased clearly in *P. deltoides*, and showed a

slight decrease in *P. trichocarpa* and G 246. The decreases in 242 and F_2 were more pronounced, indicating that even increases in SD, changes in cell area and therefore cell number are affecting the SI.

Trichome numbers are affected by environmental conditions in the same way as stomata. These single celled protrusions acts as mechanical barriers for the leaf surface (Glover 2000), and their increase could be due to less tolerance to drier conditions where the clones were more stressed (Mauricio and Rausher 1997). Apart from their value in leaf energy balance, trichomes have a role in providing protection from herbivores (Mauricio and Rausher 1997, Gutschick 1999). It is interesting to note here that while trichomes are found on the adaxial side of the female parent *P. trichocarpa* leaf, this side has very few or no stomata, and this could defend the leaf against pest attack (Gutschick 1999). Interestingly it was found that *P. trichocarpa* is highly resistant to slugs and rust in one of our field sites (Tucker 2004, comm. pers.).
The fact that trichome distribution was skewed to lower values, while in Italy distribution was more normal, suggests that the plant population may have been stressed by the environmental conditions in Italy.

6.6 Conclusion

Growth in Family 331 was strongly affected by the harsher conditions in Italy with leaf growth reduced considerably (90%) in comparison with the UK. These conditions eliminated variant phenotypes, because of the strong selection pressure. The responses observed can be considered as good indicators of the selection pressure applied, and at the same time can be used as indicators for adaptation to water deficit and high irradiance environmental conditions. Leaf shape was the least affected feature in Family 331 showing this to be a stable trait and the same applies to petiole traits.

The increases of cell number, stomata and trichome numbers in Italy relative to the UK showed that the patterns of cells differentiation were modified as well as the cell cycle.

In summary, this study has revealed that it is possible to use leaf shape and petiole traits to select the clones more suitable for drought conditions because they show less effect of harsher conditions on their biomass production. It also shows the need for molecular work to gain insight into the genetics of these traits. The results obtained for cellular traits in Italy showed that even when the mean in the population indicates no changes in the stomatal index, some of the genotypes did show big changes. Stomatal index (ratio of stomata per epidermal cell) in this particular case could be interpreted as a genotypic indicator of positive or negative response to adaptation to harsher conditions. Maintenance of the same ratios could be considered as a positive response, while increasing or decreasing of the SI could be interpreted as an stressful effects in the plants.

The role of trichomes in this particular family seems to open the possibility of further study as they have been shown to have an important role in cell fate determination in *Arabidopsis*. Even when biomass production is lower than expected, some genotypes exhibited higher yields in comparison with the same clones growing in UK conditions. The traits responsible represent good tools to be used in further work.

Chapter 7

QTL analysis and candidate genes in Family 331

7.1 Introduction

The location of polygenes is a subject distinct from Mendelian genetics in the sense that the segregation of specific genes as separate entities was not considered in Mendelian genetics (Thoday, 1961). However, when quantitative characters are studied, the continuous nature of the relevant variation naturally leads to analysis of variance and hence, to a biometrical approach. Natural variations and the study of biometric characters are the basis of the quantitative genetics which allow us to discover how genic differences contribute to phenotypic variation (Thoday 1961).

Many previous studies have taken a single gene approach to study the genetic control of leaf trait differentiation. However such studies are limited in that many interesting traits show continuous variation, controlled by many genes of small effect and the environment. Therefore, a better approach is that of Quantitative Trait Loci (QTL) analysis that can be used to dissect the genetic and environmental variation of these characters to answer fundamental questions about the nature of the QTL, and so to determine whether these QTL can be manipulated for improved breeding.

One of the main practical limitations of the QTL technique, the availability of suitable markers, is solved in the case of *Populus*, where a huge numbers of hybrids are available. The existence of several pedigree maps increases the possibilities of success in searching for the loci or genes (QTL) responsible for selected traits. QTL mapping in *Populus* aims to identify the QTL responsible for genetic variation in commercially important traits and to map them into a model forest tree. In addition, by using a three generation pedigree derived from interspecific hybridization across taxonomic sections in *Populus*, it was hoped that insights into the genetic basis of hybrid vigour itself might be found (Bradshaw and Stettler 1995, in Bradshaw and Grattapaglia 1994).

A QTL mapping pedigree was constructed by hybridization between *P. trichocarpa* and *P. deltoides* (see introduction), and their heterosis has been used as the basis of applied genetics in forest trees. Phenotypic data from the clonal trial can be used to calculate broad-sense heritabilities for all the traits, allowing estimation of the proportion of genetic variance accounted for by each mapped QTL (Bradshaw and Grattapaglia,

1994). The tacit assumption is that most of the genetic variance for the traits of interest is partitioned between the parental species rather than among individuals within the species.

Mapped QTL of *Populus* have been widely used. Bradshaw and Stettler (1994) mapped QTL for commercially important traits (stem growth and form) and an adaptive trait, (spring leaf flush), finding that 44.7% of the genetic variance in stem volume after 2 years of growth is controlled by just two QTL. The QTL governing stem basal area were found clustered with QTL for sylleptic branch leaf area, sharing similar chromosomal position and modes of action suggesting a pleiotropic effect of QTL responsible for stem diameter growth (Bradshaw and Stettler 1994).

This coincidence of QTL position, magnitude of effect, mode of action, and direction of allelic effect can explain correlations among anatomical, physiological, and gross morphological traits. An application for this would be the generation of subsets of clones differing primarily at a single QTL, with other loci held constant. This highly refined material is the forest tree equivalent of near-isogenic lines available in inbreeding crops (Bradshaw and Grattapaglia 1994).

QTL elucidation is not only useful for analysing important agronomic traits in crops. It can provide evidence that a plant characteristic of interest has a genetic component and is a good starting point for future studies on individual genes and genomic regions. The elucidation of QTL focuses on the inheritance and evolution of specific traits of interest. Provenance trials in forest trees indicate that there will be substantial environmental variance and genotype x environment (G x E) interactions in the expression of most commercial traits.

The importance of leaf size, morphology, structure and orientation in influencing tree productivity is well known. These characters can be helpful in interpreting the adaptation of a plant to environmental factors including light, water, and wind. Also, little is known about the genetic basis of leaf morphological variation in forest crops, and there is a serious lack of information on how leaf traits are genetically and developmentally correlated with one another and with stem growth (Wu et al. 1997). Taylor et al. in the UK have been studying the *Populus* F_2 hybrid Family 331 since 1999

from a biomass perspective and several putative QTL for leaf and stem growth in ambient and elevated CO_2 have been described, when this population is growing in UK conditions (Taylor et al. 2001, Ferris et al. 2002, Rae et al. 2004, Taylor et al. 2004).

The study of leaf traits from a segregating pedigree, such as an F_2, grown at differing sites should give evidence of traits that are expressed as a consequence of the environmental changes. Apart from the valuable information of traits expressed in an F_2 family, these responses could also shed light on some of the changes that occur in the epidermis - a multifunctional tissue of which the development and morphogenesis has not been completely understood - and growth. The epidermis also includes structures such as trichomes, stomata, cuticle and waxes. The differentiation processes for these structures is also of interest (Glover 2000).

Ferris et al. (2002) provided preliminary evidence that leaf stomatal and cell traits were amenable to QTL analysis and concluded that these traits are robust enough to be developed for accelerated tree breeding programs and could potentially assist in the finding of traits related to high biomass productivity. These traits appear to show a continuous range of variation in the population, which is more or less normally distributed. Consequently these traits can be used as indicators of response under different environmental conditions (Kearsey 1998).

Genome mapping work in crop plants shows that some QTL are important across all environments and others vary in their magnitude of effect in different environments (Bradshaw and Grattapaglia 1994). This work in *Populus* is intended to contribute in the same way that previous studies have yielded valuable insights into plant response to a range of environmental changes including salinity (Koyama et al. 2001), drought (Lanceras et al. 2004), nitrogen supply (Loudet et al. 2003, Hoekenga et al. 2003). It is expected that this approach prompts gene-cloning strategies and identifies candidate genes that help to reveal the basis of genetic variation in *Populus* and therefore increases our ability to answer fundamental questions about biomass production and evolution in forest species.

In the past ten years, extensive genetic maps have been established in a dozen tree species, and dozens of QTL have been identified for a variety of traits that are efficient

for constructing maps for comparison. Marker-QTL associations can be disrupted by recombination, so the association may not be robust in different genetic backgrounds (Yin et al. 2004). However, the problem of linkage equilibrium at the population level can be greatly reduced by exploiting the genome sequence and functional genomics information to directly target candidate genes putatively involved in the control of traits of interest, thus increasing the power of marker-assisted selection (Strauss et al., 1992, Yin et al. 2004). This would increase the probability of finding the genetic basis of the responses in environmental adaptation.

7.2 Aims

This study aims to identify the genetic basis of tree response to environmental stresses, particularly as water supply, through the elucidation of quantitative trait loci. In particular we are interested in analysis of the responses that the leaves in the *Populus* F_2 hybrid, Family 331, had at two levels, both macro- and microstructural (leaf blade and epidermal cell), when they were grown under extreme environmental conditions. It is expected that the knowledge of the variance in Family 331 in the UK and in Italy will show similarities and differences that could be attributed to structural and environmental effects and will give evidence of the way that genetic control occurs.

7.3 Materials and Methods

7.3.1 Leaf and cell trait distributions

All the results of leaf blade and cell traits on the adaxial and abaxial sides that were measured in the experiment described in Chapter 6 were averaged and the means were plotted in frequency histograms, to observe the distribution in the Family F_2. Trait data were tested for normal distribution using the Andersson-Darling test. In cases where data were non-normally distributed Box-Cox transformations were carried out.

7.3.2 QTL mapping

The genetic linkage map for this pedigree was produced by Tuskan et al. (personal communication, [http://www.ornl.gov/sci/ipgc/ssr_resource.htm]) and consists of 91 SSRs genotyped on 350 individuals and 92 fully informative AFLPs genotyped on 165 individuals. The primer sequence of SSR markers were blasted against the poplar genome sequence and linkage groups orientated based on the physical sequence (ie 3' to 5'). The data were analysed for QTL as indicated in Chapter 2.

7.3.3 QTL and candidate gene collocation

Once QTL were collocated using map chart software, hot-spots were detected for both leaf and cell traits. In a separate procedure candidate genes were also collocated in order to find the genes that could explain the morphological or physiological traits identified in this research. Coincidence in position and site were chosen as main criteria. Candidate genes were obtained from the *Populus* database in Prof. Taylor's Laboratory. Data was input by Harriet Trewin from several research works carried out in this laboratory. In this particular work the search for candidate genes was focussed on epidermal cell fate, stomatal and trichome responses to stress and leaf structure and growth.

7.4 Results

7.4.1 QTL mapped

Sixteen leaf and cell traits were submitted to QTL analysis for plants growing at the two experimental sites. 210 QTL were found (Table A7.1 in appendix), 94 in UK, 79 in Italy, and 37 in the percentage effect response to drought known as response Italy/UK.

The frequency of QTL in Linkage groups varied. When all traits are considered at both sites, LGs VIII and XII had the most QTL. It seems that these two LG have a loci with a multitrait control. In contrast, LG, XV, XVI and XIX seem to have less control over the traits recorded, as only one QTL for one trait was mapped in each; a QTL for SLA and a QTL for leaf length respectively, both in the UK. In addition to this group, LG XIX had a QTL exclusively for trichome density in Italy (Table 7.1).

Table 7.1 Presence of QTL per trait group in each of the LGs in *Populus*. LG IX, XV and XVI are only present in leaf traits and XIX only in cell traits. The rest of the LG include QTL mapped in leaf and cell traits.

LG	Leaf UK	Leaf Ita	Leaf Ita/UK	Cell (ad) UK	Cell (ad) Ita	Cell (ad) Ita/UK	Cell (ab) UK	Cell (ab) Ita	Cell (ab) Ita/UK
I	*		*		*	*		*	*
II		*	*		*			*	
III		*		*	*	*	*		*
IV	*						*	*	
V	*	*		*		*	*	*	
VI	*			*		*	*	*	*
VII	*		*						
VIII	*	*	*	*		*	*	*	*
IX	*	*	*						
X		*	*			*			
XI	*				*	*			
XII	*	*	*	*	*	*	*	*	
XIII	*			*	*		*	*	*
XIV	*	*		*	*		*		*
XV	*								
XVI	*								
XVII	*					*			*
XVIII	*	*		*					
XIX					*				

The frequency of QTL on LGs varied, LG IV, VII, IX, X, XI, XVII and XVIII had QTL in three or four of the groups of traits at the sites evaluated. From all these, LG IX had QTL over leaf traits that mapped in the UK, Italy and in the response, showing that the QTL controlling leaf traits does not change in different environmental conditions, but the remaining LG are present in different groups of the evaluated traits. Finally, there were five LG I, II, III, VI, XIII, XIV with a high frequency of QTL for the two sites and the response. These QTL were for both leaf and cell traits (Table 7.1).

Figures 7.1 and 7.2 show the QTL mapped in the linkage map divided per group of trait. Some of the candidate genes that have been collocated in the physical map of *P. trichocarpa* are also indicated.

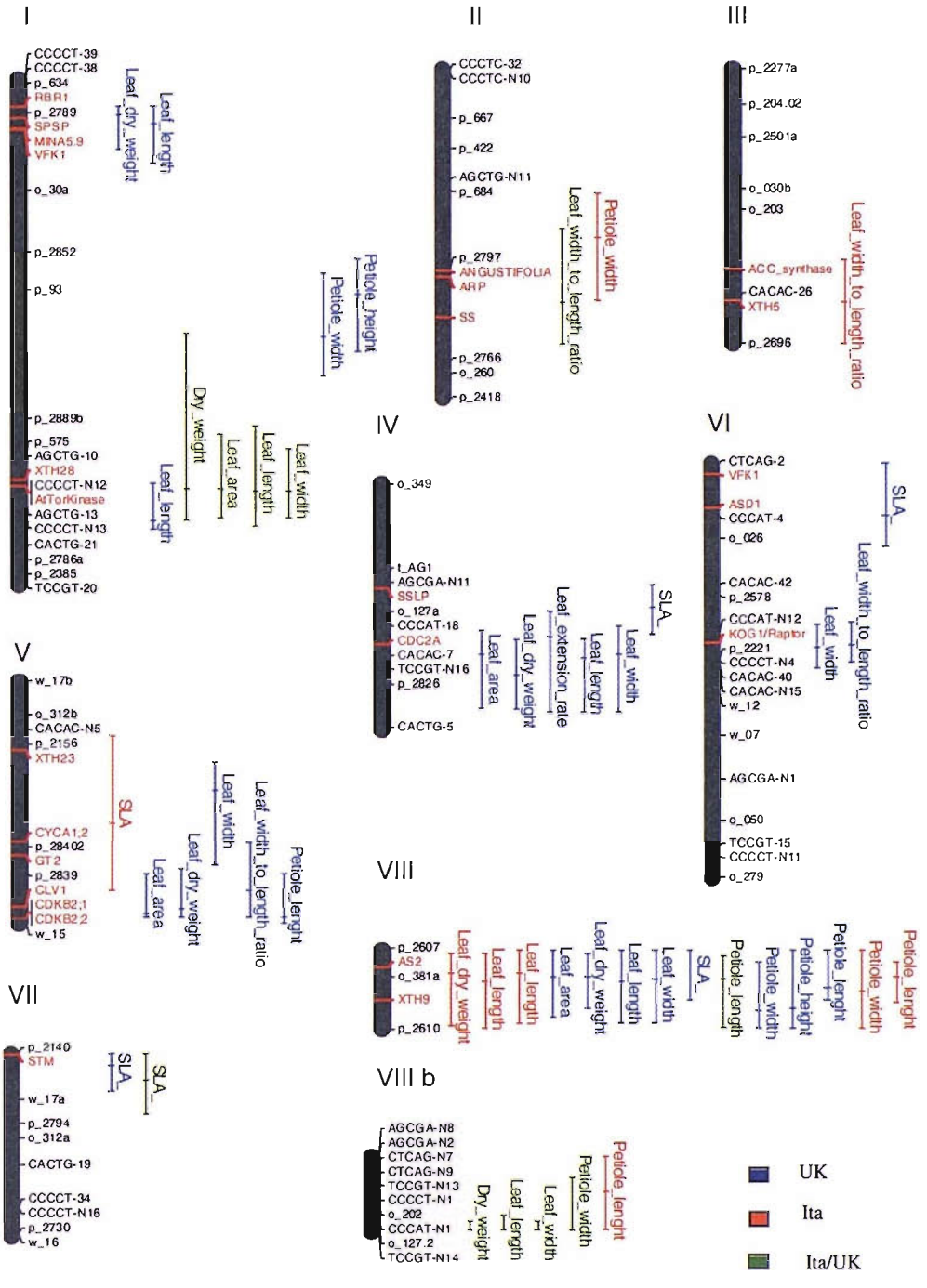


Figure 7.1 QTL locations for leaf traits in UK, Italy and the response Ita/UK (percentage effect response to drought). LG I to VIII are presented in this figure. Candidate genes are indicated in the regions of interest. LG I and VIII include QTL for several traits, while in LG IV, V and VI the QTL mapped were almost exclusively for traits in the UK. LG II, III and VII showed more specificity in the QTL mapped. QTL positions are shown ± interval as determined by bootstrap analysis. Markers names are shown to the right (in black) and so the candidate genes (in red).

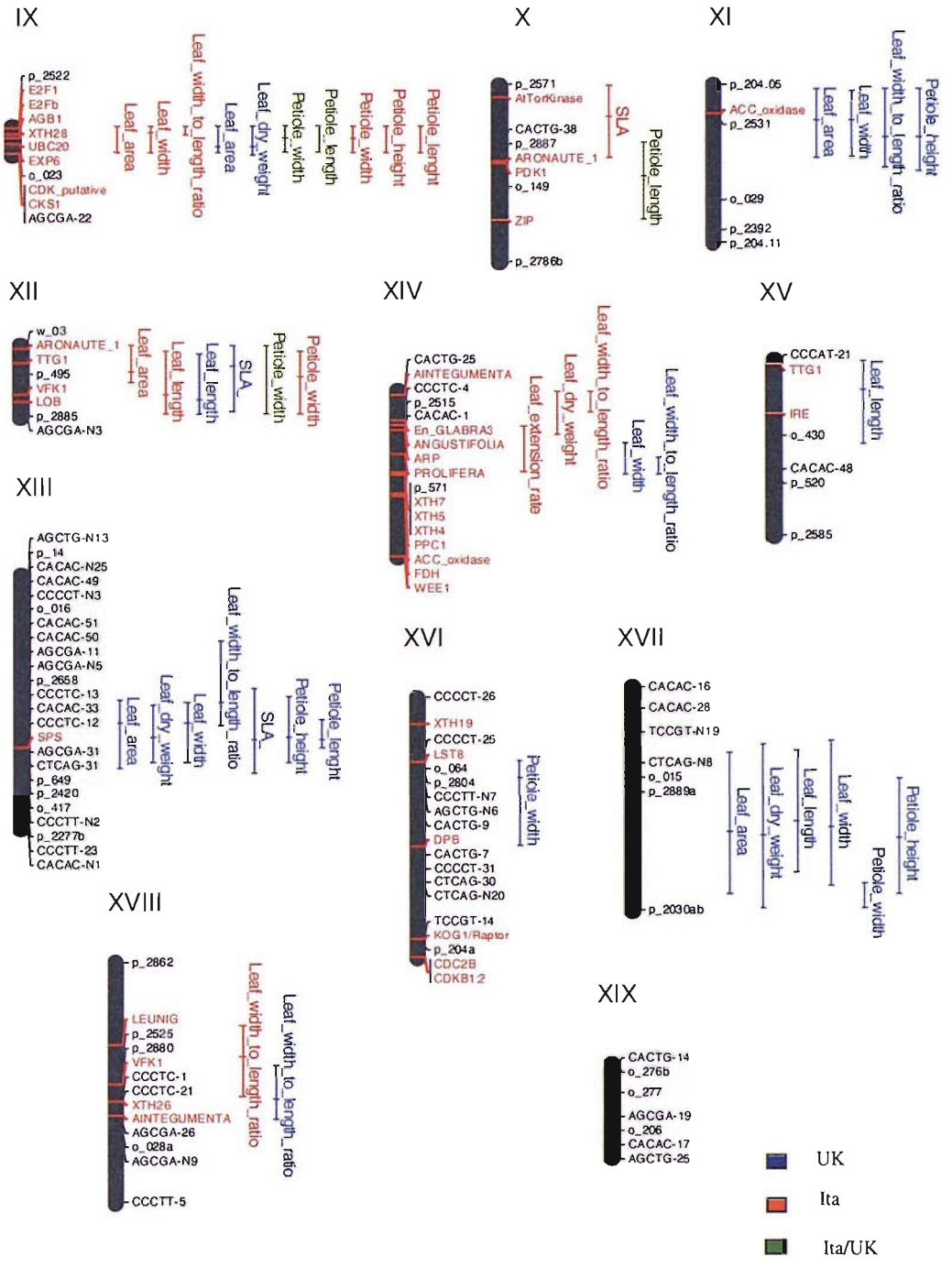


Figure 7.1 cont. QTL locations for leaf traits in UK, Italy and the response Ita/UK. LG IX to XIX are presented. Candidate genes are indicated in the regions of interest. LG IX, XII and XIV include several traits in two sites and/or in the response Ita/UK. While LG XI, XIII, XV, XVI and XVII were mapped only in the UK. LG X, included QTL for SLA and petiole length response while LG XVIII the two QTL mapped to the same effect, position and site. In LG XV and XVI only one QTL was identified in each case. QTL positions are shown ± interval as determined by bootstrap analysis. Markers names are shown to the right (in black) and so the candidate genes (in red).

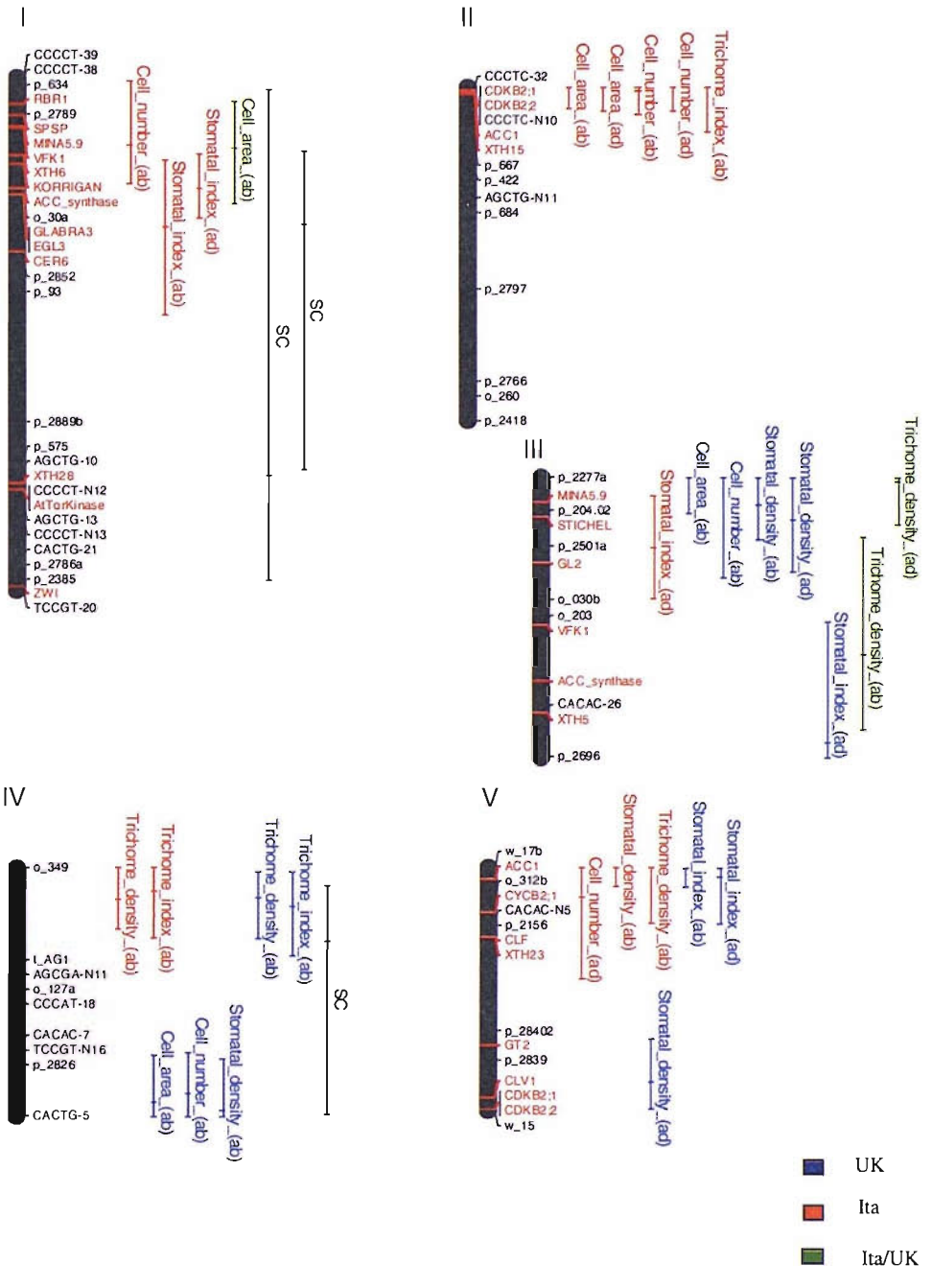


Figure 7.2 QTL locations for cell traits in UK, Italy and the response Ita/UK. Four LG are presented. Candidate genes are indicated in the regions of interest. From them LG I and II mapped QTL for cell traits in Italy basically, while III and IV combined QTL for the traits evaluated in Italy, the UK and the response. QTL positions are shown ± interval as determined by bootstrap analysis. Markers names are shown to the right (in black) and so the candidate genes (in red). QTL obtained for stomatal conductance (SC) reported in Chapter 4 are included and marked in black.

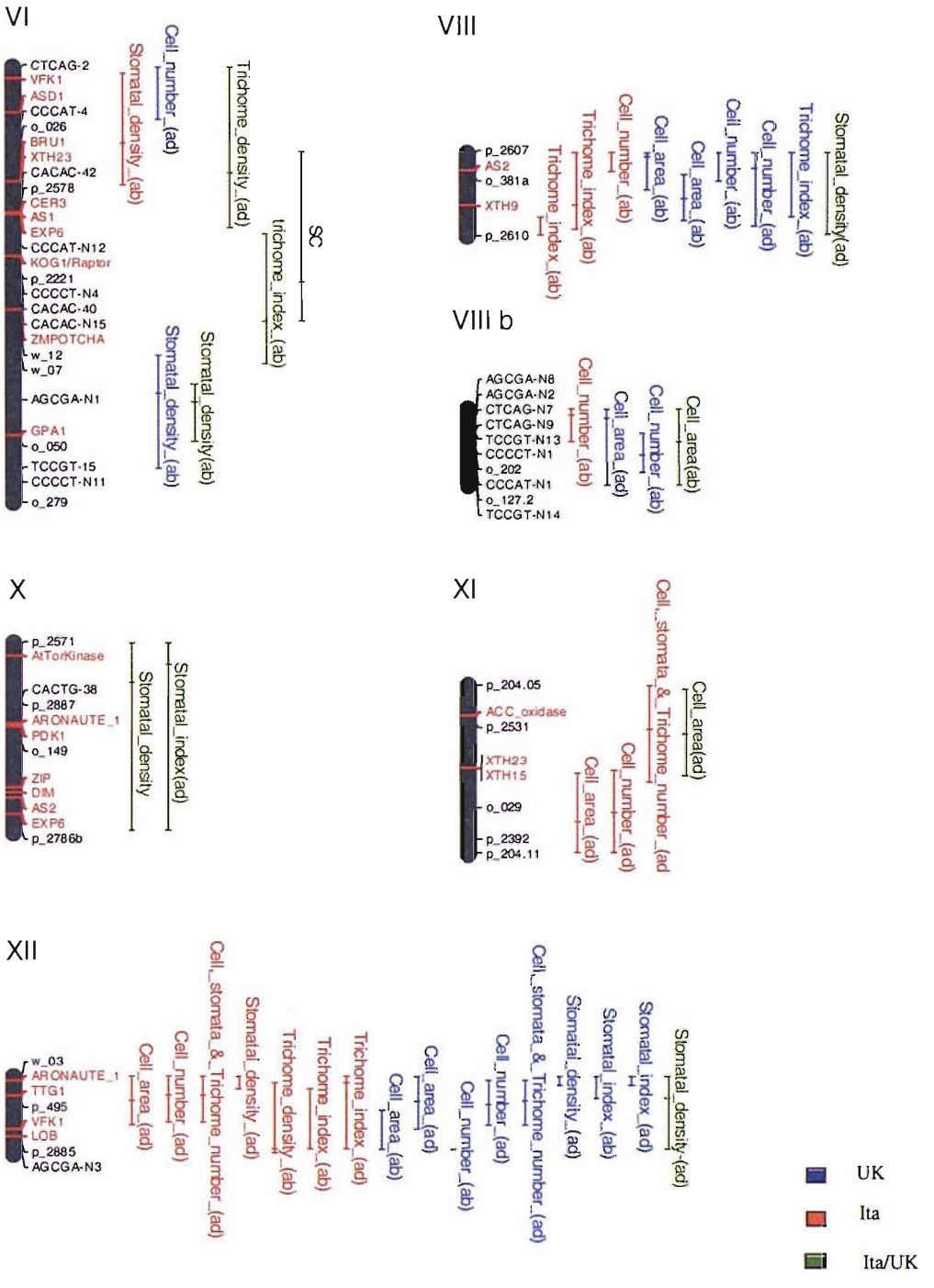


Figure 7.2 cont. QTL mapped for cell traits in UK, Italy and the response Ita/UK. Five LG are represented in this figure. Candidate genes are indicated in the regions of interest. All of them, except X, mapped QTL for the two sites and the response Ita/UK. LG X is the only LG that mapped QTL for the response Italy/UK for the same type of traits and to very similar positions. QTL positions are shown ± interval as determined by bootstrap analysis. Markers names are shown to the right (in black) and so the candidate genes (in red). QTL obtained for stomatal conductance (SC) reported in Chapter 4 are included and marked in black.

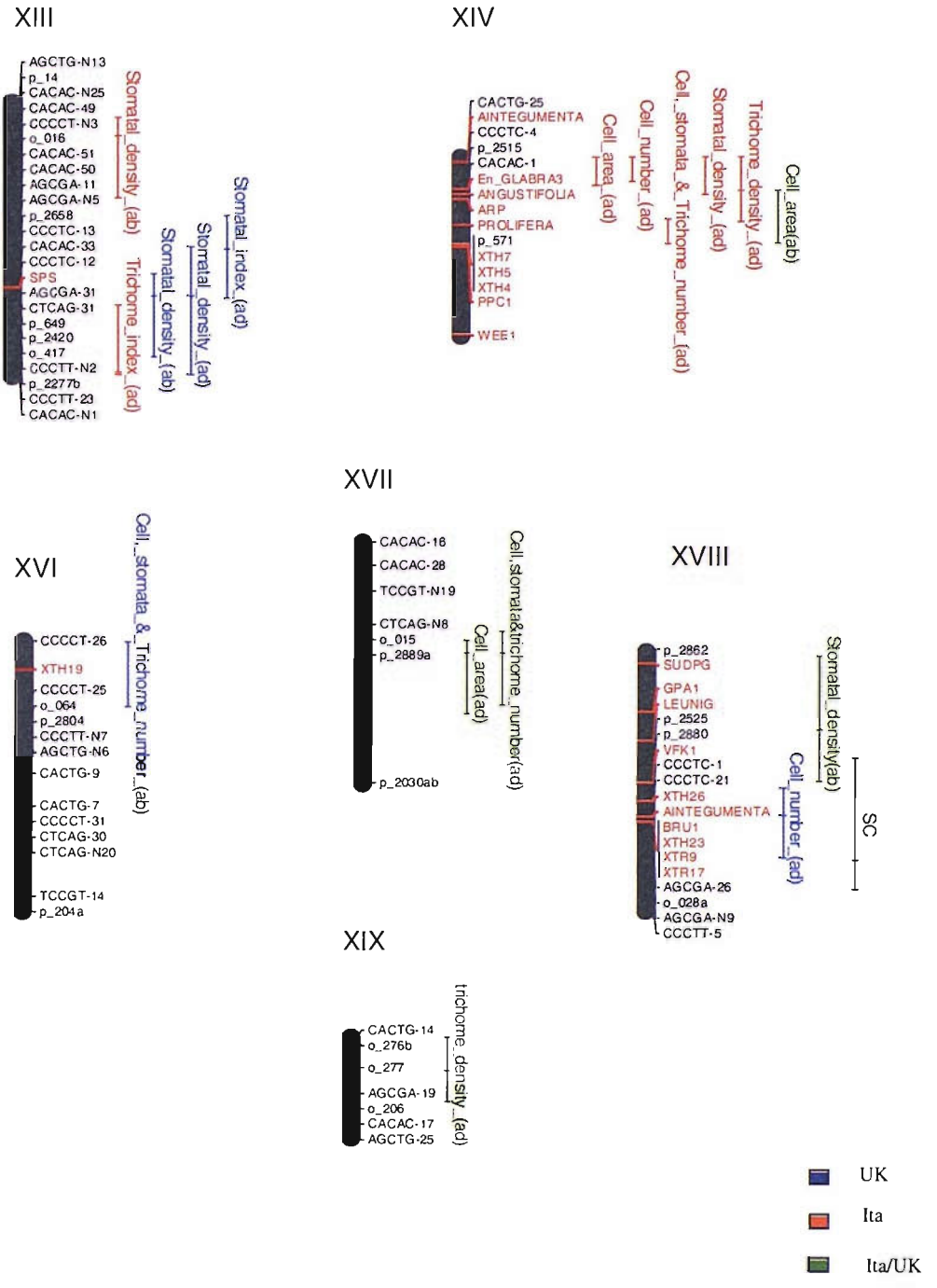


Figure 7.2 cont. QTL mapped for cell traits in UK, Italy and the response Ita/UK. Seven LG are represented in this figure. Candidate genes are indicated in the regions of interest LG XII, XIII and XIV have QTL for several traits in the two sites and in the response Ita/UK. LG XVI, XVII, XVIII and XIX had only one, two or three QTL respectively. This last set of LG responded only to UK site or the response. QTL positions are shown ± interval as determined by bootstrap analysis. Markers names are shown to the right (in black) and so the candidate genes (in red). QTL obtained for stomatal conductance (SC) reported in Chapter 4 are included and marked in black.

7.4.2 QTL analysis in leaf traits

54, 23, and 18 QTL were found in leaf traits in UK, Italy and in the response Ita/UK. Leaf area, leaf length, leaf width, dry weight, and specific leaf area were the traits with the highest percentage of phenotypic variation explained by the mapped QTL, above 30%. Leaf width/length ratio had the highest percentage of variation (44.9%). In Italy, the percentage variation in the same traits was much lower than in the UK, and leaf width/length ratio had the highest value (21.5%) together with petiole width (19.7%) (Table A7.2 in appendix 6).

At both field sites, three QTL were found for petiole length with similar percentages of variance: 18.41 and 29.31% for UK and Italy respectively. In the case of petiole width and height QTL mapped explained 29.8 and 21.99% of variance respectively, in comparison with 19.78% for petiole width and 3.58% for petiole height in Italy, which was surprisingly depressed. Broad sense heritability and clonal heritability were higher for the same traits in the UK with the difference that SLA, leaf extension rate, petiole width, height and length were higher in the plants growing in Italy. This shows that heritabilities in the case of LER and petiole width are very small (Table A7.2).

QTL identified for the response Italy vs UK (Ita/UK) were reduced in number ranging from 1-3 QTL per trait, and in percentage of variance, with the exception of the QTL identified for SLA where only one QTL was identified in LG VII responsible for the 9.09% of variance. Petiole width and length had the same number of QTL and similar variances (Table A7.2).

7.4.3 Adaxial cell traits

18, 25 and 12 QTLs for adaxial cell traits were found for the plants growing in UK, Italy and the response Italy vs UK. Variance for cell area and number were similar in the two sites, however variance explained for cell area was significantly higher in Italy (21.72%) in comparison with UK (10.67%). Heritability values for cell area, cell number, stomatal number, density and index were slightly higher in the population when grown in the UK than in Italy. The most distinct values were found for trichome number and density where broad sense heritability was 0.13 in UK and 0.27 in Italy.

However, the most remarkable difference in this group of traits occurred in trichome index, where broad sense heritability was 0.10 in UK and 0.48 in Italy.
QTL mapped for the response Ita/UK ranged between 1- 5 QTL per trait and with the lowest percentage of variance in comparison to the ones in the UK or in Italy (Table A7.2).

7.4.4 Abaxial cell traits

22, 21 and 7 QTLs were found for the abaxial cell traits in the UK , Italy and the response Ita/UK (Table A7.2). The highest percentage variance explained by the mapped QTL was for cell area, stomatal number and density in UK (25.3, 22.1 and 20.6% respectively). Values for stomatal traits in Italy were lower (18.6% for stomatal traits) and the variation explained for cell area was only 7%. Broad sense heritabilities were above 0.20 for all the cell and stomatal traits in UK, but heritabilities were much lower for trichome traits (between 0.04 and 0.13). In Italy broad sense heritabilities were found to be very similar to those observed in the UK for cell area (0.33) and cell number (0.26). Stomatal index and trichome index showed higher heritabilities in Italy. QTL mapped for the response Ita/UK ranged between 1-3 QTL per trait. These QTL were all of small effect as percentage of variance was from 3 to 6. Stomatal number variance was very similar to that one in the UK with four QTL for the same trait.

7.4.5 Candidate genes

Frewen et al. (2000) stated that QTL identification is followed by the identification of the gene or genes responsible for variation in the phenotype. In this context and in order to produce a more consistent list of candidate genes, only common QTL were selected for this analysis and divided in the following groups:

1) Common QTL to both sites and/or the response Ita/UK.
2) Correlated QTL
3) Contrasting QTL

1) QTL common to both sites and/or the response Ita/UK includes the QTL that were common in LG and position to both sites studied (Table A7.3). Candidate genes

collocated in this QTL could represent candidate genes whose expression does not depend on the environment, as their identification was constant thought the study sites. It could be speculated that the candidate genes in these cases are involved in process related to leaf morphology and development. Eleven candidate genes are proposed in this group (Table A7.4).

2) The Correlated QTL group is divided in two subgroups: **Correlated cell QTL** and **Correlated LG QTL**. **Correlated cell QTL** includes 5 QTL that had common position (or near) in both cell side, LG and position, but not across site (Table A7.5). This could be interpreted as QTL responsive to environmental effects and therefore it could be speculated that candidate genes in this case are responsive to conditions in particular environments as they were expressed at one or other of the experimental sites. Candidate genes for adaptation and damage protection would be included in this subgroup of genes. Thirteen genes were identified in this group with two of them present in the first mentioned group.

Correlated LG QTL subgroup, includes 4 cases where the QTL identified mapped in the same LG but in a different chromosomal region, without overlap on the interval confidence. Here each position corresponds to one site (Table A7.5). In could be speculated that candidates genes proposed in this subgroup could be up and down regulated in the same chromosome, depending on the environmental conditions. The response Italy versus UK QTL was also analysed in this section. Fourteen candidate genes were collocated in this category.

3) The Contrasting QTL group includes only one QTL mapped for LER , where the same trait was identified in different LG and different positions. It could be speculated that the 4 candidate genes (collocated in the same positions) have a fundamental role in leaf growth promotion and inhibition (Table A7.5).

In some cases, candidates genes that were collocated in the confidence interval were included, however this discussion is focussed to the genes collocated at the exact position or very close to it.

7.5 Discussion

7.5.1 QTL analysis

QTL analysis revealed that the leaf responses in Family 331 to the contrasting environments were strong enough to map a high number of QTL that were solely identified in both or in one of the growing conditions. Individual QTL were shown to explain relatively little of the total phenotypic variance. In this work, an average of six QTL per trait in leaf traits and three QTL in cell traits were mapped, showing that the traits assessed in Family 331 are under control of several loci, with only a few exceptions such as LER (Table A7.2), where only one QTL in each experimental site was mapped.

The higher number of QTL in the UK can be interpreted as an indicator of good growth expressed as a higher leaf area at this site and, therefore, a higher expression of traits associated with the molecular processes related to growth. In contrast, the smaller number of QTL in Italy could be interpreted as the repression of growth and an expression of genes involved in defence and injury, as the leaf trait QTL were reduced by almost half compared to those in the UK. In addition, biomass in Italy has been reported to be higher than in the UK (Rae et al. 2006), a fact that could indicate changes in carbon partitioning as occurs in other plants (Farquhar 1998, Bassman et al. 2005). These changes may be negatively related to biomass in *Populus* as suggested by Marron (2005).

The analysis of the 19 QTL mapped in common across sites and 20 QTL for correlated traits showed that there are strong morphological and physiological links between QTL and candidate genes collocated in these regions (see Tables A7.3, A7.4 and A7.5 in Apendix 6). The fact that a single locus is affecting different traits and that several loci also act on only one trait (Tonsor et al. 2005).

7.5.2 Common QTL for leaf traits and candidate genes

All QTL related to leaf shape and dimension (leaf width/length ratio, etc.) had high percentages of variance (44.85%) and heritability (0.71). Six QTL for leaf traits (leaf area, leaf length, leaf width/length ratio, dry weight, petiole width and petiole length) collocated with 13 candidate genes; *CDK-putative* (*CYCLIN-DEPENDENT KINASE PUTATIVE*), *CKS1* (*CYCLIN-DEPENDENT KINASES*), *EXP6* (*EXPANSIN PUTATIVE 6*), *REV* (*REVOLUTA*), *AS2* (*ASYMMETRIC LEAVES 2*) x 2, *XTH9* (*XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9*) x 4, *LOB* (*LATERAL ORGAN BOUNDARIES*), *LEU* (*LEUNIG*), *VKF1* (*VICIA FABA* K^+ *CHANNEL*) (Table A7.4 and Figure 7.1). QTL of the most constant trait - leaf width/length ratio - in both UK and Italy collocated with *LEU*, a gene involved floral organ identity. In general the functions of candidates genes collocated could explain a role in the leaf trait assessed, as in the case of petiole traits that collocated *XTH9* a gene involved in loosening and rearrangement of the cell wall. A high percentage of variance and high heritabilities (0.45) in these traits make them strong traits (Table A7.3 and A7.4).

7.5.3 Common QTL for cell traits and candidate genes

All five QTL identified for five traits on the adaxial side of the leaf mapped to only one LG, LG XII. The QTL collocated to *TTG1* (*TRANSPARENT TESTA GLABRA*), which functions in leaf morphogenesis, trichome development and cell fate specification. However, within the confidence interval in the trait cell area, two more candidate genes were collocated, *ARONAUTE,* which gives mutants defective in post-transcriptional gene silencing and with pleiotropic developmental and morphological defects, and *VFK1,* involved in phloem unloading, were collocated.

In the case of cell QTL on the abaxial surface, LG IV and VIII were the only two where QTL were identified. In LG IV no candidates were collocated and in LG VIII *AS2* and *XTH9* were collocated for cell number, trichome index and cell, stomata & trichome number. Trichome index on the abaxial surface had a QTL in LG VIII where *AS2* and *XTH9* were collocated. When trichome number is added to cell and stomatal number as

total number of epidermal cells, the QTL mapped in the LG XII on the adaxial surface and LG VIII on the abaxial surface, has similar positions to QTL mapped for cell number in both cases, suggesting the result is influenced by the cell number and is probably not reflecting the presence of trichome number. This would not be surprising if not for the fact that gene *TTG1* collocated to the QTL mapped for the adaxial surface, where it is more observable and that *AS2* collocated to that mapped on the abaxial surface (all candidate genes shown in Table A7.4).

It is important to mention that the traits evaluated such as stomatal number, stomatal index and the combination of cell, stomata and trichome number for the adaxial surface and cell number for adaxial and abaxial surfaces, all collocate on to similar positions on LG IV, VIII and XII. Thus, these linkage groups could be considered as "hot spots" for further investigation as they are involved in leaf morphogenesis, trichome development and cell fate specification, possible in response to drought.

7.5.4 Correlated traits

Correlated Cell QTL

For the QTL mapped for the cell traits in the UK only (cell number ad and ab), two candidate genes collocated with cell number QTL on LG VIII and XII; *AS2* on LG VIII, which was common to both the adaxial and abaxial surface, and *TTG1* in LG XII mapped for the adaxial surface at 9 cM. The position of the QTL on LG XII for the abaxial surface differed, being at 24 cM, but two further candidate genes were found to collocated here: *IRE* (*INCOMPLETE ROOT HAIR ELONGATION*), which encodes a protein with a serine/threonine kinase domain with strong presence in the root hairs and *PLE* (*PLEIADE*) which codes for a microtubule associated protein regulating cytokinesis. The differences in position of the QTL on LG XII for cell number on the adaxial surface and abaxial surface may suggest that the control of this trait occurs independently in each case (Figure 7.2), as similar differences in QTL positions can be seen for adaxial and abaxial surfaces for stomatal number and trichome traits.

The QTL for cell area mapped on LG II (Figure 7.2) mapped to a similar region of the genome as *CDKB;2*, *CDKB;1*, cyclin-dependent kinases expressed in the M phase of

mitotic cell cycle and in the regulation of the G2/M transition of the mitotic cell cycle; *ACCI* which encodes acetyl-CoA carboxylase; and *XTH15* which has a similar action to *XTH7* cell wall hydroxylase acting on glycosil bonds, and hydrolase activity.

QTL indentified for stomatal index on the adaxial and abaxial surface in Italy both mapped to LG I. Even though the positions differ slightly, their function is highly related as the QTL for SI on the adaxial surface maps at 38 cM, and collocates to *ACC_synthase*, *GL3* and *EGL3*, while the QTL identified for the abaxial surface maps at 51 cM and collocates to *CER6*. *ACC-synthase* encodes acetyl-CoA carboxylase where mutants display uncoordinated cell divisions enhanced by cytokinins, *GL3* interacts with *GL1* in trichome development and *EGL3* that encodes a *bHLHt* transcription factor redundant with *GL3* and *TT8* and interacts with *TTG1* and finally *CER6* is related with wax production. Candidate genes collocated for the adaxial traits seem to be more involved in morphological (*GL3, EGL3*) characters while the ones in the abaxial traits seems to be more related with environmental response (*CER6*).

Finally, one common QTL in LG XII for Trichome index on both the adaxial and abaxial surface mapped in two positions where *ARONAUTE_1* for adaxial, and *IRE* and *PLEIADE* for the abaxial side were collocated. This trait highlights the fact that changes in the ratio of trichomes to epidermal cell on each epidermal surface, collocated to different positions as it occurred in the same LG in the case of cell number (all candidate genes are shown in Table A7.5).

Correlated LG QTL

In LG XIII, one candidate gene was collocated in the QTL identified for stomatal density (ab) for UK, *SPS* (*SOLANESYL DIPFOSPHATE SYNTHASE*) a gene involved in isoprenoid metabolism, but it was not possible to collocate a candidate gene in the QTL identified for Italy.

In the case of stomatal index (ad) six candidate genes were collocated; three on LG III in the UK and three genes in Italy. *VKK1*, *ACC_synthase* and *XTH5* were collocated in the UK, while *MNAS 5.9* involved in threonine dehydratase biosynthesis, *STI* (*STICHEL*), which has a role in trichome differentiation, and *GL2* (*GLABRA 2*), which

regulates epidermal cell identity including trichomes and root hairs were identified in Italy.

In LG VI at positions 108 in the UK and 113 in the response Ita/UK, only one candidate gene was collocated near the QTL position identified for stomatal number (ab): *GPA1*, a gene involved in cell death and stomatal movement found in guard cells. In the same LG VI but in position 25 identified in Italy, no candidate gene was collocated in the exact position or near it. However in the range of interval confidence, 4 candidate genes were collocated: *VFK1*, *ASD1*, *BRU1*, *XTH23*. From these, *BRU1* (*ENDO-XYLOGLUCAN TRANSFERASE MERI-5*) and *XTH-23* (*XYLOGLUCAN ENDOTRANSGLYCOSYLASE 6*) involved in brassinosteroid and xyloglucan up regulatation by ABA, responses respectively (Yokoyama and Nishitani 2001) could be more related to environmental stress, as both the trait evaluated and the gene function are related to a response to water stress.

The last correlated LG is LG XIV, where 3 candidate genes were collocated in the UK: *ROT4* (*ROTUNDIFOLIA*) a gene involved in cell proliferation, *XTH5* involved in cell loosening and *PPC1* (*PHOSPHOENOL PIRUVATE CARBOXYLASE*) involved in tricarboxylic acid biosynthesis. In the same LG but in a different position, *ANT* (*AINTEGUMENTA*) a gene involved in organ initiation and growth, was the nearest gene collocated for this QTL (all candidate genes are shown in Table A7.5).

7.5.5 Contrasting QTL

It is striking that LER only had one different QTL mapped for each of the two field sites (Table A7.5). In the UK, the QTL located on LG IV explained 10.2% of variance, and *CDC2A* (*CELL DIVISION CONTROL 2*), a cyclin-dependent kinase subunit for cell division collocated to this position. This cyclin is regulated by environmental and chemical signs and part of the promoter is responsible for expression in trichomes. In contrast, the QTL mapped on LGXIV at the Italian site with a lower variance (4.4%) than the one mapped in the UK. This last QTL collocated to three xyloglucan endotrasglucosylase/hydrolase genes (*XTH4*, *XTH5*, *XTH7*). Each of these may play a specific role. For example, *At-XTH4* is mainly expressed in young, developing tissues,

and *At-XTH5* is mainly expressed in the roots of *Arabidopsis* (Akamatsu et al. 1999, Yokoyama and Nishitani 2001). However, it has also been reported that *AT-XTH4* is increased by auxin, touch and darkness, which can stimulate cell elongation and growth (Xu et al., 1996). The presence of a QTL mapped to this region at the Italian site, could be interpreted as an involvement in the decrease of leaf growth. This work has identified LG XIV as particularly interesting as several of the genes seen to collocate with QTL are involved in the regulation of cell fate, trichome and stomatal formation.

Overall, fourty four candidate genes were collocated in the physical map of *P. trichocarpa* and can be divided in three groups: 1) 10 genes presented in leaf traits only, 2) 5 genes shared with leaf and cell traits and 3) 22 in cell traits, from which 3 were shared with common and correlated cell QTL traits, and one with Correlated LG QTL. Correlated LG QTL also share one gene with Common QTL and two with Contrasting QTL (Table 7.2).

Table 7.2 Summary of candidate genes divided in groups, according to their level of action and degree closeness. Genes names in bold indicated that they are shared in 2 or more groups.

Trait level	Common QTL	Correlated cell QTL	Correlated LG QTL	Contrasting QTL
Leaf traits	*CDK-putative*		*PROLIFERA*	*XTH16*
	CKS1		*ANT*	*CDC2A*
	EXP6			
	REV			
	LOB			
	LEU			
Leaf and cell traits	***AS2***			
	XTH9			
	VFK1			
			XTH5	***XTH5***
			PPC1	***PPC1***
Cell traits	***TTG1***	***TTG1***		
	AGO1	***AGO1***		
		ACC synthase	***ACC synthase***	
		AS2		
			VKF1	
		CER6	*GL2*	
		EGL3	*SPS*	
		GL3	*XTH23*	
		ACC1	*MINA5.9*	
		CDKB;2	*GPA1*	
		CDKB2;1	*ASD1*	
		IRE	*BRU1*	
		PLE	*STI*	
		XTH15		

This natural separation indicates a clear division between the traits assessed and the candidate genes that could control them (e.g. leaf traits and cell traits). Few of the candidate genes were shared in the control of the traits and their study needs different approaches.

For the aims of this research work, the use of changes in morphology and physiology when the F_2 population grows in conditions were key to the revealing of genetic basis of drought tolerance in *Populus*. The approach of common, correlated and contrasting QTL to identify candidate genes that could control this trait was successful. Now, further research is needed in the cloning of some of the candidate genes presented here to prove the accuracy and reliability of this work.

7.6 Conclusions

This research identified the adaptive responses that occurred in the leaves of the parental species and a hybrid F_2 family. The favourable and harsh conditions of the UK and Italy respectively allowed identification of certain candidate genes that could be up-regulated or over-expressed at each location. The data showed how important site conditions are in influencing gene expression and allowed genetic dissection by QTL analysis based on an understanding of the morphology and physiology of the genotypes present.

QTL analysis of Family 331 identified polygenic control of most of the traits evaluated with differences at each site, and highlights the environmental influence. Leaf extension rate, the trait related most directly with growth rate, was strongly affected by site/environment and the candidate genes associated with them seems to be related with promotion and depression of growth. The latter can be considered as a response to injury.

One of the main findings was the collocation of QTL and candidate genes for cell traits in LG I, III, VIII and XII, where the complex *TTG1*, *GL1*, *GL2*, *GL3* and *EGL3,* seems to be frequently expressed. This complex is closely related to the control of cell fate specification showing a strong link between morphology, environment, QTL and candidate genes. As far as I know, this is the first time that trichome number has been assessed as a trait in *Populus trichocarpa* and *P. trichocarpa* x *deltoides* hybrids, and it can be expected that the cloning of the trichome-stomata genes complex could give insight into the mechanisms by which cell fates are determined.
In the same way, the coincidence in the mapping of QTL for cell area and number (LG II) in Italy and the relationship of this QTL with the candidate genes involved in cyclin regulation is interesting and worthy of further investigation, as well as the differential positional control on adaxial and abaxial surfaces in LG XII in the UK and Italy. The responses observed in stomatal number point toward the investigation of LG VI as the two genes collocated are related with the response to the stress hormone ABA. From these, *GPA1* is one of the most interesting to further investigation, because of its involvement in the cell death process.

In the work reported here, QTL analysis allowed the dissection of the genetics of complex characters and showed that stomatal traits are good indicators of adaptation to stress. Significant progress has been made here by using poplar Family 331 to understand the genetic basis of responses to environmental conditions as the results also suggest that the cellular traits assessed on the abaxial side are more involved with morphological features while the abaxial ones were more involved with environmental responses, a direction that needs immediate attention. The work is of considerable interest not only in poplar breeding programmes but also because it helps us to understand the mechanisms responsible for the interactions between genetics and environment in other forest trees.

Chapter 8

General Discussion and Conclusions

General Discussion

The experimental work presented here on the physiology and morphology of *P. trichocarpa* and *P. deltoides* provides good inside into the ecophysiology and the genetic basis of drought tolerance in *Populus* and in particular, in their F_2 hybrid Family. Studies of these and other genotypes have identified their remarkable yields when grown in short rotation coppice, and intensive work has concentrated on finding the genetic and molecular basis of how biomass can be increased at a individual and species level (Tuskan 1998, Stettler et al. 1988).

Gas exchange

The study of gas exchange revealed the different strategies that the parental species develop when they face a complete lack of water. No significant difference in photosynthetic rate was found between the two species, *P. trichocarpa* (93-968) and *P. deltoides* (Ill-129) growing in well irrigated conditions (Figure 3.1, 3.2), but marked differences in the response were found in droughted plants. Stomatal closure does not occur or is partial in *P. trichocarpa*, but total and rapid in *P. deltoides* (Figure 3.3). Similar effects have been reported by Scarasccia-Mugnozza et al. (1986) and Bassman et al. (2001). However, it is important to mention that:

1) Photosynthetic rates during water deficit decreased more rapidly in *P. deltoides* than in *P. trichocarpa.*
2) The decreases in photosynthetic rate in *P. deltoides* are proportional to decrease in stomatal conductance and both decrease first in the older leaves.
3) Photosynthetic rates in *P. trichocarpa* decrease from the basal leaves, but increase in the top leaves of the plant, where an important decrease in stomatal closure occurs in the youngest leaf.
4) Similar gas exchange regulation occurs in leaves of different position between genotypes, revealing differences between genotypes in the physiology of leaves of leaves of the same LPI.
5) After two weeks of drought, mature leaves of *P. deltoides* show changes in leaf colour and the onset of senescence, while *P. trichocarpa* show much earlier impacts with leaves starting to be lost at 9 DAT.

Leaf loss and branching sacrifice are physiological responses to drought and can last from weeks to months. They are adaptive traits for drought avoidance, because these responses eliminate transpirational demand, thus improving water balance and contributing to the survival of the youngest leaves (Rood et al. 2003, Munne-Bosch and Alegre 2004, Taiz and Zeiger 2002, Ridolfi et al. 1996).

The results obtained show differences related to species and secondly, physiological differences related to leaf ageing. Similar results in *Populus* were obtained by Scarascia-Mugnozza et al. (1986) who explained changes in photosynthesis by a reduction in CO_2 fixation in juvenile leaves caused by protoplast volume shrinkage, as reported by Berkowitz and Gibbs (1983). In the current experiment, the results suggest 1) that limitation of photosynthesis in juvenile leaves by drought is delayed because translocation from source to sink leaves allowing them to continue maintaining high rates of photosynthesis in comparison to older leaves, and 2) that rapid stomatal closure in *P. deltoides* and loss of older leaves in *P. trichocarpa* help to maintain high water potential in the young leaves. As stress caused by drought develops slowly and increases with time and intensity (Munné-Bosch et al. 2001), this response allows sustained grow in the early period of drought.

The results in juvenile leaves indicate that during the early stages of water stress, WUE may increase, i.e. more CO_2 may be taken up per unit of water transpired and growth continues. What is not clear is why do the youngest leaves of *P. trichocarpa* continue having much higher rates of photosynthesis than in *P. deltoides*? An explanation may be because in young leaves of *P. deltoides*, photosynthesis is limited due to low stomatal conductance, low chlorophyll content, immature chloroplasts and reduction of light caused by anthocyanins (Choinski et al. 1993, 2003). As a reddish colour is observed in young leaves of *P. deltoides* and not in *P. trichocarpa*, differences in ontogeny are suggested but they need to be proved.

Responses to CO_2 and Light

A/*Ci* responses showed a better performance of *P. trichocarpa* than *P. deltoides* at moderate PAR, attributed to a very high carboxylation efficiency and a lower dark respiration rate in the former. They also revealed that *P. deltoides* had a lower Vc_{max} than *P. trichocarpa*.

Contrary to expectation, *P. trichocarpa* showed a better performance in response to light than *P. deltoides*. An explanation for this is that *P. deltoides* was growing in a shaded site. In the wild, it is a pioneer species establishing on exposed sites usually in riparian areas and requires full exposure to sunlight for best growth (Cooper and Van Haverbeke 1990). Regehr et al. (1975) reported that *P. deltoides,* at conditions of 30 °C and 2000 PAR, had Vc_{max} of 59 ($\mu mol\ m^{-2}\ s^{-1}$) and a Jmax of 117 ($\mu mol\ m^{-2}\ s^{-1}$), contrasting with the values obtained here of Vc_{max} of 18.5 ($\mu mol\ m^{-2}\ s^{-1}$) and a Jmax of 96.8 ($\mu mol\ m^{-2}\ s^{-1}$) (see Table 3.6). As sun and shade leaves differentiate according to the light environment in which they are grown (Terashima et al. 2005), it is concluded that plants used for this particular experiment were adapted to shaded conditions and showed how *P. deltoides* is affected when grown in less sunny conditions.

P. trichocarpa however showed a higher Vc_{max} and a lower compensation point than in *P. deltoides*, as well as a higher photosynthetic rate in light at a moderate temperature. This feature could be a link to leaf anatomical and biochemical adaptations that allow a higher Rubisco concentration and a lower compensation point in *P. trichocarpa*. Ridolfi et al. (1997) showed for the cultivar 'Peace' (*P. koreana* x *P. trichocarpa*) that drought induced a sharp and parallel decrease in A and in g_c. The decline in A and even the onset of senescense, in 'Peace' was accompanied by a perfect stability of predawn photochemical efficiency of PSII, confirming that PS II is insensitivity to severe drought stress. In 'Peace', f_c/f_o did not change and this would indicate that the drought-induced decrease of net assimilation could not be ascribed to a limitation by CO_2 availability in the chloropasts, showing that that response is not the typical one that occurs in C_3 plants.

Stomatal dynamics and functioning

Following the main findings in the first chapter as summarized above, which showed the importance of leaf development in the contrasting stomatal responses, the study of stomatal dynamics in *P. trichocarpa* and *P. deltoides* aimed to identify the patterns followed in stomata according to leaf age and level of insertion in order to separate ontogenetic and developmental differences of each species, from responses to drought. Diurnal and midday/ midnight conductance showed a very similar pattern in the two grandparental species. g_s values were similar in the two genotypes, but variations

occurred in the responses at different leaf insertion level and an opposite response occurred at night in the two species. The lack of stomatal closure and high rates of transpiration at midday conductance in *P. trichocarpa* (Figure 4.1), and the contrasting stomatal response during the night in *P. trichocarpa* which does not close stomata, stresses the differences between them.

The stomatal responses during midday and midnight are correlated to the soil water potential and leaf level of insertion. Even though the g_s values of young leaves were always lower than in the mature leaves, young leaves in *P. deltoides* at night have higher conductances indicating the existence of higher turgor in comparison with the mature leaves (Figure 4.2). This result coincides with Stiles and Van Volkenburg (2002) who have explained the presence of high turgor in young leaves of *P. deltoides* as a cause for growth, when the Em (resting mesophyll) is insensitive to decade changes in external [K^+].

At night, while mature leaves of *P. deltoides* show conductance near to 0, *P. trichocarpa* had g_s values higher than 150 mmol m^{-2} s^{-1}. There are several reports of high conductance at night in poplars and these include observations on *P. balsamifera* spp. *trichocarpa* as well as other hybrids (Reich 1984, Snyder 2003). Although an explanation of this behavior has not been found, Snyder (2003) mentioned that night time stomatal opening appears to be potentially widespread in plants, which leads to questions about the relative costs and/or benefits of night time water loss. Control of night time g and E are more complicated than initially predicted and further work is needed to explain it. Open stomata in *P. trichocarpa* at night could represent an advantage to concentrate carbon in other ways and that would explain the high CE obtained in A/*Ci* in the drought experiment. *P. trichocarpa* also show the lack of stomatal response to light and these results agree with those reported for 'Peace' by Furukawa et al. (1990) and later by Ridolfi et al. (1997) who also conclude that response to CO_2 in drought conditions does not correspond to the response of a typical C_3 plant. The results presented here suggest that this 'abnormal' stomatal behaviour is more common than previously thought, as it is also found in *P. koreana* and other hybrids such as *P. trichocarpa* x *maximowiczii*, all members of the same section Tacamahaca.

The combination of stomatal conductance with cell sap osmolarity and cell wall extensibility, two properties that have been strongly related with the stomatal behaivour, showed the existence of two well defined groups of leaves; young, from insertion levels 1-5, and mature leaves (levels of insertion 6-12) in the profile studied. These results are consistent with those reported in *P. deltoides* by (Isebrand and Larson 1973) and in other angiosperms (Catsky and Ticha 1982, Ticha 1982). In these reports when there are differences between genotypes, they occur in the youngest and oldest leaves.
The main results of this analysis (Figure 4.3) can be summarized as:

1) Stomatal conductance varies according to leaf insertion level and it is significantly different between *P. trichocarpa* and *P. deltoides* in young but not in mature leaves. However the pattern followed by each genotypes reveals from the ontogenetic changes that are occurring in leaf development.
2) Cell extensibility (plasticity and elasticity) does not differ between both genotypes with the exception of plasticity in the youngest leaf and elasticity in the oldest leaf, both in *P. trichocarpa*, where their values were higher than in *P. deltoides*.
3) Significant differences in cell sap osmolarity were found in mature leaves in well irrigated conditions, where the values for *P. deltoides* were significantly higher that those of *P. trichocarpa*.

Developmental differences between leaves of *P. trichocarpa* and *P. deltoides* are clear, indicating that cell division is completed very early in the development of the former. Van Volkenburgh and Taylor (1996) have mentioned that *P. trichocarpa* leaves stop cell division when leaves were less than 5% full size, in contrast *P. deltoides* cell number continue increasing until the leaf reached full size and therefore the number of stomata also increase. Recently, a profile done by Laura Graham (unpublished data) in our laboratory, showed that once leaves finish extension (Leaf 5), there are no changes in the number of cells with increasing leaf insertion level, so changes in stomata number should not be expected. These results agree with work on CO_2 relationships in drought that showed that growth and physiological differences are related to the developmental state of growing leaf cells (Stiles and Volkenburgh (2002).

Stomatal number in *P. trichocarpa* would remain fixed in young and mature leaves so big differences in g_s should not be expected. As maturation is reached very soon, leaf

ageing would causes senescence and ageing of chloroplasts (Monesch-Boesch et al. 2001) seen as decreases in gas exchange. On the contrary, *P. deltoides* shows the typical pattern reported in the literature for most angiosperms (Catsky and Ticha 1982, Ticha 1982) where leaf gas exchange is related to stomatal densities, development, ontogeny and metabolite changes (Catsky and Ticha 1982, Ticha 1982, Isebrands and Larson 1973). Leaf LPI 5 in *P. deltoides* reached maturity responding in the same way as leaf LPI 1 in *P. trichocarpa* revealing that leaf development and physiology at that point would be equal between the two species, and maximum gas exchange values were shown. Similar responses in WUE for juvenile leaves has been found *Populus deltoides* when submitted to other stresses such as UB-B irradiance (Bassman et al. 2001). From these data it is concluded that leaf age does not necessarily reflect the same ontogenetic changes in the leaves and therefore physiological parameters should be compared with caution.

The patterns observed in cell plasticity and elasticity suggest that cell extensibility is not responsible for the lack of stomatal response in *P. trichocarpa*. However they also shown that extensibility of mature leaves decrease in *P. trichocarpa* before they do in *P. deltoides*, supporting in part the explanation that it is cell wall stiffened what determines the lack of response in *P. trichocarpa* stomata. However, maximum load (N) increases with age in *P. deltoides*, but decreases in juvenile and mature leaves of *P. trichocarpa*, indicating that higher pressures could be resisted by *P. deltoides* epidermal cell (Van Volkenburgh and Taylor 1996). According to Corcuera et al. (2002) Mediterranean oak species avoid an excessive loss of cell water through high cell-wall rigidity.

Profiles of cell sap osmolarity showed that values in young and mature leaves of *P. trichocarpa* were significantly lower than those in *P. deltoides*. When these values were transformed to $\Psi\pi$ and compared to their equal in guard cell protoplast it was clear that values in the cell sap and guard cells of young leaves of genotypes did not differ. However, the same did not occurred in mature leaves where cell sap $\Psi\pi$ and guard cell protoplast in *P. deltoides* only differ by 0.35 while in *P. trichocarpa* the difference is 0.85; a difference of more than 142%. This result is very significant as these differences have a major implication for turgor pressure, membrane functionality and xylem conductivity (see review by Gebre and Tschaplinski, 2000). As osmotic potential

reached by the guard cell in drought is lower than that obtained in well irrigated conditions, this would affect turgor pressure and membrane integrity in epidermal and guard cells, and xylem conductivity. To avoid this the strategy in *P. trichocarpa* in response to drought is the shedding of leaves in order to maintain Ψ_w in the younger leaves, rather than osmotic adjustment. The results shown here would explain the reported by Schulte et al. (1987) that even at water potentials of -7.2 Mpa guard cell does not close, but cells plasmolysis occurs.

Response to ABA and mechanical dehydration

Several publications have attemped to explain the reasons for the lack of response to ABA in *P. trichocarpa* in comparison with the fast response in *P. deltoides*. The most accepted explanation has been that young leaves in *P. trichocarpa* are able to acclimate to water deficit and response to ABA after several periods of drought, but mature leaves do not. The results obtained in this work proved that 1) the response of young leaves to ABA in each genotype is the same as in mature leaves, 2) Stomatal responses in young leaves of both genotypes are masked by the common response to turgor and cuticular transpiration and 3) stomata in mature leaves in *P. trichocarpa* do not respond to ABA nor turgor, even when [ABA] is present at similar concentrations to those which occur in *P. deltoides*. This result does not support the findings of Schultes et al. (1987) regarding changes in stomatal response for water stress acclimatization of young leaves in *P. trichocarpa*, in the same way that those mentioned by Ridolfi et al. (1997)

The results also showed that after ABA application, young leaves (LPI 4) in *P. trichocarpa* and *P. deltoides* continue with good rates of CO_2 assimilation for almost two hours after excision, particularly *P. trichocarpa* (up to 7 μmmol m^{-2} s^{-1}). This result agrees with that obtained in the first experiment for young and juvenile leaves in *P. trichocarpa*, and juvenile leaves of *P. deltoides* (Chapter 1). However, on this occasion, increases of photosynthetic rates are not due to photosynthate translocation from old to young leaves, but result from a lack of ABA effect in *P. trichocarpa* young leaves and in the control of *P. deltoides*, and to a partial reduction in ABA treated *P. deltoides*, showing again that WUE increases with the partial stomatal reduction. Phosynthesis decrease in mature leaves, but result from a lack of ABA response was clearer in *P. trichocarpa* as no change in g_s occurred and CO_2 assimilation rates were keeping high. After one hour, A probably decreased by substrates depletion and not because lack of

water. In the case of *P. deltoides* the same occurred, but the ABA treatment caused a strong reduction of A independently of its concentration.
Thermal imaging analysis showed the response to ABA in detached leaves in young and mature leaves of *P. trichocarpa* and *P. deltoides*, and in the mature leaves in the hybrids G 242 and G 246, parents of the F_2 generation. The results for grandparents' young leaves showed that both genotypes respond to dehydration. Leaf wilting indicates that cuticular transpiration helps to cool the leaf in order to maintain temperatures in the same range as in mature leaves (26.5°C to 29.5 °C). They also showed that *P. deltoides* respond to ABA but not *P. trichocarpa.*

The response in mature leaves confirmed the results obtained with the ABA application previously mentioned and the lack of response in *P. trichocarpa* to dehydration treatment. The F_2 parents G 242 and G 246 showed a delay and a less intensive response to ABA with time, but a more accentuated response to dehydration with lower values of relative conductance reached without complete stomatal closure. However, in both cases leaf temperatures ranged from 25 °C to 30 °C, a wider range than in the grandparents. These delays in ABA response, lack of complete closure during dehydration and wider temperature range could be interpreted as greater tolerance to water deficit, thus explaining the greater drought resistance of these hybrids.

The thermal imaging shows the presence of two mechanisms for stomatal regulation in the genotypes studied; turgor regulation that is a universal feature of terrestrial embryophytes (Raven 2002) and hormonal regulation for ABA which has been identified during the last decade as a mechanism controlled by root-shoot signalling. Both mechanisms have been found frequently in plants, but the mechanism which links turgor to stomatal opening is still unknown. However a basis for this is presented here.

Leaf morphology in *P. trichocarpa* and *P. deltoides*

The grandparental genotypes showed differences in leaf morphology consistent with those of the taxonomical sections Tacamahaca and Aeigeros, to which they belong. The most striking difference was in epidermal cell size (with those of *P. trichocarpa* almost twice those of *P. deltoides*) including that of guard cells, and this determined cell and stomatal density and leaf shape in both genotypes. In the mesophyll of *P.*

trichocarpa, the most distinctive feature was the presence of big intercellular spaces in the spongy parenchyma, the origin and function of which is unknown (Van Volkenburgh and Taylor 1996). Transverse sections in young and mature leaves of both genotypes showed that LPI 3 leaf in *P. trichocarpa* has a well defined mesophyll structure that is similar in *P. deltoides* in LPI 6, proving the developmental differences showed in the drought and stomatal dynamics experiments. In addition, the microscopic evaluation of the transverse sections made it possible to see the apparent extension of the spongy parenchyma in the juvenile leaves of *P. trichocarpa*, and its complete dissolution (as mentioned in Chapter 5) in mature leaves *P. trichocarpa*, but not in *P. deltoides*. Both genotypes showed differences in development, agreeing with the results found in the profiles mainly in stomatal development, intercellular spaces formation, and with previous work on *P. deltoides* (Isebrands and Larson 1973).

Other important morphological features are that mesophyll in *P. deltoides* is thicker when grown in Italy, while *P. trichocarpa* maintains practically the same thickness but shows increased wax and SLA compared to *P. deltoides*. The palisade cells in *P. trichocarpa* seems to reduce their width, but maintain their length in Italy, while the same cells increase in length. However, one of the most interesting changes observed was the size of guard cell in *P. trichocarpa* growing in the UK, and the extremely reduced volume when they were grown in Italy (Figure 5.7), while the cell wall thickness seems to increase considerably, something that was not observed in *P. deltoides*.

These distinctive features mentioned above (Ilustrated graphically in Figure 8.1), are strongly related to CO_2 influx and diffusion, and could be considered as the reason for the carboxylation efficiency shown in *P. trichocarpa* for all age leaves. It could be speculated that a reason for the formation of the large intercellular spaces in mesophyll of *P. trichocarpa* is that, coming from a riparian habitat subjected to intermittent water table oscillations, plants need to increase oxygen intake when flooded as mentioned for the grass *Miscanthus* (Drew et al. 2000). Presence of abundant intercellular spaces in the dorsoventral leaves of some *Populus* hybrids of section Tacamahaca has been reported by Reich (1984).

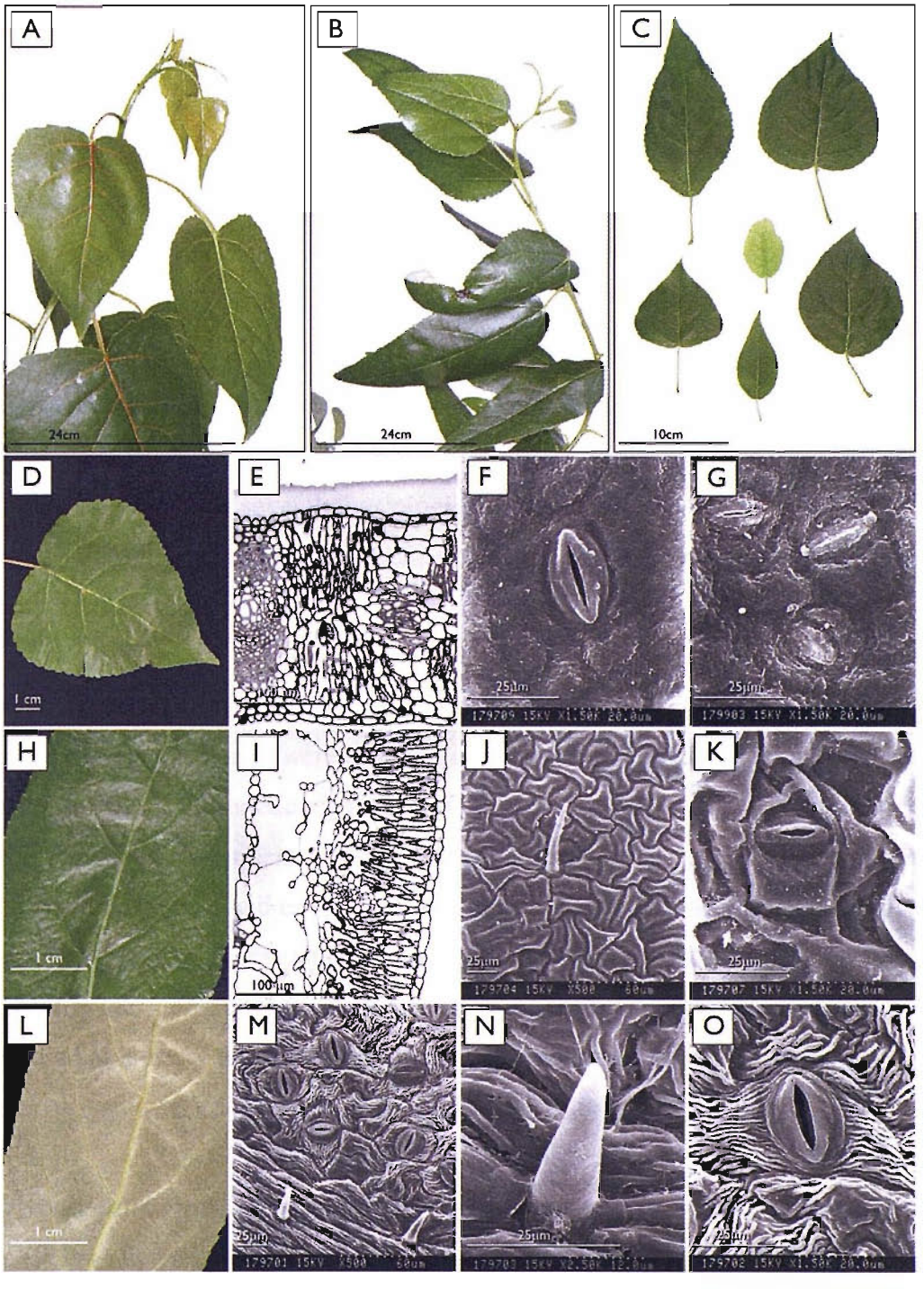


Figure 8.1. Leaf features in *P. trichocarpa and P. deltoides*. The figure shows the leaves of four month old plants of (A) *P. deltoides* and (B) *P. trichocarpa* , (C) six selected F_2 clones and details of leaf shapes in the grandparents; (D) *P. deltoides* , (H) Upper side and (L) lower side in *P. trichocarpa* showing the presence of a whitish wax in the second. Microscopic views of mesophyll and epidermis are showing: *P. deltoides* transversal section of (E) mesophyll , stomata in (F) adaxial and (G) abaxial side showing wax aspect; *P. trichocarpa* (I) mesophyll transversal section , (J) trichome in adaxial and (K) stomata in the abaxial sides. Stomata and mesophyll view of *P. trichocarpa*: (I) mesophyll, (J) trichome in adaxial side , (K) stomatal adaxial side Adaxial side of *P. trichocarpa*: (M) epidermis (N) trichome , (C) stomata (O). Scales as indicated by bars.

Intercellular spaces in leaves are considered one of the selective pressures in the evolution of stomata in the same way as the cuticle is regarded in the evolution of aquatic to terrestrial plants (for a review see Raven 2002). Their presence on the abaxial side could also serve to increase C*i* as a way to compensate for the lack of stomata on the adaxial side of the leaf in *P. trichocarpa* and to increase leaf conductance, as it is reported that hypostomatous leaves have lower conductances than amphistomatous ones (Ridolfi et al. 1997).

Presence of trichomes presence on the adaxial side of the leaf also represented a distinctive characteristic in *P. trichocarpa* which was not found in *P. deltoides*. As this structure is involved in several physiological process including cell fate differentiation, it was considered one of the most useful traits to evaluate responses to contrasting environmental conditions.

Another important trait in the grandparents was stomatal density, as it showed large contrasts when the species were grown in harsh environmental conditions (especially in *P. trichocarpa*). The genetic regulation of the presence or absence of stomata in different parts of the epidermis is not well understood, but progress is being made in understanding genetic and environmental control of stomatal indices. The genotypically determined stomatal index (and/or density) modulated by environmental factors (especially atmospheric CO_2 partial pressure) has been related to past CO_2 levels, and even used as a proxy for these CO_2 abundances (Raven 2002). The presence of cuticular waxes also showed contrasting patterns that deserve to be studied deeply.

Environmental stress effects in the grandparents, parents, and Family 331

The effects caused in the grandparents, parents, and Family 331, by harsher environmental conditions when grown in Italy, was expressed in the strong growth reduction in all the genotypes. This study made it clear that leaf shape, measured as the ratio of leaf width/leaf length, is one of the strongest genetic features segregated in the F_2, and gave a complete panorama of the variation in a second generation in response to environmental stresses as poor soil humidity and high sun irradiance were observed, in comparison to UK conditions.

Leaf shape has been studied in *Arabidopsis*, where the *AN* (*ANGUSTIFOLIA*) gene, a member of CtBP (C Terminal Binding Protein) gene family (Kim et al. 2002), regulates

width of leaf cells and the *ROT3* (*ROTUNDIFOLIA3*) gene, a member of cytochrome P450 family (Kim et al. 1998), regulates length of leaf cells. On the other hand, in seed plants, natural diversity of leaf shape is mainly attributable to diversity of cell number along a particular axis of leaf lamina, not to diversity of cell shape. Thus, polarity-dependent control of cell proliferation genes, involved in the processes of leaf-shape control have been found. *ROT4* gene is involved in control of cell proliferation in the leaf lamina only along the longitudinal axis (Narita et al. 2004) and *AN3* gene, a gene encoding a co-activator, regulates leaf width via regulation of leaf cell proliferation (Horiguchi et al. 2004). Taken together, both cell proliferation and cell elongation in the leaf lamina are controlled in a two-dimensional, polar-dependent manner (Tsukaya 2004).

There was another important response observed in the F_2 when grown in Italy. Petiole traits were less affected than other traits, especially petiole width. It was observed that the same occurred with petiole traits in *P. deltoides*, so it is clear that this response in petiole traits could be attributed to the male grandparent *P. deltoides.*

Stomatal density is also subject to genetic (endogenous) control, as illustrated by the differences between varieties of the same species (Reich 1984, Buttery et al. 1992, 1993; Ramos et al. 1992) or F_1 hybrids (Abak and Yanmaz 1985). The multigenic, oligogenic, or monogenic control of stomatal characteristics has also been demonstrated (Jones 1987). The molecular mechanisms controlling stomatal differentiation, however, are poorly understood and no information about the control mechanisms involved is available.

Because stomatal density may be affected by alterations in epidermal cell size and/or changes in the ratio of pavement cells to stomata, stomatal indices (SI) are very important. SI increased in the grandparents, as an expected response, but decreased in the F_2 in response to drought, an unexpected response as it has been reported that SD increases. This decrease in SI could be interpreted as a pleiotropic effect of leaf growth effect and clonal effect interaction that should be studied further. The study of mutant *sdd1-1* has shown that most of the leaf epidermis is composed of stomatal complexes (Berger and Altmann 2000), modifying the minimum distance between guard cells, what is known as the major principle of order (Sachs et al. 1993).

Adaxial stomatal index was reduced in the F_2 and in G 246, but increased in *P. deltoides* and in G 242. In *P. trichocarpa* the increase in SI and TI on the adaxial side was about 500%, suggesting that there was a cell fate modification, promoting trichome differentiation. However on the abaxial side, stomatal index only increased clearly in *P. deltoides*, and showed a slight decrease in *P. trichocarpa* and G 246. The decreases in G 242 and F_2 were more pronounced, indicating that even when SD increases, changes in cell area and therefore cell number affect the SI. These results can be explained by the fact that stomatal index does not take into account the number of trichomes, which increased in *P. trichocarpa* and in the F_2. The SI values for the adaxial and abaxial sides give some evidence of a different control in SI, that would be very important to investigate. They also show important differences between male and female parents and grandparents that could be influencing these results.

Trichome density increased in the F_2 growing in Italy showing that to express this feature plants need to be submitted to stressful conditions. This could be explained by a mechanism that allows more cells to be recruited to the trichome cell fate pathway because they are not required for the stomatal cell fate. Its increase is linked to the SI decreases observed in F_2 and could be explained by the selective pressure of soil humidity and sun irradiance, in order to improve cooling and diseases protection in the leaf. According to Quarrie and Jones (1977), changes in trichome numbers following ABA or water stress may be due to the reduction in stomata, allowing more protodermal cells to be recruited into the trichome development pathway. Recruitment of protodermal cells to various epidermal cell fates has been shown to be competitive and an increase in one cell type, such as trichomes, leads to a concomitant decrease in another cell type, such as stomata (Glover et al. 1998, Bird and Gray 2003). Trichome patterning has been postulated to be fully positionally dependant, and pre-existing epidermal cells are recruited to become part of the trichome complex (as socket cells) rather than part of the trichome developmental cell division programme.

The multigenic, oligogenic, or monogenic control of stomatal characteristics has also been demonstrated in the past (Jones 1987). The molecular mechanisms controlling stomatal differentiation, however, are poorly understood and no information about the control mechanisms involved is available. It could be considerd that stomata and trichome formation are part of the same mechanism, but as trichome initiation ends

before stomatal initiation begins, the mechanism by which this pathway would function is still unclear (Bird and Gray 2003). Whatever, its origin, this trait could be used as an stress indicator.

QTL and candidate genes

QTL mapped allowed a comprehensive dissection of the genetic components. It showed which LG are multi and uni control in the traits evaluated. For example, LG VIII and XII were the ones with more traits, while VII and XV were specific to only one trait.

Few QTL were coincident in the position, effect and site (Table 7.4), which represent more possibilities than that part of the chromosome regulates the trait mapped. In addition, in some cases the QTL mapped in leaf traits is in agreement with the trait at a cellular level, as in the case of LG X where QTL for SLA is in the same position as the QTL for SD and SI, suggesting an interaction between them.
In general, heritability - defined as the proportion of the total phenotypic variation of a trait that has a genetic basis - showed higher values in leaf traits in the UK, and values were lower in Italy than in the UK, indicating a major effect of environmental factors in Italy (Table 7.2). Values of up to 0.87 were obtained for traits such as leaf area, length, width, leaf width/length in the UK. Heritability values were also slightly higher in the adaxial cell traits than in the abaxial traits, a fact that could show that abaxial cell traits are more influenced by environment than the adaxial traits. This environmental control could partially explain why, in the case of SLA and petiole traits, heritabilities are higher in Italy than in the UK.

For trichome traits differences of heritability also showed between site but in the opposite direction. Trichome heritabilities were much higher in Italy than in the UK, indicating that these traits were not expressed in the UK environmental conditions. This agrees with the normal distribution of trichomes in the F_2, and a not normal distribution obtained in the UK. In summary, a good understanding of the physiology and morphology of the original parental species (grandparents) of Family 331 and their response in contrasting environmental conditions has been extremely helpful in the interpretation of the QTL mapped in this work.

As Frewen et al. stated (2000), once the QTL are identified, the gene or genes responsible for that variation need to be identified. In this research work, common, correlated and distinctive QTL were used to propose them, in order to increase the probability that the traits are regulated by the candidate genes proposed here.

The natural separation between the 42 candidate genes collocated in the physical map of *P. trichocarpa* presented in Table 7.6, indicates a clear division between the traits assessed and the biological processes that each gene could controls. Few of the candidate genes shared in the control of the traits and therefore its study needs different approaches. For the aims of this research work, the approach of common QTL to identify candidate genes that could control this trait was successful in the sense of number, function and level of action. It also indicates the suitability of the use of changes in morphology and physiology when the F_2 population grows in different conditions as a key to identification of the genetic basis of drought tolerance in *Populus*. Now, further research is needed in the cloning of some of the candidate genes presented here to prove the accuracy and reliability of this work.

Conclusions

A comprehensive study is presented here of two genotypes of *Populus*, with distinctive morphological features derived from their distinct phylogenetic origin. Important differences in leaf ontogeny and development are responsible for the contrasting stomatal behaviour which give rise to different physiological responses and differences in gas exchange and leaf conductance.

An important contribution has been made here in revealing stomatal behaviour, especially in *P. trichocarpa*, where the response is a function of water potential and leaf maturity, but not of the stress hormone ABA. As mentioned by Raven (2002), turgor regulation is a universal feature of terrestrial embryophytes. Therefore the existence in a close ancestor of regulation of internal osmolarity equaling turgor regulation, opens up the possibility that these two mechanism are present in *Populus* and therefore represent a fundamental key in the understanding of their guard cell function. Of the responses to water deficit, the increase of WUE during the first stages of drought, whether natural or caused by externally applied ABA, was highly marked, especially in young leaves, showing the direction in which biomass could be improved in *Populus*.

The results also show a greater water deficit tolerance in the hybrids (F_1) than in their parents. This is based on a clear reduction in responsiveness to ABA, delaying the time of response and increasing the minimum threshold values of g_s, and increasing the response to dehydration, and lowering the energetic cost of leaf area per dry weight (SLA). In addition, the hybrids also showed strong similarities to the grandparents, stressing the differences influenced by sex.

Family 331 responses to contrasting environmental conditions gave strong evidence for the ecophysiology and genetic basis of drought tolerance in *Populus* using a morphological and physiological approach, opening many possibilities for further research in *Populus*. In the practical sense what is suggested here is that biomass increase in *Populus* depends not only on genetic and environmental factors, but also on how the microenvironment can be manipulated through cultural practices performed

according to the different stages of plant development and specific planting sites. In other words major attention must be given to the ecotypes instead of a single ideotype.

The insight given in the stomatal function in the grandparents reveals three main problems that deserve further attention.

1) From an evolutionary perspective, the questions are: Whether the stomatal regulation mechanism in *P. trichocarpa* is more widely distributed in vascular plants and in particular angiosperms? Has the control of stomata by ABA in *P. deltoides* evolved in poplars to allow growth in drier habitats?. The fact that the same stomatal behaviour shown by *P. trichocarpa* has been reported in other genotypes or hybrids in the Tacamahaca section, or other vascular plants such as *Gunnera* (a typical plant of wet habitats) and *Salix*, suggests that this could be the case. However evidence for this should be sought. In addition to this, *P. deltoides* is the only species in its section that has been reported to have slow rooting properties, therefore, it would be very interesting to know if its fast response to ABA could be linked to this poor rootability.

2) From a physiological and biochemical prespective, the questions raised are: What is the advantage of night transpiration in *P. trichocarpa*? Does this feature occur only in Tacamachaca section members? Further studies are needed in order to answer these questions.

3) In a hydraulic and molecular sense, what is the exact mechanism of the conversion of turgor to stomatal regulation? Do younger leaves in *P. trichocarpa* ever reach a low water potential when the leaf shedding process exits? If yes, how does the stomata closure process occur? Is it possible that *P. trichocarpa* closes stomata when leaf water potential reaches lower negative values than those found in the guard cell protoplast? If not, is it that osmolality in the guard cell is a mechanism that indicates the limits of the xylem functionality? The investigation of this problem in a developmental way would show how this process changes with age and when accompanied by genomics could help to reveal the mechanism involved in the conversion of turgor, from a mechanical stimulus to a chemical signal and to stomatal closure.

Undoubtedly, a complete understanding of this mechanism could contribute to a better control of transpiration and water use efficiency in plants when water deficit conditions are present and therefore in biomass production in marginal agricultural lands.

References

Aasamaa K, Sõber A, Hartung W, Niinemets Ü. (2002). Rate of stomatal opening, shoot hydraulic conductance and photosynthetic characeristics in relation to leaf abscission concentration in six temperate deciduous trees. Tree physiology **22** 267-276

Abak K, Yanmaz R. 1985. Investigation on the stomatal density in certain pepper lines and their F1 hybrids. Capsicum Newsletter **4:** 22

Aitken S, Adams T. (1996). Meeting Report, Western Forest genetics association Annual meeting newport, Oregon July 29-August 1. Dendrome. http://dendrome.ucdavis.edu/Newsletter/wfgareport.html . 08/04/2003.

Akamatsu T, Hanzawa Y, Ohtake Y, Takahashi T, Nishitani K, Komeda Y . 1999 . Expression of Endoxyloglucan Transferase Genes in *acaulis* Mutants of Arabidopsis. Plant Physiol. 121: 715-722

Allan AC, Trewavas AJ. (1994). Abscisic acid and giberellin perception: inside or out? Plant Physiology **104** 1107-1008

Allen GJ, Kuchitsu K, Chu SP, Murata Y, Schroeder JI. (1999). Arabidopsis abi1-1 and abi2-1 phosphatase mutations reduce abscisic acid-induced cytoplasmic calcium rises in guard cells. *Plant Cell* **11**, 1785-1798

Allen GJ, Chu SP, Schumacher K, Shimazaki CT, Vadefeados D, Kemp A, Hawke SD, Tallman G, Tsien RY, Harper JF, Chory J, Schroeder J. (2000). Alteration of stimulus-specific guard cell calcium oscillations and stomatal closing in Arabidopsis det3 mutant. Science **289 (5488)** 2338-42

Allen MT, Pearcy RW. (2000). Stomatal versus biochemical limitations to dynamic photosynthetic performance in four tropical rainforest shrub species. Oecologia **122** 479-486

Alonso-Blanco C, Koornneef M. (2000). Naturally occurring variation in Arabidopsis: an underexploited resource for plant genetics.Trends in Plant Science **5** 22-29

Altman A, Alegrand T, Pelah D, Tzifira T, Vinocur B, Wang W, Yamitzky O. (1996). Molecular Biology of Drought Tolerance and transformation of Populus and Pinus at the Hebrew University of Jerusalem. Dendrome **3 (2)**:5-7

Andersson A, Keskitalo J, Sjödin A, Bhalerao R, Sterky F, Wissel K, Tandre K, et al. (2004). **A transcriptional timetable of autumn senescence.** Genome Biology 5(4) R24

Arisi AC, Noctor G, Foyer CH, Jouanin L. (1997). Modification of thiol contents in poplars (*Populus tremula* x *P. alba*) overexpressing enzymes involved in glutathione synthesis. Planta 203 362-372

Arndt SK, Wanek W, Clifford SC, Popp M. (2000). Contrasting adaptations to drought stress in field-grown *Ziziphus mauritiana* and *Prunus persica* trees: water relations, osmotic adjustment and carbon isotope composition. Australian Jornal of Plant Physiology **27** 985-996

Assman SM. (2003). OPEN STOMATA1 opens the door to ABA signaling in Arabidopsis guard cells. Trends in Plant Science **8** (**4**) 151-153

Augé RM, Duan X, Croker JL, Witte WT, Green GD. (1998). Foliar dehydration tolerance of twelve deciduous tree species. Journal of Experimental Botany 49 (321) 753-759

Baggerly KA, Coombes KR, Hess KR, Stivers DN, Abruzzo LV, Zhang W. (2001). Identifying Differentially Expressed genes in cDNA Microarray Experiments. Journal of Computational Biology **8** (**6**) 639-659

Baldocchi DD, Verma SB, Anderson DE. (1987). Canopy photosynthesis and water-use efficiency in a deciduous forest. Journal of Applied Ecology **24** 251-260

Ball MC, Hodges V, Laughlin CP. (1991). Cold-induced photoinhibition limits regeneration of snow gum at treeline. Functional Ecology **5** 663-668

Ball MC, Egerton JJG, Leuning R, Cunningham RB, Dunne P. (1997). Microclimate above grass adversely affects spring growth of seedling snow gum (*Eucalyptus pauciflora*). Plant, Cell and Environment **20** 155-166

Balocchi CE, Bridgwater FE, Zobel BJ, Jahromi S. 1993. Age trends in genetic parameters for tree height in a non-selected population of loblolly pine. Forest Science **39**:231-251

Barrero RA, Umeda M, Yumamura S, Uchimiya H. (2002). Arabidopsis CAP Regulates the Actin Cytoskeleton Necessary for Plant Cell Elongation and Division. The Plant Cell **14** 149-163

Bassman JH, Robberecht R, Edwards GE. (2001). Effects of Enhanced UV-B Radiation on Growth and Gas Exchange in *Populus deltoides* Bartr. ex Marsh. International Journal of Plant Sciences **162** 103–110

Bassman JH, Zwier JC. (1991). Gas exchange characteristics of *Populus trichocarpa, Populus deltoides* and *Populus trichocarpa* x *P. deltoides* clones. Tree Physiology **8** 145-159

Beadle CL, Ludlow MM, Honeysett JL. 1993. Water relations. In Hall DO, Scurlock JMO, Bolhar-Nordenkampf HR, Leegood RC, Long SP eds. Photosynthesis and Production in a changing environment. 1st ed. 113-128

Beerling DJ, Kelly CK. (1996). Evolutionary comparative analyses of the relationship between leaf structure and function. New Phytologist **134** 35-51

Berger D, Altmann T. (2000). A subtilisin-like serine protease involved in the regulation of stomatal density and distribution in Arabidopsis thaliana . Genes & Development **14 (9)** 1119-1131

Bertrand A, Robitaille G, Nadeau P, Boutin R. (1994). Effects of soil freezing and drought stress on abscisic acid content of sugar maple sap and leaves. Tree Physiology **14** 413-425

Bird SM, Gray JE. (2003). Signals from the cuticle affect epidermal cell differentiation. New Phytologist **157** 9-23

Blake TJ, Tschaplinski TJ, Eastham A. (1984). Stomatal Control of Water Use Efficiency in Poplar Clones and Hybrids. Canadian Journal of Botany **62** 1344-1351

Bond BJ, Kavanagh KL. (1999). Stomatal behaviour of four woody species in relation to leaf-specific hydraulic conductance and threshold water potential. Tree Physiology **19** 503-510

Bolhar-Nordenkampf HR, Draxler G. (1993). Functional leaf anatomy. Chapter 7 in **Hall DO, Scurlock JMO, Bolhar-Nordenkampf HR, Leegood RC, Long SP eds.** (1993). Photosynthesis and Production in a changing environment 1st ed. Chapman & Hall. UNEP. 91-112

Boston RS, Viitanen PV, Vierling E. (1996). Molecular chaperones and protein folding in plants. Plant molecular Biology **32 (1-2)** 191-222

Boyer JS. (1982). Plant productivity and environment. Science **218** 443-448

Braatne JH, Hinckley TM, Stettler RF. (1992). Influence of soil water on the physiological and morphological components of plant water balance in *Populus trichocarpa*, *Populus deltoides* and their F1 hybrids. Tree Physiology **11** 325-229

Bradshaw HD, Ceulemans R, Davis J, Stettler R. (2000). Emerging model systems in plant biology: poplar (*Populus*) as a model forest tree. Journal of Plant Growth Regulation **19** 306-313

Bradshaw HD, Grattapaglia D. (1994). QTL mapping interspecific hybrids of forest trees. Forest Genetics **1 (4)** 191-196

Bradshaw HD Jr., Stettler RF. (1995) Molecular genetics of growth and development in *Populus* IV. Mapping QTLs with large effects on growth, form and phenology in a forest tree. Genetics **139**: 963-973.

Bradshaw HD, Stettler RF. (1997). Molecular genetics of growth and development in *Populus* (Salicaceae). 5. Mapping quantitative traits affecting leaf variation. American Journal of Botany **84 (2)** 143-153

Bradshaw HD Jr, Strauss SH. (2001). Breeding strategies for the 21st Century: domestication of Poplar. In Poplar Culture in North America. Part B, Chapter 14. Edited by D.I. Dickmann, J.G. Isebrands, J.H. Eckenwalder, and J. Richardson. NRC Research press, National Research Council of Canada, Ottawa, ON KIA 0R6, Canada pp. 383-394

Bradshaw T, Villar M. (1996). International Poplar Symposium, University of Washington, Seattle, 5 August 1995. Dendrome **3 (1)** 14-15

Bradshaw HD Jr., Villar M, Watson BD, Otto KG, Stewart S, Stettler RF. (1994). Molecular genetics of growth and development in *Populus*. III. A genetic linkage map of a hybrid poplar composed of RFLP, STS and RAPDS markers. Theoretical and Applied Genetics 89:167-178

Bradshaw HD, Stettler R F. (1994). Molecular genetics of growth and development in *Populus*. II. Segregation distortion due to genetic load. TAG Theoretical and Applied Genetics 89 551-558

Bray EA. (1997). Plant responses to water deficit. Trends in Plant Science **2** 48-54

Bray EA. (2002a). Abscicic acid regulation of gene expression during water-deficit stress in the era of the *Arabidopsis* genome. Plant, Cell and Environment **25** 153-161

Bray EA. (2002b). Classification of Genes Differentially Expressed during Water-deficit Stress in *Arabidopsis thaliana*: an Analysis using Microarray and Differential Expression Data. Annals of Botany **89** 803-811

Brown RH, Hettersley PW. (1989). Leaf anatomy of C_3-C_4 species as related to evolution of C_4 photosynthesis. Plant Physiology **91** 1543-1550

Brooks A, Farquhar GD. (1985). Effects of temperature on the CO_2/O_2 specificity of ribulose-1,5- biphosphate carboxylase/oxygenase and the rate of respiration in the light. Planta **165** 397-406

Buckley TN, Farquhar GD, Mott KA. (1997). Qualitative effects of patchy stomatal conductance distribution features on gas-exchange calculations. Plant, Cell and Environment **19** 131-135

Bunn, SM, Rae AM, Herbert CS, Taylor G. (2004). Leaf-level productivity traits in Populus grown in short rotation coppice for biomass energy. Forestry **77** 307-323

Burssens S, Himanen K, van de Cotte B, Beeckman T, Van Montagu M, Inze D, Verbruggen N. (2000). Expression of cell cycle regulatory genes and morphological alterations in response to salt stress in *Arabidopsis thaliana*. Planta **211** 632-640

Buttery BR, Tan CS, Buzell RI, Gaynor JD, Mactavish DC. (1993). Stomatal numbers of soybean and response to water stress. Plant Soil **149:** 283-288

Buttery BR, Gaynor JD, Buzell RI, Mactavish DC, Armstrong RJ. (1992). The effect of shading on kaempherol content and leaf characteristics of five soybean lines. Physiol. Plant. **86:** 279-284

Cameron KD, Teece MA, Bevilacqua E, Smart L B. (2002). Diversity of cuticular wax among *Salix* species and *Populus* species hybrids. Phytochemistry **60** 715-725

Catsky J, Ticha I. (1982). Photosynthetic Characteristics during Ontogenesis of Leaves 6. Intracellular Conductance and its Components. Photosynthetica **16** 253-284

Catsky J, Ticha I. (1980). Ontogenetic Changes in the Internal Limitations to Bean-Leaf Photosynthesis.5. Photosynthetic and Photo-Respiration Rates and Conductances for CO_2 Transfer as Affected by Irradiance. Photosynthetica **14** 392-400

Cervera MT, Storme V, Ivens B, Gusmão J, Liu BH, Hostyn V, Slycken JV, Van Montagu, Boerjan W.2001. Dense Genetic Linkage Maps of Three *Populus* Species (*Populus deltoides*, *P. nigra* and *P. trichocarpa*) Based on AFLP and Microsatellite Markers. Genetics **158** 787-809

Ceulemans R, Impens I. (1980). Leaf exchange processes and related characteristics for several poplar clones based on their gas exchange variables determined under laboratory conditions. Photosynthetica **14** 355-362

Ceulemans R , Impens I. (1983). Net CO_2 Exchange Rate and Shoot Growth of Young Poplar (*Populus*) Clones. Journal of Experimental Botany **34** 866-870

Ceulemans R, Impens I, Imler R. (1988). Stomatal conductance and stomatal behavior in *Populus* clones and hybrids. Canadian Journal of Botany **66** 1404-1414

Ceulemans R, Jiang X N, Shao B Y. (1995). Growth and Physiology of One-year Poplar (*Populus*) Under Elevated Atmospheric CO_2 Levels. Annals of Botany **75**: 609-617

Ceulemans R, Mousseau M. (1994). Effects of elevated atmospheric CO_2 on woody plants. New Phytologist **127** 425-446

Ceulemans R, Van Praet L, Jiang XN. (1995). Effects of CO_2 enrichment, leaf position and clone on stomatal index and epidermal cell density in poplar (*Populus*). New Phytologist **131 (1)** 99-107

Chae HS, Lee WS. (2001). Ethylene- and enzyme-mediated superoxide production and cell death in carrot cells grown under carbon starvation. Plant Cell Reports **20** 256-261

Chen SL, Wang SS, Altman A, Huttermann A. (1997). Genotypic variation in drought tolerance of poplar in relation to abscisic acid. Tree Physiology **17** 797-803

Chen SL, Wang SS, Huttermann A, Altman A. (2002). Xylem abscisic acid accelerates leaf abscission by modulating polyamine and ethylene synthesis in water –stressed intact poplar. Trees-Structure and Function **16 (1)** 16-22

Clifford SC, Black CR, Roberts JA, Stronach IM, Singleton-Jones PR, Mohamed AD, Azam-Ali SN. (1995). The effect of elevated atmospheric CO_2 and drought on stomatal frequency in groundnut [*Arachis hypogea* L.]. Journal of Experimental Botany **46:** 847-852

Cochard H, Ridolfi M, Dreyer E. (1996). Responses to water stress in an ABA-unresponsive hybrid poplar (*Populus koreana* x *trichocarpa* cv. Peace). New Phytologist **134** 455-461

Coder KD. (1999). Tree selection for dought resistance. Daniel B. Warnell School of Forest Resources. University of Georgia. 4/99. HTML www.forestry.uga.edu/efr

Corcuera L, Camarero JJ, Gil-Pelegrín E. (2002). Functional groups in *Quercus* species derived from the analysis of pressure-volume curves. Trees - Structure and Function **16** 465 – 472

Choinski JS Jr.,Johnson JM. (1993). Changes in photosynthesis and water status of developing leaves of Brachystegia spiciformis Benth. Tree Physiology **13** 17–27

Choinski JS Jr., Ralph P, Eamus D. (2003). Changes in photosynthesis during leaf expansion in Corymbia gummifera. Australian Journal of Botany **51** 111-118

Corcuera L, Camarero JJ, Gil-Pelegrin E. (2002). Functional groups in *Quercus* species derived from the analysis of pressure-volume curves. Trees **16:**465-472

Cowan IR, Farquhar GD. (1977). Stomatal diffusion in relation to leaf metabolism in environment. Symposia of the Society for Experimental Biology **31** 471-505

Croker JL, Witte WT, Augé RM. (1998). Stomatal sensitivity of six temperate, deciduous tree species to non-hydraulic root-shoot signaling of particle soil drying. Journal of Experimental Botany **49 (321)** 761-774

Davies WJ, Tardieu F, Trejo CL. (1994). How do chemical signals work in plants that grow in drying soil? Plant Physiology **104** 309-314

Davies WJ, Mansfield TA, Hetherington AM. (1990). Sensing of soil water status and the regulation of plant growth and development. Plant, Cell and Environment **13** 709-719

Davies WJ, Wilkinson S, Loveys B. (2002). Stomatal control by chemical signaling and the exploitation of this mechanism to increase water use efficiency in agriculture. New Phytologist **153** 449-460

Dawson TE, Ehleringer JR. (1993) Gender-specific physiology, carbon isotope discrimination, and habitat distribution in box-elder, *Acer negundo*. Ecology **74** 798-815

Delfine S, Alvino A, Villani MC, Loreto F. (1999). Restrictions to Carbon Dioxide Conductance and Photosynthesis in Spinach Leaves Recovering from Salt Stress. Plant Physiology **119** 1101-1106

Dickmann DI. (1971). Photosynthesis and respiration by developing leaves of cottonwood (*Populus deltoides* Bartr.) Botanical Gazzette **132** 253-259

Dickmann DI, Liu Z, Nguyen PV, Pregitzer K. (1992). Photosynthesis, water relations, and growth of two hybrid *Populus* genotypes during a severe drought. Canadian Journal of Forest Research **22** 1094-1106

Dickmann DI, Stuart KW. (1983). The culture of poplars in eastern North America. East Lansing, MI: Michigan State University, Department of Forestry. 168 pp

Dickson RE. (1987). Diurnal changes in leaf chemical constituents and ^{14}C partitioning in cottonwood. Tree Physiology **3** 157-171

Donovan LA, Ehleringer JR. (1994). Potential for selection in plants for water-use efficiency as estimated by carbon isotope discrimination. American Journal of Botany **81** 927-935

Donovan LA, Grise DJ, West JB, Pappert RA, Alder NN, Richards JH. (1999). Predawn disequilibrium between plant and soil water potentials in two cold-desert shrubs. Oecologia **120** 209-217

Drew MC, He CJ, Morgan PW. (2000). Programmed cell death and aerenchyma formation in roots. Trends in Plant Science. **5** (**3**) 123-127

Dunlap JM, Braatne JH, Hinckley TM, Stettler RF. (1993). Intraspecific variation in photosynthetic traits of *Populus trichocarpa*. Canadian Journal of Botany **71** 1304-1311

Dunlap JM, Heilman PE, Stettler RF. (1994). Genetic variation and productivity of Populus trichocarpa and its hybrids. VII. Leaf and crown morphology of native *P. trichocarpa* clones from four river valleys in Washington. Canadian Journal of Forestry Research **24** 1539-1549

Dunlap JM, Heilman PE, Stettler RF. (1995). Genetic variation and productivity of Populus trichocarpa and its hybrids. VIII. Leaf and crown morphology of native

P. trichocarpa clones from four river valleys in Washington. Canadian Journal of Forestry Research **25** 1710-1724

Dunlap JM, Stettler RF. (1998). Genetic variation and productivity of *Populus trichocarpa* and its hybrids. X. Trait correlations in young black cottonwood from four river valleys in Washington. Trees **13** 28-39

Dunlap JM, Stettler RF. (2001). Variation in leaf epidermal and stomatal traits of Populus trichocarpa from two transects across the Washington Cascades. Canadian Journal of Botany **75** 528-536

Edwards D, Kerp H, Hass H. (1998). Stomata in early plants: anatomical and ecophysiological approach. Journal of Experimental Botany **49** 255-278.

Erickson R, Michelini F. (1957). The Plastochron Index. American Journal of Botany **44** (**4**) 297-305

Falconer DS, Mackay TFC. (1996). Introduction to Quantitative Genetics, 4th ed. Harlow, UK: Longman

Farquhar GD, Ehleringer JR, Hubick KT. (1989). – Carbon Isotope Discrimination and Photosynthesis. Annual Review of Plant Physiology and Plant Molecular **40** 503-537

Farquhar GD, Sharkey TD. (1982). Stomatal conductance and photosynthesis. Annual Review in Plant Physiology **133** 317-345

Farquhar GD, Von Caemmerer S, Berry JA. (1980). Biochemical model of photosynthesis CO_2 assimilation in leaves of C_3 species. Planta **149** 78-90

Ferris R, Sabatti M, Miglietta F, Mills RF, Taylor G. (2001). Leaf area is stimulated in *Populus* by free air CO2 enrichment (POPFACE), through increased cell expansion and production. Plant Cell and Environment **24** 305-315

Ferris R, Taylor G. (1994). Stomatal characteristics of four native herbs following exposure to elevated CO_2. Annals of Botany **73** 477-453

Ferris R, Long L, Bunn SM, Robinson KM, Bradshaw HD, Rae AM, Taylor G. (2002). Leaf stomatal and epidermal cell development: identification of putative quantitative trait loci in relation to elevated carbon dioxide concentration in poplar. Tree Physiology **22** 633-640

Finkelstein RR, Gampala SSL, Rock CD. (2002). Abscisic Acid Signalling in Seeds and Seedlings. The Plant Cell S15-S45

Fiscus EL, Reid CD, Miller JE, Heagle AS. (1997). Elevated CO_2 reduces O_3 flux and O_3 induced yield losses in soybeans: possible implications for elevated CO_2 studies. Journal of Experimental Botany **48** 307-313

Franks PJ, Farqhuar GD. (2001). The effect of exogenous abscisic acid on stomatal development, stomatal mechanics and leaf gas exchange in Tradescantia virginiana. Plant Physiology 125:935-942

Frewen BE, Chen THH, Howe GT, Davies J, Rohde A, Boerjan W, Bradshaw HD Jr. (2000). Quantitative Trait Loci and Candidate Gene Mapping of Bud Set and Bud Flush in *Populus*. Genetics **154** 837-845

Furukawa A, Park SY , Yasumi F. (1990). Hybrid poplar stomata unresponsive to changes in environmental conditions. Trees - Structure and Function **4** 191-197

Gates DM. (1980). Biophysical ecology. New York, USA: Springer-Verlag

Geber MA, Dawson TE. (1990). Genetic-variation in and covariation between leaf exchange, morphology, and development in *Polygonum arenastrum*, an annual plant. Oecologia **85** 153-158

Geber MA, Dawson TE. (1997). Genetic variation in stomatal and biochemical limitations to photosynthesis in the annual plant, *Polygonum arenastrum*. Oecologia **109** 535-546

Gebre GM, Brandle JR, Kuhns MR. (1997). Influence of rewatering and time of sampling on solute accumulation of two *P. deltoides* clones. Tree Physiology **17** 341-346

Gebre GM, Kuhns MR. (1991). Seasonal and Clonal variations in drought tolerance of *Populus deltoides*. Canadian Journal of Forest Research **21** (**6**) 910-916

Gebre GM, Kuhns MR. (1993). Effects of water-stress preconditioning on gas-exchange and water relations of *Populus deltoides* clones. Canadian Journal of Forest Research **23** (**7**) 1291-1297

Gebre GM, Kuhns MR, Brandle JR. (1994). Organic solute accumulation and dehydration tolerance in 3 water-stressed *Populus deltoides* clones. Tree Physiology **14** (**6**) 575-587

Gebre GM, Tschaplinski TJ, Shirshac TL. (1998). Water relations of several hardwood species in response to throughfall manipulation in an upland oak forest during a wet year. Tree Physiology **18** 299-305

Gebre GM, Tschaplinski TJ. (2000). Role of Osmotic Adjustment in Plant Productivity. Environmental Sciences Division Publication No. 4961. Oak Ridge National Laboratory ORNL/TM-2000\6. 24 pp.

Gebre GM, Tschaplinski TJ, Tuskan GA, Todd DE. (1988). Clonal and seasonal differences in leaf osmotic potential and organic solutes of five poplar clones grown under field conditions. Tree Physiology **18** 645-652

Givnish TJ. (1986). On the economy of plant form and function. Cambridge, UK: Cambridge University Press

Glover B. (2000). Differentiation in plant epidermal cells. Journal of Experimental Botany **51 (344)** 479-505

Glover BJ, Perez-Rodriguez M, Martin C. (1998). Development of several epidermal cell types can be specified by the same MYB-related plant transcription factor. Development **125**(17): 3497-3508.

Griffin DH, Schaedle M, Manion PD, DeVit M. (1991). Clonal variation in amino acid contents of roots, stems, and leaves of aspen (Populus tremuloides Michx.) as influenced by diurnal drought stress. Tree Physiology **8** 337-350

Gutschick VP. (1999). Biotic and abiotic consequences of differences in leaf structure. New Phytologist **143** 3-18

Hacke U, Sauter JJ. (1995). Vulnerability of xylem to embolism in relation to leaf water potential and stomatal conductance *in Fagus sylvatica* f. *purpurea* and *Populus balsamifera*. Journal of Experimental Botany **46 (290)** 1177-1183

Haefner JW, Buckley TN, Mott KA. (1997). A spatially explicit model of patchy stomatal responses to humidity. Plant, Cell and Environment **20** 1087-1098

Hall DO, Scurlock JMO, Bolhar-Nordenkampf HR, Leegood RC, Long SP eds. (1993). Photosynthesis and Production in a changing environment 1st ed. Chapman & Hall .UNEP. 464 pp

Han KH, Bradshaw HD Jr., Gordon MP. (1994). Adventitious root and shoot regeneration in vitro is under major gene control in an F2 family of hybrid poplar (*Populus trichocarpa* x *P. deltoides*). Forest Genetics **1 (3)** 139-146

Hansen EA. (1988). Irrigating short rotation intensive culture hybrid poplars. Biomass **16** 237-250

Hare PD, Cress WA, Van Staden J. (1999). Proline synthesis and degradation: a model system for elucidating stress-related signal transduction. Journal of Experimental Botany **50** 413-434

Harley PC, Thomas RB, Reynolds JF, Srain BR. (1991). An improved model of Photosynthesis at high CO_2: Reversed O_2 sensitivity explained by lack of glycerate re-entry into the chloroplast. Photosynthesis Research **27** 169-178

Harley PC, Thomas RB, Reynolds JF, Strain BR. (1992). Modelling photosynthesis of cotton grown in elevated CO_2. Plant Cell and Environment **15** 271-282

Hartung W, Wilkinson S, Davies DW. (1998). Factors that regulate abscisic acid concentrations at the primary site of action at the guard cell. Journal of Experimental Botany **49** 361-367

Harvey HP, Van Den Driessche. (1997). Nutrition, xylem cavitation and drought resistance in hybrid poplar. Tree Physiology **17** 647-654

Heilman PE, Stettler RF. (1985). Genetic variation and productivity of *Populus trichocarpa* and its hybrids. II Biomass production in a 4-year plantation. Canadian Journal of Forest Research **15** 384-388

Herschbach C, Kopriva S. (2002). Transgenic trees as tools in tree and plant physiology. Trees **16** 250-261

Hetherington AM, Woodward FI. (2003). The role of stomata in sensing and driving environmental change. Nature **424** (**21**) 901-907

Hall DO, Scurlock JMO, Bolhar-Nordenkampf HR, Leegood RC, Long SP eds. (1993). Photosynthesis and Production in a changing environment 1st ed. 129-168

Hillier J, Coombes AJ. eds.(2002). The Hillier Manual of Trees & Shrubs. David & Charles. UK. 512 pp

Hinckley TM, Ceulemans R, Dunlap JM et al. (1989). Physiological, morphological, and anatomical components of hybrid vigor in Populus. In Structural and funtional responses to environmental stresses. Edited by Krebb KH, Richter H, and Hinckley TM. SPB Academic Publications, The Hague, The Netherlands. pp. 119-217

Hinckley TM, Braatne J, Ceulemans R, Clum P, Dunlap J, Newman D, Smit B, Scarascia-Mugnozza G, van Volkenburgh E. (1992). 1. Growth dynamics and canopy structure. *In* Mitchell, C.P.; Ford-Robertson, J.B.; Sennerby-Forsse, L. (eds). Ecophysiology of short rotation forest crops. Elsevier Applied Science Publishers Ltd., London: 1-34.

Hinckley TM, Roberts DA, Ustin SL. (1999). Scaling up from leaf to stands: coupling ecophysiological models with remote sensing in plantation *Populus*, deciduous broadleaf and old-growth Pseudotsuga menziesii, evergreen needleleaf. http://nigec.ucdavis.edu/publications/annual99/western/WRHinckley0.html

Horiguchi G, Kin GT, Tsukaya H. (2004). ANGUSTIFOLIA3 encodes a homolog of synovial sarcoma translocation protein and mediates local cell proliferation for lateral expansion of leaf blade in Arabidopsis thaliana" by **Gorou Horiguchi**, National Institute

for Basic Biology .15TH International Conference on Arabidopsis Research. TO2 Development 2 Shoot=-root (T02-006). July 11-14 Berlin, Germany. p. 143

Hoekenga OA, Vision TJ, Shaff Jon E, Monforte Antonio J, Lee GP, Howell SH, Kochian LV. (2003). Identification and Characterization of Aluminum Tolerance Loci in Arabidopsis (Landsberg *erecta* x Columbia) by Quantitative Trait Locus Mapping. A Physiologically Simple But Genetically Complex Trait. Plant Physiology **132** 936-948

Iacobelli A, McCaughey JH. (1993). Stomatal conductance in a northern temperate deciduous forest: temporal and spatial patterns. Canadian Journal of Forest Research **23** 245-252

Idso SB, Kimball BA, Anderson MG, Mauney JR. (1987). Effects of atmospheric CO_2 enrichment on plant growth: the interactive role of air temperature. Agricultural Ecosystems & Environment **20** 1-10

Isebrands JG, Larson P. (1973). Anatomical changes during leaf ontogeny in *Populus deltoides*. American Journal of Botany **60** 199-208

Jarvis AJ, Davies WJ. (1997). Whole-plant ABA flux and the regulation of water loss in *Cedrella odorata*. Plant, Cell and Environment **20** 521-527

Jarvis AJ, Davies WJ. (1998). The coupled response of stomatal conductance to photosynthesis and transpiration. Journal of Experimental Botany **49** 399-406

Jensen AB, Busk PK, Figueras M, Mar Albà, Peracchia G, Messeguer R, Goday A, Pagès M. (1996). Drought signal transduction in plants. Plant Growth Regulations 20 105-110

Jia W, Wang Y, Zhang S, Zhang J. (2002). Salt-stress-induced ABA accumulation is more sensitively triggered in roots that in shoots. Journal of Experimental Botany **53 (378)** 2201-2206

Jones HG. (1987). Stomatal function (ed. E. Zeiger, et al.), pp. 431-443. Stanford University Press, Stanford, CA.

Jones HG. (1999). Use of thermography for quantitative studies of spatial and temporal variation of stomatal conductance over leaf surfaces. *Plant, Cell and Environment* **22** 1043-1055

Jouanin L, Goujon T, de Nadai V, Martin MT, Mila I, Vallet C, Pollet B, Yoshinaga A, Chabbert B, Petit-Conil M, Lapierre C. (2000). Lignification in transgenic poplars with extremely reduced caffeic acid O-methyltransferase activity. Plant Physiology **123 (4)** 1363-1373

Kamaluddin M, Zwiazek JJ. (2002). Naphthenic acids inhibit root water transport, gas exchange and leaf growth in aspen (*Populus tremuloides*) seedlings. Tree Physiology **22** 1265-1270

Kearsey MJ. (1998). The principles of QTL analysis (a minimal mathematics approach). Journal of Experimental Botany **49 (327)** 1619-1623

Kim GT, Tsukaya H, Uchimiya H. (1998). The ROTUNDIFOLIA3 gene of *Arabidopsis thaliana* encodes a new member of the cytochrome P-450 family that is required for the regulated polar elongation of leaf cells. Genes & Development **12** 2381- 2391

Kim GT, Shoda K, Tsuge T, Cho KH, Uchimiya H, Yokoyama R, Nishitani K, Tsukaya H. (2002). The ANGUSTIFOLIA gene of *Arabidopsis*, a plant CtBP gene, regulates leaf-cell expansion, the arrangement of cortical microtubules in leaf cells and expression of a gene involved in cell-wall formation. The EMBO Journal **21** 1267-1279.

Kohler A, Delaruelle C, Martin D, Encelot N, Martin F. (2003). The poplar root transcriptome: analysis of 7000 expressed sequence tags. FEBS Letters **542** 37-41

Koyama ML, Levesley A, Koebner RMD, Flowers TJ, Yeo AR. (2001). Quantitative Trait Loci for Component Physiological Traits Determining Salt Tolerance in Rice. Plant Physiology **125** 406-422

Kwak JM, Moon J-H, Murata Y, Kuchitsu K, Leonhardt N, DeLong A, Schroeder JI. (2002). Disruption of a Guard Cell-Expressed Protein Phosphatase 2A Regulatory Subunit, *RCN1*, Confers Abscisic Acid Insensitivity in *Arabidopsis*. The Plant Cell **14** 2849-2861

Lake JA, Woodward FI, Quick WP. (2002). Long-distance CO2 signalling in plants. Journal of Experimental Botany **53:** 183-193

Lambers H., Poorter H and Van Vuuren MMI. (1998). Inherent variation in Plant growth. Backhuys Publishers, Leiden, The Netherlands.

Lanceras JC, Pantuwan G, Jongdee B, Toojinda T. (2004). Quantitative Trait Loci Associated with Drought Tolerance at Reproductive Stage in Rice. Plant Physiology **135** 384-399

Larkin J, Brown ML, Schiefelbein J. (2003). How do cells know what they want to be when they grow up? Lessons from Epidermal Patterning in Arabidopsis. Annual Review of Plant Biology **54** 403-430

Larson PR, Isebrands JG. (1971). The Plastochron Index as Applied to Developmental Studies of Cottonwood. Canadian Journal of Forest Research **1** 1-11

Leinonen I, Jones HG. (2004). Combining thermal and visible imagery for estimating canopy temperature and identifying plant stress. *Journal of Experimental Botany* 55 (401) 1423-1431

Liu L, McDonald AJS, Stadenberg I, Davies WJ. (2001). Abscisic acid in leaves and roots of willow: significance for stomatal conductance. Tree Physiology **21** 759-764

Liu Z, Dickmann DI. (1992a). Abscisic-Acid accumulation in leaves of two contrasting hybrid poplar clones affected by nitrogen-fertilization plus cyclic flooding and soil drying. Tree Physiology **11** (**2**) 109-122

Liu Z, Dickmann DI. (1992b). Responses of two hybrid *Populus* clones to flooding, drought, and nitrogen availability. I. Morphology and growth. Canadian Journal of Botany **70** 2265-2270

Liu Z, Dickmann DI. (1992c). Responses of two hybrid *Populus* clones to flooding, drought, and nitrogen availability. II. Gas exchange and water relations. Canadian Journal of Botany **71** 927-938

Liu Z, Dickmann DI. (1996). Effects of water and nitrogen interaction on net photosynthesis, stomatal conductance and water-use efficiency in two hybrid poplar clones. Physiologia Plantarum **97** (**3**) 507-512

Lockhart JA. 1965. An analysis of irreversible plant elongation. Jpurnal of Theoretical Biology **8** 264-275

Long SP, Hällgren J-E. (1993). Measurement of CO_2 assimilation by plants in the field and the laboratory. In Hall DO, Scurlock JMO, Bolhar-Nordenkampf HR, Leegood RC, Long SP eds. Photosynthesis and Production in a changing environment 1rst ed. 129-168

Loreti E, De Bellis L, Alpi A, Perata D. (2001). Why and How Do Plant Cells Sense Sugars?.Annals of Botany **88** 803-812

Loudet O, Chaillou S, Merigout P, Talbotec J, Daniel-Vedele F. (2003). Quantitative Trait Loci Analysis of Nitrogen Use Efficiency in Arabidopsis. Plant Physiology **131** 345-358

McAinsh MR, Brownlee C, Hetherington AM. (1990). Abscisic acid-induced elevation of guard cell cytosolic Ca^{2+} precedes stomatal closure. Nature **343** 186-88

Mantyla E, Lang V, Palva E. (1995). Role of abscisic-acid in drought-induced freezing tolerance, cold-acclimation, and accumulation of LT1 78 and RAB18 Proteins in Arabidopsis-thaliana. Plant Physiology **107** (**1**) 141-148

Marron N, Delay D, Petit J-M, Dreyer E, Kahlem G, Delmotte FM, Brignolas F. (2002). Physiological traits of two Populus x euramericana clones, Luisa Avanzo and Dorskamp, during a water stress and rewatering cycle. Tree Physiology **22** 849-858

Mauricio R, Rausher MD. (1997). Experimental manipulation of putative selective agents provides evidence for the role of natural enemies in the evolution of plant defense. Evolution **51 (5):** 1435-1444

Mathur J, Spielhofer P, Kost B, Chua NH. (1999). The actin cytoskeleton is required to elaborate and mantain spacial patterning during trichome cell morphogenesis in *Arabidopsis thaliana.* Development **126** 5559-5568

Mazzoleni S, Dickmann DI. (1988). Differential physiological and morphological responses of two hybrid *Populus* clones to water stress. Tree Physiology **4** 61-70

Merlot S, Mustill AC, Genty G, North H, Lefebvre V, Sotta B, Vavasseur B, Giraudat J. (2002). Use of infrared thermal imaging to isolate *Arabidopsis* mutants defective in stomatal regulation. The Plant Journal **30** 601- 609

Mohiuddin M, Jarvis PG, Russel G. (1997). Study on growth and stomatal response of young poplar plants subjected to partial soil drying. Journal of Tropical Forest Science **10 (1)** 50-57

Monclus R, Dreyer E, Villar M, Delmotte FM, Delay D, Petit JM, Barbaroux C, Le Thiec D, Bréchet C, Brignolas F. (2006). Impact of drought on productivity and water use efficiency in 29 genotypes of *Populus deltoides*×*Populus nigra.* New Phytologist **169** 765-777

Monneveux P, Belhassen E. (1996). The diversity of drought adaptation in the wide. Plant Growth Regulations **20** 85-92

Mott KA, Woodrow JE. (1993). Effects of O_2 and CO_2 on nonsteady-state photosynthesis –further evidence for ribulose-1,5-biphosphate carboxylase oxygenase limitation. Plant Physiology **102** 859-866

Mullet JE, Whitsitt MS. (1996). Plant cellular responses to water deficit. Plant Growth Regulations **20** 119-124

Muraoka H, Uchida M, Mishio M, Nakatsubo T, Kanda H, Koizumi H. (2002). Leaf photosynthetic characteristics and net primary production of the polar willow (*Salix polaris*) in a high artic polar semi-desert, Ny-Alesund, Svalbard. Canadian Journal of Botany **80** 1193-1202

Munné-Bosch S, Alegre L. (2004). Die and let live: leaf senescence contributes to plant survival under drought stress. Functional Plant Biology **31** 203 - 216

Munné-Bosch S, Jubany-Marí T, Alegre L. (2001). Drought-induced senescence is characterized by a loss of antioxidant defences in chloroplasts. Plant, Cell and Environment **24** 1319-1327

Narita NN, Moore S, Horiguchi G, Kubo M, Demura T, Fukuda H, Goodrich J, Tsukaya H. (2004). Overexpression of a novel small peptide ROTUNDIFOLIA4 decreases cell proliferation and alters leaf shape in Arabidopsis thaliana. Plant Journal **38** 699-713

Nardini A, Salleo S. (2000). Limitation of stomatal conductance by hydraulic traits: sensing or preventing xylem cavitation?. Trees **15** 14-24

Neuman DS, Rood SB, Smit BA. (1990). Does cytokinin transport from root-to-shoot in the xylem sap regulate leaf responses to root hypoxia. Journal of Experimental Botany **41** (**231**) 1325-1333

Niklas KJ. (1999). A mechanical perspective on foliage leaf form and function. New Phytologist **143** 19-31

Nilsson O. (2002). The Swedish *Populus* EST project. Abstract. Plant, Animal & Microbe Genomes X Conference. January 12-16. San Diego, CA, p. W127

Noormets A, Sober A, Pell EJ, Dickson RE, Podila GK, Sober J, Isebrands JG, Karnosky DF. (2001). Stomatal and non-stomatal limitation to photosynthesis in two trembling aspen (*Populus tremuloides* Michx.) clones exposed to elevated CO_2 and/or O_3. Plant, Cell and Environment **24** 327-336

Norby RD, Wullschleger SD, Gunderson, Johnson DW, Ceulemans R. (1999). Tree responses to rising CO_2 in field experimentss implications for the future forest. Plant, Cell and Environment **22** 683-714

Pandey R, Agarwal RM. (1998). Water Stress-induced Changes in Proline Contents and Nitrate Reductase Activity in Rice Under Light and Dark Conditions. Physiology Molecular Biology in Plants **4** 53-57

Papadakis J. (1984). Advances in the analysis of field experiments. Comm. Acad. Athenes **59** 326-342.

Parkhurst DF, Loucks OL. (1972). Optimal leaf size in relation to environment. Journal of Ecology **60** 505-537

Parsons R, Ogston S. (1998). Photosynthesis Assistant. Software for analysis of photosynthesis version 1.1. Dundee Scientific. Scotland, UK.

Pei ZM, Kutchitsu JM, Ward JM, Schwarz M, Schroeder JI. (1997). Differential Abscisic Acid Regulation of Guard Cell Slow Anion Channels in Arabidopsis Wild-Type and abi1 and abi2 Mutants. The Plant Cell **9** (**3**) 409-423

Pei ZM, Murata Y, Benning G, Thomine S, Klüsener B, Allen GJ, Grill E, Schroeder JI. (2000). Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature **406** 731-734

Pelah D, Shoseyov O, Altman A. (1995). Characterization of Bspa, a Major Boiling-Stable, Water-Stress-Responsive Protein in Aspen (*Populus tremula*). Tree Physiology **15** (**10**) 673-678

Pelah D, Shoseyov O, Altman A, Bartels D. (1997a). Water-stress response in Aspen (*Populus tremula*): Differential accumulation of dehydrin, sucrose synthase, GAPDH homologues, and soluble sugars. Journal of Plant Physiology **151** (**1**) 96-100

Pelah D, Wang WX, Altman A, Shoseyov O, Bartels D. (1997b). Differential accumulation of water stress-related proteins, sucrose synthase and soluble sugars in *Populus* species that differ in their water stress response. Physiologia Plantarum **99** (**1**) 153-159

Pezeshki SR, Hinckley TM. (1988). Water relations characteristics of *Alnus rubra* and *Populus trichocarpa*- responses to field drought. Canadian Journal of Forest Research **18** (**9**) 1159-1166

Poorter H. & van der Werf A. (1998). Is inherent variation in RGR determined by LAR at low irradiance and by NAR at hight irradiance? A review of herbaceous species. In Physiological Mechanisms and Ecological Consequences (eds. H. Lambers, H. Poorter & M.M.I. Van Vuuren), pp. 309-306. Backhuys Publishers, Leiden. The Netherlands.

Press M. (1999). Research review The functional significance of leaf structure: a search for generalizations. New Phytology **143** 213-219

Price AH, Cairns JE, Horton P, Jones HG, Griffiths H. (2002). Linking drought-resistance mechanisms to drought avoidance in upland rice using a Q.T.L. approach: progress and new opportunities to integrate stomatal and mesophyll responses. Journal of Experimental Botany **373** 989-1004

Prioul JL, Chartier P. (1977). Partitioning of transfer and carboxylation components of intracellular resistance to photosynthetic CO_2 fixation: A critical analysis of the methods used. Annals of Botany 41: 789-800

Pritchard SG, Rogers HH, Prior SA, Peterson CM. (1999). Elevated CO_2 and plant structure: a review. Global Change Biology **5** 807-837

Quarry SA, Jones HG. 1977. Effect of Abscisic acid and water stress on development and morphology of wheat. Journal of experimental Botany **28:** 192-203

Quarrie S A, Whitford PN, Appleford NEJ, Wang TL , Cook S K, Henson IE, Loveys B R. (1988). A monoclonal antibody to (S)-abscisic acid: its characterisation and

use in a radioimmunoassay for measuring abscisic acid in crude extracts of cereal and lupin leaves. Planta **173** 330 - 339

Rae AM, Robinson KM, Street N, Taylor G (2004). Structural, morphological and biochemical traits linked to fast growth in short-rotation coppice poplar for biomass Canadian Journal of Forest Research, 34(7): 1488-1498.

Rae AR, Pinel MPC, Bastien C, Sabatti M, Street NR, Tucker J, Dixon C, Marron N, Dillen SY, Taylor G. (2006). QTL for yield in bioenergy /Populus/: Identifying G x E interactions from growth at three contrasting sites. Accepted in *Tree Genetics* & Genomes

Ramos LJ, Nayaranan KR, McMillan RT Jr. (1992). Association of stomatal frequency and morphology in Lycopersicon species with resistance to *Xanthomonas campestris* pv. *vesicatoria*. Plant Pathol. **41:** 157-164

Häusler RE, Hirsch HJ, Kreuzaler F, Peterhänsel C. (2002). Overexpression of C_4-cycle enzymes in transgenic C_3 plants: a biotechnological approach to improve C_3-photosynthesis. Journal of Experimental Botany **53** 591-607

Raskin I, Ladyman JAR. (1988). Isolation and characterization of a barley mutant with abscisic-acid-insensitive stomata. Planta **173** 73-78

Raven JA. (2002). Selection pressures on stomatal evolution. New Phytologist **153** 371-386

Rawsthorne S, Hylton CM, Smith AM, Woolhouse HW. (1988). Photorespiratory metabolism and immunogold localization of photorespiratory enzymes in leaves of C_3 and C_3-C_4 intermediate species of *Moricandia*. Planta **173** 298-308

Reich PB. (1984a). Leaf stomatal density and diffusive conductance in 3 amphistomatous hybrid poplar cultivars. New Phytol. **98** 231-240.

Reich PB. (1984b). Loss of Stomatal Function in Ageing Hybrid Poplar leaves. Annals of Botany **53** 691-698

Regehr DL, Bazzaz FA, Boggess WR. (1975). Photosynthesis, transpiration and leaf conductance in *Populus deltoides* in relation to flooding and drought. Photosynthetica **9** 52-61

Ridolfi M, Fauveneau P, Label P, Garrec JP, Dreyer E. (1996). Responses to water stress in an ABA-unresponsive hybrid poplar (*Populus koreana* x *trichocarpa* cv. 'Peace'). New Phytologist **134** 445-454

Riera M, Valon C, Fenzi F, Giraudat J, Leung J. (2005). The genetics of adaptive responses to drought stress: abscisic acid-dependent and abscisic acid-independent signalling components. Physiologia Plantarum **123** 111-119

Robinson KM, Karp A, Taylor G. (2004). Defining leaf and canopy traits linked to high yield in short rotation coppice willow. Biomass and Bioenergy **26** 417-431

Rohde A, Ardilez-Diaz W, Van Montagu M, Boerjan W. (1998). Isolation and expression analysis of an ABSCISIC ACID-INSENSITIVE 3 (*ABI3*) homologue from *Populus trichocarpa*. Journal of Experimental Botany **49** (**323**) 1059-1060

Rogers DL, Stettler RF, Heilman PE. (1989). Genetic variation and productivity of *Populus trichocarpa* and its hybrids. III. Structure and pattern of variation in a 3-year field test. Canadian Journal of Forest Research **19** 372-377

Rood SB, Braatne JH, Hughes FMR. (2003). Ecophysiology of riparian cottonwoods: stream flow dependency, water relations and restoration. Tree Physiology **23** 1113-1124

Rood SB, Patino S, Coombs K, Tyree MT. (2000). Branch sacrifice: cavitation-associated drought adaptation of riparian cottonwoods. Trees **14** 248-257

Rowland DL, Beals L, Chaudhry AA, Evans AS, Grodeska L. (2001). Physiological, Morphological, and environmental variation among Geographically isolated cottonwood (*Populus deltoides*) Populations in New Mexico. Western North American Naturalist **61**(4) 452-462

Sachs, T., N. Novoplansky, and M. Kagan. (1993).Variable development and cellular patterning in the epidermis of *Ruscus*. Annals of Botany **71** 237-243

Sage RF. (2004). The evolution of C_4 photosynthesis. New Phytologist **161** 341-370

Sauter A, Hartung W. (2002).The contribution of internode and mesocotyl tissues to root-to-shoot signalling of abscisic acid. Journal of Experimental Botany **53** 297-302

Sauter A, Davies WJ, Hartung, W. (2001). The long-distance abscisic acid signal in the droughted plant: the fate of the hormone on its way from root to shoot. Journal of Experimental Botany **52** 1991-1997

Scarascia-Mugnozza G, Hinckley TM, Stettler RF. (1986). Evidence for nonstomatal inhibition of net photosynthesis in rapid dehydrated shoots of *Populus*. Canadian Journal of Forest Research **16** 1371-1375

Schoch PG, Zinsou C, Sibi M. 1980. Dependence of the stomatal index on environmental factors during stomatal differentiation in leaves of Vigna sinensis L. Journal of Experimental Botany **31**: 1211-1216

Schroeder JI, Allen GJ, Hugouvieux V, Kwak JM, Waner D. (2001a). Guard Cell Signal Transduction. Annual Review in Plant Physiology and Plant Molecular Biology **52** 627-58

Schroeder JI, Kwak JM, Allen GJ. (2001b). Guard cell abscisic acid signalling and engineering drought hardiness in plants. Nature **410** 327-330

Schulte PJ, Hinckley TM. (1987a). Abscisic acid relations and the response of *Populus trichocarpa* stomata to leaf water potential. Tree Physiology **3**, 103-113.

Schulte PJ, Hinckley TM. (1987b). The relationship between guard cell water potential and the aperture of stomata in *Populus*. Plant, Cell and Environment **10** 313-318

Schulte PJ, Hinckley TM, Stettler RF. (1987). Stomatal responses of *Populus* to leaf water potential. Canadian Journal of Botany **65** 255-260

Schürmann, B. (1959). Über den Einfluss der Hydratur und des Lichtes auf die Ausbildung der Stomata-Initialen. Flora **147:** 471-520.

Schwartz A, Wu WH, Tucker EB, Assmann SM. (1994). Inhibition of inward K^+ channels and stomatal response by abscisic acid: an intracellular locus of phytohormone action. Proceedings of the Natural Academy of Sciences. USA. **91 (9)** 4019-4023

Scott P. (2000). Resurrection Plants and the secrets of eternal leaf. Annals of Botany **65** 159-166

Seaton G, Haley CS, Knott SA, Kearsey M, Visscher PM. (2002). QTL Express: mapping quantitative trait loci in simple and complex pedigrees. Bioinformatics **18** 339-340

Sharkey TD. (1985). Photosynthesis in intact leaves of C3 plants: Physics, physiology and rate limitations. The Botanical Review **51** 53-105

Shinozaki K, Yamahuchi-Shinozaki K. (2000). Molecular responses to dehydration and low temperature: differences and cross talk between two stress signaling pathways Current Opinion in Plant Biology **3** 217-223

Sheppard LA, Brunner AM, Krutovskii, Rottmann WH, Skinner JS, Vollmer SS, Strauss SH. (2000). A DEFICIENS Homolog from the Dioecious Tree Black Cottonwood Is Expressed in Female and Male Floral Meristems of the two-whorled, Unisexual Flowers. Plant Physiology **124** 627-639

Smith E. (1937). The influence of light and carbon dioxide on photosynthesis. General Physiology, 20, 807-830

Snyder KA, Richards JH, Donovan LA. (2003). Night-time conductance in C_3 and C_4 species: Do plants lose water at night? Journal of Experimental Botany **54 (383)** 861-865

Somerville C, Bauer S, Brinistool G, Facette M, Hamann T, Milne J, Osborne E, Paredez A, Persson S, Raab T, Vorwerk S, Youngs H. (2004). Toward a System Approach to Understanding Plant Cell Walls. Science 306 (24) 2206-2211

Sparks JP, Black RA. (1999). Regulation of water loss in populations of *Populus trichocarpa*: the role of stomatal control in preventing xylem cavitation. Tree Physiology **19** 453-459

Sperry JS, Donnelly JR, Tyree MT. (1988). A method for measuring hydraulic conductivity and embolism in xylem. Plant Cell and Environment 11: 35-40

Sperry JS, Hacke UG, Oren R, Comstock JP. (2002). Water deficit and hydraulic limits to leaf water supply. Plant, Cell and Environment **25** 251-263

Srivastava A, Lu Z , Zeiger E. 1995. Modification of guard cell properties in advanced lines of pima cotton bred for higher yields and heat resistance. *Plant Sci.* **108:** 125-131

Stettler RF. (1968). Irradiate mentor pollen: its use in remote hybridization of Black cottonwood. Nature **219** 746-747

Stettler RF, Bradshaw HD Jr., Heilman PE, Hinckley TM. (1996). Biology of *Populus* and its Implications for Management and Conservation. NRC Research Press, Ottawa, Ontario, Canada. 539 p.

Stettler RF, Fenn RC, Heilman PE, Stanton BJ. (1988). *Populus trichocarpa* x *P. deltoides* hybrids for short rotation culture: variation patterns and 4-year field performance. Canadian Journal of Forest Research **18** 745-753

Stiles KA,Volkenburgh E Van. (2002). Light-regulated leaf expansion in two Populus species: dependence on developmentally controlled ion transport. Journal of Experimental Botany **53** 1651-1657

Street NR, Skogstrom O, Sjodin A, Tucker J, Rodríguez-Acosta M, Nilsson P, Jansson S, Taylor G. (2006). The genetics and genomics of drought response in *Populus.* The Plant Journal 48 (3) 321-483

Strauss S, Lande R, Namkoong G. (1992). Limitations of molecular marker-aided selection in forest tree breeding. Canadian Journal of Forest Research **22** 1050-1061

Taiz L, Zeiger E. (2002). Plant Physiology. 3rd Ed. Sinauer Associates, Inc., Publishers. Massachusetts, USA. Chapters 3, 4, 25,

Tang Y, Liang N. (2000). Characterization of the photosynthetic induction response in a *Populus* species with stomata barely responding to light changes. Tree Physiology **20** 969–976

Tang XD, Marten I, Dietrich P, Ivashikina N, Hedrich R, Hoshi T. (2000). Histidine[118] in the S2-S3 Linker Specifically Controls Activation of the KAT1 Channel Expressed in Xenopus Oocytes. Biophysical Journal **78** 1255-1269

Tardieu F. (1994). Growth and functioning of roots and of root systems subjected to soil compaction: towards a system with multiple signaling. Soil Tillage Research **30** 217-243

Tardieu F, Davies WK. (1994). Integration of hydraulic and chemical signalling in the control of stomatal conductance and water status of droughted plants. Plant Cell and Environment **16** 341-349

Tardieu F, Simmoneau T. (1998). Variability among species of stomata control under fluctuating soil water and evaporative demand: modelling isohydric and anysohydric behaviours. Journal of Experimental Botany **49** 419-432

Taylor G. (1998). Heritable physiological traits in hybrid poplars related to drought tolerance. Abstracts SEB York meeting

Taylor G. (2002). *Populus*: *Arabidopsis* for Forestry. Do we Need a Model Tree? Annals of Botany **90** 681-689

Taylor G, Beckett KP, Robinson KM, Stiles K, Rae AM. (2001a). Identyfing QTL for yield in UK biomass poplar. Aspects of Applied Biology **65** 173-182

Taylor G, Ceulemans R, Ferris R, Gardner SDL, Shaoo BY. (2001b). Increased leaf area expansion of hybrid poplar in elevated CO_2. From controlled environments to open-top chambers and to FACE. Environment Pollution **115** 463-472

Taylor G, Tricker P, Zhang FZ, Alston VJ, Miglieta F, Kuzminsky E. (2003). Spatial and Temporal Effects of Free-Air CO_2 enrichment (POPFACE) on leaf Growth, Cell Expansion, and Cell Production in a Closed Canopy of Poplar. Plant Physiology **131** 177-185

Terashima I, Araya T, Miyazawa S-I, Sone K, Yano S. (2005). Construction and Maintenance of the Optimal Photosynthetic Systems of the Leaf, Herbaceous Plant and Tree: an Eco-developmental Treatise. Annals of Botany **95** 507-519

Thoday JM. 1961. Location of Polygenes. Nature **191** (478): 368-370

Ticha I. (1982). Photosynthetic Characteristics During Ontogenesis of Leaves .7. Stomata Density and Sizes. *Photosynthetica* **16**, 375-471.

Tonsor SJ, Alonso-Blanco C, Koornneef M. (2005). Gene function beyond the single trait: natural variation, gene effects, and evolutionary ecology in *Arabidopsis thaliana* . Plant, Cell & Environment **28** 2-20

Trejo C L, Davies WJ, Ruiz LdMP. (1993). Sensitivity of Stomata to Abscisic Acid (An Effect of the Mesophyll). Plant Physiology **102** 497-502

Tschaplinski TJ, Blake TJ. (1989a). Correlation between early root production, carbohydrate metabolism, and subsequent biomass production in hybrid poplar. Canadian Journal of Botany **67** 2168-2174

Tschaplinski TJ, Blake TJ. (1989b). Water relations, photosynthetic capacity, and root/shoot partitioning of photosynthate as determinant of productivity in hybrid poplar. Canadian Journal of Botany **67** 1689-1697

Tschaplinski TJ, Blake TJ. (1989c). Water-stress tolerance and late-season organic solute accumulation in hybrid poplar. Canadian Journal of Botany **67** 1681-1688

Tschaplinski TJ, Blake TJ. (1994). Carbohydrate mobilization following shoot defoliation and decapitation in hybrid poplar. Tree Physiology **14** 141-151

Tschaplinski TJ, Blake TJ. (1995) Growth and carbohydrate status of coppice shoots of hybrid poplar following shoot pruning. Tree Physiology **15** 333-338

Tschaplinski TJ, Gebre GM, Dahl JE, Roberts GT, Tuskan GA. (1995a). Growth and Solute Adjustment of Calli of Populus Clones Cultured on Nutrient Medium Containing Polyethylene-Glycol. Canadian Journal of Forest Research **25 (9)** 1425-1433

Tschaplinski TJ, Gebre GM, Shirshac TL. (1988a). Osmotic potential of several hardwood species as affected by manipulation of trough fall precipitation in an upland oak forest during a dry year. Tree Physiology **18 (5)** 291-298

Tschaplinski TJ, Stewart DB, Hanson PJ, Norby RJ. (1995b). Interactions between drought and elevated CO_2 on growth and gas-exchange of seedlings of 3 deciduous tree species. New Phytologist **129 (1)** 63-71

Tschaplinski TJ, Stewart DB, Norby RJ. (1995c). Interactions between drought and elevated CO2 on osmotic adjustment and solute concentrations of tree seedlings. New Phytologist **131 (2)** 169-177

Tschaplinski TJ, Tuskan GA. (1994). Water stress tolerance of black and eastern cottonwood clones and four hybrid progeny. II. Metabolites and inorganic ions that constitute osmotic adjustment. Canadian Journal of Forest Research **24** 681-687

Tschaplinski TJ, Tuskan GA, Gebre GM, Todd DE. (1998a). Drought resistance of two hybrid *Populus* clones grown in a large-scale plantation. Tree Physiology **18** 645-652

Tschaplinski TJ, Tuskan GA, Gebre GM, Todd DE, Bradshaw HD. (1998b). Limits of Drought Tolerance in *Populus*. Annual Report. Biofuels Feedstock Development Program. Department of Energy. Oak Ridge National Laboratory. 9 pp.

Tschaplinski TJ, Gebre GM, Shirshac TL. (1998c). Osmotic potential of several hardwood species as affected by manipulation of throughfall precipitation in an upland oak forest during a dry year. *Tree Physiology* **18**, 291-298

Tschaplinski TJ, Tuskan GA, Gunderson CA. (1994). Water-stress tolerance of black and eastern cottonwood clones and four hybrid progeny. I. Growth, water relations, and gas exchange. Canadian Journal of Forest Research **24** 364-371

Tschaplinski TJ, Tuskan GA, Gebre GM, Todd DE, Bradshaw HD. (1999). Limits of Drought Tolerance in *Populus*. Annual Report. Biofuels Feedstock Development Program. Department of Energy. Oak Ridge National Laboratory. 8 pp.

Tschaplinski TJ, Tuskan GA , Sewell MM, Gebre GM, Todd DE, Pendley CD.. (2005). Phenotypic variation and quantitative trait locus identification for osmotic potential in an interspecific hybrid inbred F_2 poplar pedigree grown in contrasting environments. Tree Physiology **26** 595–604

Tsukaya H. (2003). Organ shape and size: a lesson from studies of leaf morphogenesis. Current Opinion in Plant Biology **6** 57-62

Tsukaya H. (2004). Length and width: Both cell proliferation and cell elongation are controlled in a polar-dependent manner in a leaf, two-dimensional and determinate organ. 15TH International Conference on Arabidopsis Research.

Tuskan GA. (1998). Short-rotation woody crop supply systems in the United States: What do we know and what do we need to know?. Biomass and Bioenergy **14** 307-315

Tuskan GA. (2003). Limits of Drought Tolerance in *Populus*. U.S. Department of Energy (DOE), Office of Fuels Development trough Oak Ridge National Laboratory. http://bioenergy.ornl.gov/doeofd/96_97summaries/woody.html

Tuskan GA, Wullschleger SD, Bradshaw HD, Dalham RC. (2002). Sequencing the *Populus* genome: Applications to the energy-related missions of DOE. Abstract. Plant, Animal & Microbe Genomes X Conference. January 12-16. San Diego, CA. p. W128

Tuskan GA, DiFazio SP, Teichmann T. (2004). Poplar genomics is getting popular: the impact of the poplar genome project on tree research. Plant Biology **6** 2-4

Tyree MT, Kolb KJ, Rood SB, Patino S. (1994). Vulnerability to drought induced cavitation of riparian cottonwoods in Alberta: a possible factor in the decline of the ecosystem? Tree Physiology **14** 455-466

Tzifira T, Ben-Meir H, Vainstein A, Altman A. (1996). Highly efficient transformation and regeneration of aspen plants through shoot-bud formation in root culture. Plant Cell Reports **15 (8)** 566-571

Tzfira T, Vainstein A, Altman A. (1999). rol-Gene expression in transgenic aspen (*Populus tremula*) plants results in accelerated growth and improved stem production index. Trees **14** 49-54

Uddin MR, Meyer MM, Jokela JJ. (1988). Plantlet production from anthers of eastern cottonwood (Populus deltoides). Canadian Journal of Forest Research. **18** 937-941

U.S. Department of Energy. (2002). Bioenergy Feedstock Development Program at Oak Ridge National Laboratory. P.4. http://bioenergy.ornl.gov/forum/94summer.html

U.S. Environmental Protection Agency. (1999). Biological Aspects of Hybrid Poplar Cultivation on Floodplains in Western North America –A Review. 9EPA Document No. 910-R-99-002

Van Volkenburgh E, Cleland RE. (1986). Wall yield threshold and effective turgor in growing bean leaves. Planta **167** 37 - 43

Van Volkenburgh E, Schmidt MG, Cleleland RE. (1985). Loss of capacity for acid-induced wall loosening as the principal cause of the cessation of cell enlargement in light-grown bean leaves. Planta **163** 500-505

Van Volkenburgh E, Taylor G. (1996). Leaf growth physiology. In Biology of *Populus* and its implications for management and conservation. Part II. Ch. 19. Edited by Stettler RF, Bradshaw Jr, Heilman PE, and Hinckley TM. NRC Research Press, National Research Press, National Research Council of Canada, Ottawa, Ont. pp. 283-299.

Von Caemmerer, Farquhar GD. (1981). Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta **153** 376-387

Wada T, Tachibana T, Shimura Y, Okada K. (1977). Epidermal Cell Differentiation in *Arabidopsis* determined by a *Myb* Homolog, CPC. Science **277:** 1113-1116

Wang WX, Pelah D, Alergand T, Shoseyov O, Altman A. (2002). Characterization of SP1, a stress-responsive, boiling-soluble, homo-oligomeric protein from aspen. Plant Physiology **130** 865-875

Wang WX, Pelah D, Altman A, Shoseyov O. (1997). Clonal differences in the expression of a water stress related protein (BSPA) in *Populus* spp. Acta Horticola (ISHS) **447** 467-470

Weber JC, Stettler RF, Heilman PE. (1985). Genetic variation and productivity of Populus trichocarpa and its hybrids. I. Morphology and Phenology of 50 native clones. Canadian Journal of Forest Research **15** 376-383

Wilcox JR & Farmer RE, Jr. 1965. Heritability and C effects in early root growth of eastern cottonwood cuttings. Heredity **23:** 239-246

Wisniewski M, Sauter J, Fuchigami L, Stepien V. (1997). Effects of near-lethal heat stress on bud break, heat-shock proteins and ubiquitin in dormant poplar (*Populus nigra* Charkowiensis x *P. nigra* incrassata). Tree Physiology **17** 453-460

Woodward FI. (1987). Stomatal numbers are sensitive to increases in CO_2 from pre-industrial levels. Nature **327** 627-618

Woodward FI, Bazzaz FA. (1988). The responses of stomatal density to CO_2 partial pressure. Journal of Experimental Botany **39** 1717-1721

Wright JW. (1976). Introduction to Forest Genetics. Academic Press, New York. 463 pp

Wu RL, Bradshaw HD, Stettler RF. (1997). Mapping quantitative trait loci affecting leaf variation. American Journal of Botany **84** 143-153

Wu R, Stettler R. (1998). Quantitative genetics of growth and development in *Populus*. III. Phenotypic plasticity of crown structure and function. Heredity **81** 299-310

Wu RL, Han YF, Hu JJ, Fang, JJ, Li L, Li ML, Zeng ZB. (2000). An integrated genetic map of Populus deltoides based on amplified fragment length polymorphisms. Theoretical Applied Genetics **100** 1249-1256

Wu RL, Wang MX, Huang MR. (1992). Quantitative genetics of yield breeding for *Populus* short rotation culture. I. Dynamics of genetic control and selection model of yield traits. Canadian Journal of Forest Research **22** 175-182

Wullschleger SD. (1993). Biochemical Limitation to Carbon Assimilation in C_3 Plants – A Retrospective Analysis of the A/Ci Curves from 109 species. Journal of Experimental Botany **44** (**262**) 907-920

Wullschleger S, Jansson S, Taylor G. (2002). Genomics and forest biology – *Populus* emerges as the perennial favorite. The Plant Cell **14** 2651–2655

Wullschleger SD, Tschaplinski TJ, Norby RJ. (2002). Plant water relations at elevated CO_2 – implications for water-limited environments. Plant, Cell & Environment **25** (2), 319-331.

Wyckoff G, Zasada J. (2002). Populus L. URL http:/wpsm.net/Populus.pdf . March 18th.

Xiong L, Zhu JK. (2002). Molecular and genetic aspects of plant responses to osmotic stress. Plant, Cell and Environment **25** 131-139

Xu W, Campbell P, Vargheese AK, Braam J. (1996). The Arabidopsis XET-related gene family: environmental and hormonal regulation of expression . The Plant Journal **9** 879–889

Yin TM, DiFazio SP, Gunter LE, Riemenschneider D, Tuskan GA. (2004). Large-scale heterospecific segregation distortion in *Populus* revealed by a dense genetic map. Theor. Appl. Genet. **109** 451-463

Yokoyama R, Nishitani K. (2001). A Comprehensive Expression Analysis of all Members of a Gene Family Encoding Cell-Wall Enzymes Allowed us to Predict *cis*-Regulatory Regions Involved in Cell-Wall Construction in Specific Organs of Arabidopsis. Plant Cell Physiology 42: 1025-1033

Yoshida S. (2003). Molecular regulation of leaf senescence. Current Opinion in Plant Biology **6** 79-84

Zeiger E, Farqhuar GD, Cowan IR. (1987). Stomatal Function. Stanford University Press, Standford, California. 503 pp.

Zhang JX, Nguyen HT, Blum A. (1999). Genetic analysis of osmotic adjustment in crop plants. Journal of Experimental Botany **50** (**332**) 291-302

Zobel B, Talbert JT. (1984). Applied Forest Tree Improvement. John Wiley and Sons, NY

Appendices

Appendix 1 Publications arising from this work

Journal Papers

Street NR, Skogstrom O, Sjodin A, Tucker J, Rodríguez-Acosta M, Nilsson P, Jansson S, Taylor G. (2006). The genetics and genomics of drought response in *Populus.* The Plant Journal 48 (3) 321-483

Oral presentations

The physiology of drought tolerance in *Populus.* (2003). BED Seminar. University of Southampton. Maricela Rodríguez-Acosta.

The genetic basis of drought tolerance in *Populus.* (2004). II. Leaf morphology and structure: a functional approach. BED Seminar. Maricela Rodríguez-Acosta.

More about *Populus*..... (2004). Seminar Taylor's Lab Internal. University of Southampton. Maricela Rodríguez-Acosta.

The genetic basis of drought tolerance in *Populus.* (2005). Post Graduate Conference. University of Southampton. Maricela Rodríguez-Acosta.

Thermal Imaging of leaf responses to environment. (Apr 2006). Morison J (University of Essex), Rodriguez-Acosta M, Taylor G. (University of Southampton). P3.20 Society for Experimental Biology Conference. The University of Kent at Canterbury, UK.

Conference posters

Transcriptional and Physiological Analysis Following Acute and Chronic Drought stress in Two *Populus* Species from a Three Generation Mapping Pedigree (2003). Street NR, Rodríguez-Acosta M, Skogström O, Sjödin A, Jansson S, Nilsson P, Bradshaw HD, and Taylor G. Tree Biotechnology Conference.

Drought survival strategies in two *Populus* species: an adaptive approach. Jun 2004. Maricela Rodríguez-Acosta. Post Graduate Conference. University of Southampton.

Abiotic stress: Linking microarrays and ELPs. Oct 2004. 12th New Phytologist Symposium: Functional genomics of environmental Adaptation in *Populus.* Gatlinburg, Tennessee, USA.

Leaf growth and development of *Populus* -is cell expansion or cell production most important? Jul 2005. Rodríguez-Acosta M, Rae AM, Graham LE, Tricker PJ, Hemersley A., Street N R, Trewin H and Taylor G (University of Southampton, UK). Society of Experimental Botany. Barcelona, Spain.

Linking physiological and genetical Approaches to understand drought Tolerance in a fast growing tree crop, *Populus*. Sep 2005. Rodriguez-Acosta M, Street NR, Janssen S, Trewin H, Rae AM, Taylor G. Interdrought II. The 2nd International Conference on Integrated Approaches to Sustain and Improve Plant Production Under Drought Stress. Rome, Italy.

Appendix 2 Transverse sections and SEM technique

The General TEM and SEM preparation Schedule was done by Dr. Anton Page from the Biomedical Imaging Unit at Southampton Hospital.

Hazards in fixing process.

Glutaraldehyde is **Harmful** by inhalation, ingestion and on skin contact. Wear gloves and use in a fume cupboard.

Formaldehyde is **Toxic** by inhalation and ingestion. Wear gloves and use in a fume cupboard.

Acetonitrile is **Harmful** by inhalation, ingestion and on skin contact. Wear gloves and use in a fume cupboard.

Epoxy resin is **Harmful** by inhalation, ingestion and on skin contact. Wear gloves and use in a fume cupboard.

Scanning Electronic Microscope fixation.

General SEM

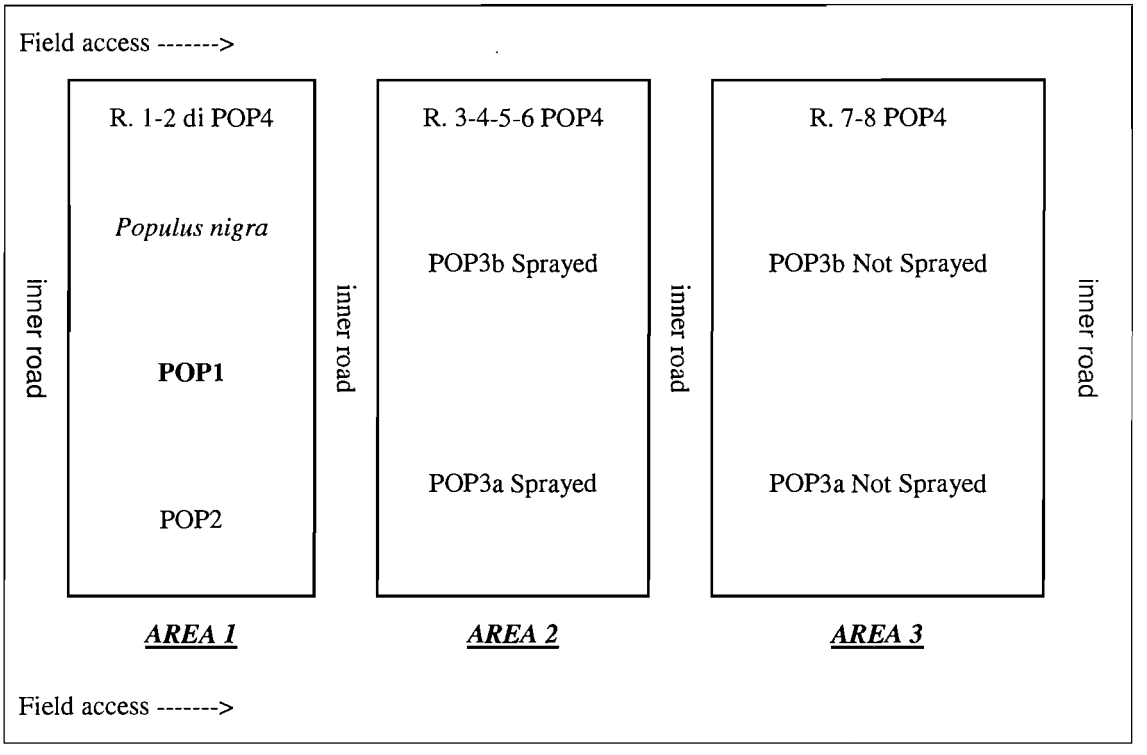
Fixative	1 hour
Buffer rinse	10 mins
Buffer rinse	10 mins
30% ethanol	10 mins
50% ethanol	10 mins
70% ethanol	10 mins
95% ethanol	10 mins
Absolute ethanol	20 mins
Absolute ethanol	20 mins
Critical point dry	
Mount specimens on stubs	
Sputter coat	

Notes: The fixative used is 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.2.

Appendix 3 Italy plantation map

POPYOMICS

Exp. Plantation - Cavallermaggiore (CN)



POPYOMICS
Experimental plantation
Cavallermaggiore (Cuneo province)

Coordinates:
Latitude
North 44°42'07"

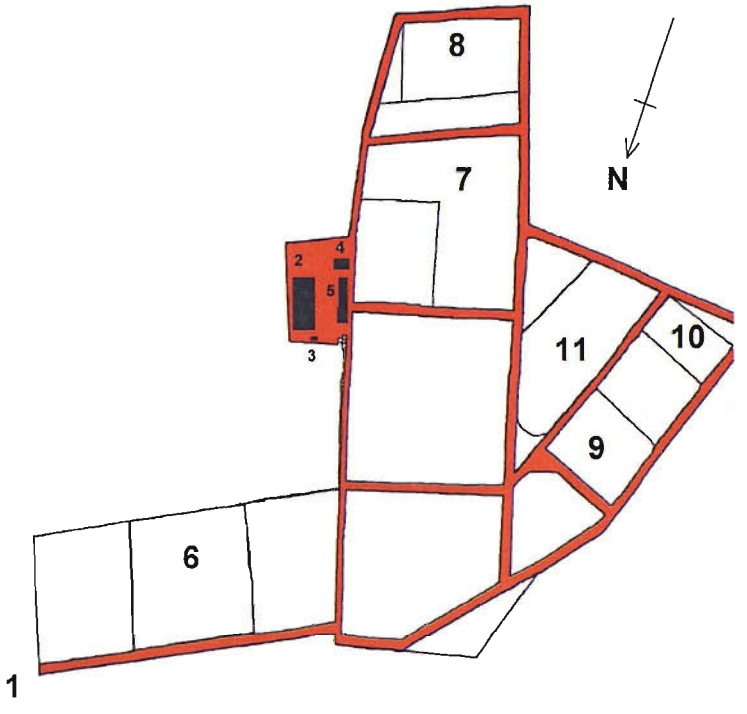
Longitude:
East 7°40'31"

Planting date: 7-12 April 2003

Soil category: alluvial soil, texture sandy loam

Appendix 4 UK plantation map

Headley Research Nursery Map



Key:
1 Entrance gate
2 Vehicle Storage
3 Tool Storage Shed
4 Cold Storage Shed
5 Main Hut (with Toilet)
6 Population 1
7 Population 2
8 Population 3a
9 Population 3b
10 Population 3b
11 Population 4

Appendix 5 Radio-immunoassay (RIA) for ABA

Procedure used in Bill Davies' lab, written by S Wilkinson, with modifications by J.Theobald.

The Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK.
E-mail: j.theobald@lancaster.ac.uk

Based on the method described in Quarrie *et al.*, (1988) Planta 173: 330-339.

Equipment and reagents:

1. 2 ml vials and foam racks
2. Pipettes and tips
3. Microcentrifuge (40 tube capacity)
4. Phosphate buffered saline (PBS)
5. ABA antibody MAC 252 dissolved in PBS containing 5 mg/ml bovine serum albumin (BSA) and 4 mg/ml PVP
6. ^{3}H-ABA dissolved in PBS containing 5 mg/ml gamma-globulin
7. Saturated ammonium sulphate
8. Unlabelled ABA standards
9. Scintillation cocktail (Ecoscint-H) and counter

Preparation of material:

1. Harvest plant tissue (leaves/roots) and immediately wrap in labelled silver foil and plunge into liquid nitrogen.

2. Freeze dry leaf tissue for 48 hours.

3. Grind up sample to a fine powder and store in a suitable airtight vial/container.

4. Weigh out approximately 60 mg of sample into a 1.5 ml microcentrifuge tube.

5. Add deionised distilled water (dd H_2O) at a ratio of 1:25 (dry weight:water). If the tissue is expected to be especially high in ABA (species specific/very droughted) a 1:50 dilution may be required. Ideally with any previously untested species, a range of dilutions should initially be tried. Further, an ABA spike dilution test (see below) should also be performed for each new species, to test for any compounds that the plant may contain that might interfere with the assay, for example by preferentially binding with the MAC 252. If interference is detected it will be necessary to clean the sample by passing it through Sep-Pak columns, and checking RIA tested ABA values against those obtained by GC/MS.

6. Place a small ball bearing in each sample tube to aid mixing/shaking (samples can be stored frozen at this point).

7. Place all tubes on a shaker in a dark cold room (< 10°C) overnight to extract ABA from the leaf/root material.

8. Next morning, transfer approximately 1 ml of each extract to a clean microcentrifuge tube, and centrifuge at 2500-3000 rpm for 5 min.

9. Transfer supernatant to a further clean microcentrifuge tube for immediate use in the assay, or storage in a freezer.

Assay:

1. Make a chart of the layout of the treatment tubes in the foam racks. Since each microcentrifuge takes 40 tubes, we have 40 tubes per foam rack, 10 per row. Eighty tubes are a comfortable number to assay per day. The first 14 tubes will be duplicated ABA standards (0-2000 pg ABA), and the remainder samples, either 26 or 13 in duplicate etc.

H_2O	H_2O	125	125	250	250	500	500	1000	1000
2000	2000	ABA	ABA	1	1	2	2	3	3
4	4	5	5	6	6	7	7	8	8
9	9	10	10	11	11	12	12	13	13

2. Place 2 ml plastic centrifuge tubes into foam rack and place 200 ul of 50% PBS (see solution preparation below) into each tube.

3. Lift up all tubes in the racks so that they can be pushed down as you fill each with standards or treatments, basically so that you don't lose your place (as the tubes are not labelled).

4. Place 50 ul of standard (see below for preparation) or 50 ul of sample into tubes as dictated by the chart, keeping them refrigerated until immediately before use.

5. Place 100 ul of ^{3}H-ABA into ALL tubes (mix before use – see below for preparation).

6. Place 100 ul of MAC 252 antibody into ALL tubes.

7. Place caps on tubes and mix. Place tubes and rack in fridge for 45 minutes.

8. Carefully remove tubes in order from foam rack and place in order in numbered microcentrifuge racks. Make sure that the microcentrifuge racks are balanced, and centrifuge for 1 minute. Carefully replace tubes back into foam rack, keeping them in their original order.

9. Add 0.5 ml of saturated ammonium sulphate to every tube to precipitate the ABA-antibody complex.

10. Leave for 30 minutes in the dark at room temperature.

11. Centrifuge tubes (as in 8) for 4 minutes. Should see a small white/green pellet at the base of each tube.

12. Remove lids and tip supernatant into sink (tubes will stay in foam rack when rack is inverted), blotting off excess liquid by gently tapping inverted rack onto blue roll.

13. Add 1.0 ml 50% ammonium sulphate as a second wash to remove excess unbound radioactivity. Replace lids on tubes, and bash upside down on bench to re-suspend pellets.

14. Re-centrifuge for 5 minutes, remove lids, tip out excess liquid, and blot again on blue roll.

15. Add 100 ul of ddH_2O to each tube. Mix gently without replacing lids to begin resuspension of pellet, by holding the rack against the side of a whirlimixer.

16. Add 1.5 ml of Ecoscint-H (scintillant) to all tubes.

17. Replace lids and whirlimix tubes individually to resuspend pellet thoroughly.

18. Place each tube inside a clean glass scintillation vial (20 ml) in a counting rack. Count on Protocol 7 for 6 minutes. Theoretically this can be done immediately, but it is preferable to store the tubes overnight and count the activity the following day.

19. Standard curves can be constructed with the highest radioactive counts being given by the H_2O standards, as these contain no unlabelled ABA to compete with the antibody/^{3}H-ABA binding reaction. Typically this Bmax will have a count of 1500-2000 dpm, whilst the Bmin value (being those standards with an excess of unlabelled ABA) will be 80-100 dpm, and representative of non-specific binding. The counts given by each sample tube can be interpolated from this curve as pg ABA per tube, and converted to a per unit dry weight value. When lots of ABA is present in the sample the counts obtained will be low, as unlabelled ABA will have preferentially bound to the antibody in place of the ^{3}H-ABA.

Preparation of PBS

1. Prepare a 50mM solution of $NaH_2PO_4.2H_2O$ (7.80g in 1l dd H_2O).

2. Prepare a 50mM solution of Na_2HPO_4 (1.42g in 200ml dd H_2O).

3. Mix approximately 877ml of the former with 123ml of the latter. Adjust pH to 6.0 if necessary.
4. Add 5.844g NaCl.

5. Dilute to 50% for use in the assay only.

6. Store in fridge for up to 1 month.

Dilution of original stock ^{3}H-ABA

1. Purchase 1.85 MBq (in 250ul ethanol) DL-*cis, trans*-[G-^{3}H] abscisic acid from Amersham International at a specific activity of approximately 1.96 TBq/mmol (specific activity will vary). Store at -20°C.
2. The contents of the bottle should be removed and diluted in 100ml of 100% PBS containing 0.5g of gamma globulin. This is divided into 10 aliquots of 10ml and stored as master stock in the -20°C freezer. One hundred micro litres of this master stock should give a count of approximately 60000 dpm.

3. Each master stock bottle is diluted as required: each is sub-divided into 7 aliquots of 1.43ml and made up to 10ml in PBS/gamma globulin. One hundred microlitres of this working stock solution should count at 8000-9000 dpm. Store frozen for up to two years.

Preparation of MAC 252 antibody (this may vary depending on supplier and the specific activity of each batch).

1. Obtained from Geoff Butcher (Babraham Institute, UK) as a 250ul aliquot of ascitic fluid.

2. Dilute the 250ul of ascites to 1:80 using 19.75ml of buffer (100% PBS containing 500mg BSA and 400mg PVP per 100 ml), and store at -20°C as 20 x 1 ml aliquots of master stock.

3. Dilute 1ml of master stock in 99ml of the same buffer to give a 1:8000 dilution. Subdivide into ten aliquots of 8.33ml and store in a -20°C freezer. These are the working stocks ready to use in the assay, and each aliquot will be sufficient for two racks of 40 assay tubes each.

4. Aliquots can be stored for up to a year in the freezer.

Making unlabelled ABA standards

1. Weigh out 8mg of (+/-) ABA.

2. Dissolve in a few drops of 1M KOH in a glass weighing boat.

3. Transfer to a 100ml volumetric flask using ddH_2O, and make up to the mark. Decant 5ml of this to a glass vial to use as the saturating 'ABA' standard (i.e. Bmin at 80ug/ml ABA or 4ug/50ul as used in the assay).

4. Take 1ml of the Bmin and make up to 100ml in another volumetric flask to give 800ng/ml. Take 1ml of this and add 9ml ddH_2O to give 80ng/ml or 4000pg/50ul as used in the assay.

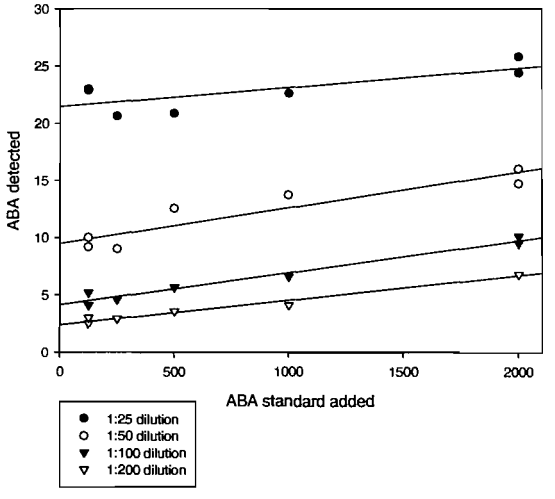
5. Take 5ml of the 4000pg/50ul standard, and add 5ml ddH_2O to give the 2000pg/50ul standard.

6. Continue with 1:1 serial dilutions to give 1000, 500, 250, and 125pg/50ul standards.

7. Use ddH_2O for Bmax. Store standards in a refrigerator for up to one month.

ABA spike dilution test for cross reactivity/interference in new species

Spike dilution tests involve spiking a range of serial dilutions of a tissue extract with known amounts of ABA to generate a series of plots of ABA detected against ABA added. In the absence of interference the lines generated should be parallel (Rosher *et al.*, 1985 (Planta) 165: 91-99)), and the absolute values obtained where the plots intercept the y-axis should decrease in concert with the increase, and in proportion to dilution, as in the figure below.



Results from a spike dilution test on leaves of citrus (J.Theobald, July 2002)

Method

1. Prepare a series of dilutions of sample tissue e.g. 1:25, 1:50, 1:100, 1:200.

2. The radioimmunoassay proceeds as normal, except when samples are added: (1) 25ul of sample are added to the 200ul of 50% PBS in tubes (2) 25ul of different known concentrations of ABA standards are then added to these same tubes. For example, 250, 500, 1000, 1500, 2000pg/50ul standards might be used. In illustration, a typical chart that would be prepared for the spike dilution ABA-RIA would look like this:

H_2O	H_2O	125	125	250	250	500	500	1000	1000
2000	2000	ABA	ABA	125a	125a	250a	250a	500a	500a
1000a	1000a	1500a	1500a	2000a	2000a	125b	125b	250b	250b
500b	500b	1000b	1000b	1500b	1500b	2000b	2000b		

Where *x*a & *x*b are 1:25 & 1:50 sample dilutions respectively, and the numbers represent the ABA standards added, noting that the first 14 tubes are the standards used for the assay and 50ul of these are used as normal for the assay.

Appendix 6

Tables included in appendix

Table A4.1 Diurnal leaf conductance and transpiration in *P. trichocarpa* and *P. deltoides*.

G	Day time	Irrigated		Droughted	
		g_s (mmol $m^{-2}s^{-1}$)	E (mol $m^{-2}s^{-1}$)	g_s (mmol $m^{-2}s^{-1}$)	E (mol $m^{-2}s^{-1}$)
P. trichocarpa	4	405 ± 9.2	2.25 ± 0.21	380 ± 22	2.08 ± 0.03
	5	434 ± 121	0.61 ± 0.17	455 ± 95	0.66 ± 0.13
	6	543 ± 92	0.67 ± 0.10	513 ± 21	0.69 ± 0.01
	7	707 ± 143	0.57 ± 0.02	672 ± 4.2	0.69 ± 0.27
	8	555 ± 9.2	0.81 ± 0.03	524 ± 110	0.82 ± 0.13
	10	407 ± 18.4	3.43 ± 0.36	435 ± 21	3.57 ± 0.33
	12	396 ± 13.4	5.41 ± 0.55	402 ± 0	5.70 ± 0.14
	14	305 ± 49.5	6.92 ± 0.19	401 ± 0.7	7.74 ± 0.37
	16	373 ± 20.5	5.68 ± 0.88	354 ± 21.2	5.53 ± 0.39
	18	327 ± 19	4.25 ± 0.35	312 ± 7.8	4.20 ± 0.14
	20	288 ± 33.2	2.29 ± 0.02	313 ± 59.4	2.29 ± 0.05
	21	340 ± 67.8	1.08 ± 0.09	316 ± 59	1.08 ± 0.12
	22	367 ± 29.7	0.79 ± 0.12	373 ± 33.2	0.78 ± 0.05
	23	415 ± 143	0.75 ± 0.16	381 ± 143	0.71 ± 0.18
P. deltoides	4	189 ± 74.3	1.10 ± 0.35	63 ± 5.6	0.37 ± 0.03
	5	210 ± 97.6	0.32 ± 0.23	54 ± 14	0.11 ± 0.01
	6	280 ± 134	0.43 ± 0.18	48 ± 0.0	0.08 ± 0.00
	7	539 ± 110	0.62 ± 0.50	188± 46.7	0.19 ± 0.08
	8	721 ± 59	0.93 ± 0.23	262±104	0.43 ± 0.14
	10	308 ± 212	2.47 ± 1.30	74 ± 61	0.65 ± 0.58
	12	168 ± 17	2.70 ± 0.42	17 ± 5.4	0.33 ± 0.10
	14	66 ± 31	1.40 ± 0.75	18 ± 0.5	0.36 ± 0.02
	16	44 ± 35	1.11 ± 1.12	10 ± 3.13	0.17 ± 0.01
	18	39 ± 29	0.53 ± 0.31	16 ± 5.94	0.24 ± 0.04
	20	66 ± 82	0.13 ± 0.08	21 ± 7.99	0.15 ± 0.04
	21	10 ± 2.9	0.03 ± 0.01	12 ± 5.4	0.04 ± 0.02
	22	9 ± 2.6	0.02 ± 0.00	20 ± 13.6	0.05 ± 0.04
	23	17 ± 0.0	0.03 ± 0.00	9 ± 0.0	0.02 ± 0.00

Tabla A4.2 Profile of physiological properties measured in the grandparents of hybrid Family 331. **LN** leaf number, $\mathbf{g_s}$ stomatal conductance (mmol m^{-2} s^{-1}), **O** sap osmolality (mmol kg^{-1}), $\Psi\pi$ (MPa), **P** epidermal cell plasticity and **E** epidermal cell elasticity.

Genotype	LN	g_s	se ±	O	se ±	-Ψπ	se ±	P	se ±	E	se ±
P. trichocarpa	1	156	22	447	18.3	1.11	0.04	18.1	1.31	8.27	0.41
	2	226	17.6	509	31.9	1.27	0.07	19.5	4.56	8.39	1.68
	3	235	16.5	565	14.7	1.41	0.03	17.8	7.46	7.32	2.09
	4	232	7.39	609	15.9	1.52	0.04	16.9	7.51	7.38	2.70
	5	227	11.5	586	13.2	1.46	0.03	6.57	3.34	4.36	1.19
	6	224	6.64	608	16.4	1.52	0.03	4.60	2.45	3.12	1.12
	7	224	7.71	586	14.4	1.46	0.04	4.36	2.18	2.84	0.88
	8	224	11.5	580	14.9	1.20	0.24	2.40	0.71	2.59	0.39
	9	219	16.5	596	13.2	1.49	0.03	1.89	0.19	2.98	0.50
	10	212	14.9	589	8.9	1.47	0.03	2.90	1.12	2.53	0.77
	11	204	10.9	590	20.6	1.47	0.05	2.37	0.86	2.76	0.54
	12	188	17.8	593	15.6	1.48	0.04	2.57	0.77	2.83	0.63
P. deltoides	1	15.1	4.81	518	15.1	1.29	0.03	11.2	0.49	5.88	0.23
	2	90.2	8.32	542	10.4	1.13	0.23	16.4	1.80	6.65	0.36
	3	130	15.3	582	18.5	1.45	0.04	10.1	3.36	4.72	0.65
	4	157	20.1	680	18.2	1.7	0.04	2.32	0.90	2.59	0.63
	5	193	24.7	660	18.1	1.65	0.04	1.21	0.19	1.50	0.37
	6	255	35.7	643	24.9	1.60	0.06	1.75	0.42	2.14	0.58
	7	264	10.0	624	22.5	1.56	0.05	1.34	0.02	1.53	0.48
	8	257	11.5	643	19.6	1.60	0.04	1.11	0.26	1.6	0.75
	9	258	13.2	626	17.2	1.56	0.04	0.55	0.17	1.34	0.44
	10	244	20.3	659	27.8	1.64	0.06	0.94	0.27	0.80	0.19
	11	230	11.8	661	16.7	1.65	0.04	0.50	0.26	0.97	0.52
	12	228	13.2	633	24.4	1.58	0.06	0.55	0.05	0.82	0.12

Table A6.1 Leaf traits in the grandparents of Family 331 growing in two constrasting enviromental conditions. Values were obtained from 3 replicates. Standard deviation is shown. Percentage variation was calculated as % variation = ((mean Italy-mean UK)/(mean UK))*100.

Trait	UK mean *P. deltoides*	sd ±	UK mean *P. trichocarpa*	sd ±	Italy mean *P. deltoides*	sd ±	Italy mean *P. trichocarpa*	sd ±	% difference *P. deltoides*	% difference *P. trichocarpa*
Leaf										
Leaf area (mm^2)	24947	5422	11109.07	1993.73	17125.3	3682.4	3665.3	648.4	-31.35	-67.01
Leaf length (mm)	197.8	31.8	165.95	27.61	159.87	20.17	102.8	15.97	-19.18	-38.05
Leaf width (mm)	161.05	27.11	103.83	18.56	142.56	14.23	55.72	3.73	-11.48	-46.34
Width/length ratio	0.814	0.006	0.625	0.007	0.89	0.02	0.54	0.05	9.34	-13.60
Dry weight (g)	1.83	1.05	0.99	0.3	2.39	0.54	0.43	0.09	30.60	-56.57
SLA ($mm^2\ g^{-1}$)	14271.4	2235.427	15728.06	5007.39	7173.52	72.27	8424.18	239.41	-49.73	-46.44
Petiole width (mm)	1.6	0.28	1.43	0.15	1.9	0.56	1.33	0.4	18.75	-6.99
Petiole height (mm)	2.95	1.06	2.36	0.66	4.5	0.7	1.36	0.06	52.54	-42.37
Petiole length (mm)	99.06	20.7	55.13	8.61	84.65	1.48	29.5	0.36	-14.55	-46.49
LER (mm)	6.29		13	4.4	2	0.95	2.83	0.23	-68.20	-78.23
Adaxial cell traits										
Cell area (μm^2)	463.5	55.47	822.17	111.4	349.24	134.8	489.9	141.9	-24.65	-40.41
Cell number (mm^{-2})	303.5	36.32	172.14	25.3	432.07	166.8	301.5	86.4	42.36	75.15
Total cell number per leaf (10^6)	7.6		1.9		7.4		1.1		-2.27	-42.21
Stomatal density (mm^{-2})	118.2	65.84	9.55	16.54	247.13	86	21.48	12.4	109.08	124.92
Stomatal Index	5.33	2.35	0.83	1.44	5.9	0.45	4.84	0.98	10.69	483.13
Trichome density (mm^{-2})	0	0	11.93	4.13	0	0	85.96	31.22		620.54
Trichome index	0	0	0.95	0.3	0	0	3.8	0.91		300.00
Abaxial cell traits										
Cell area (μm^2)	470.8	20.4	1038.98	92.3	387	107.8	847.01	224.8	-17.80	-18.48
Cell number (mm^{-2})	296.9	12.85	135.65	15.8	375.3	104.5	171.9	39.6	26.41	26.72
Total cell number per leaf (10^6)	7.4		1.5		6.4		.63		-13.23	-58.19
Stomatal density (mm^{-2})	210.12	76.6	145.7	58.3	329.5	37.9	303.25	28.9	56.82	108.13
Stomatal Index	8	3.49	12.95	4.84	9.5	2.72	12.08	11.84	18.75	-6.72
Trichome density (mm^{-2})	0	0	11.93	4.13	0	0	19.1	4.13		60.10
Trichome index	0	0	1.22	0.46	0	0	1.53	0.03		25.41

Table A6.2 Leaf and cell traits in the F_1 parents growing in two constrasting enviromental conditions. Values were obtained from 3 Replicates. Standard deviation is shown. Percentage variation was calculated as % variation = ((mean Italy-mean UK)/(mean UK))*100.

Trait	UK mean G 242	sd ±	UK mean G 246	sd ±	Italy mean G 242	sd ±	Italy mean G 246	sd ±	%difference G 242	G 246
Leaf										
Leaf area (mm^2)	29251.27	2484.56	28589.00	318.70	19198.90	5207.00	14035.30	148.60	-34.37	-50.91
Leaf length (mm)	235.90	6.90	261.15	4.90	189.60	21.90	186.14	14.74	-19.63	-28.72
Leaf width (mm)	182.60	16.20	171.21	1.12	147.86	20.00	118.44	2.39	-19.03	-30.82
Width/length ratio	0.77	0.04	0.66	0.01	0.78	0.02	0.64	0.05	1.30	-3.03
Dry weight (g)	2.29	0.40	2.21	0.04	2.58	0.89	1.75	0.20	12.66	-20.81
SLA ($mm^2\ g^{-1}$)	12921.60	1103.13	12921.52	385.17	7570.65	654.65	8095.52	951.00	-41.41	-37.35
Petiole width (mm)	1.60	0.78	1.96	0.35	2.26	0.60	2.56	1.20	41.25	30.61
Petiole height(mm)	2.90	0.96	3.70	1.05	6.63	0.90	2.86	0.84	128.62	-22.70
Petiole length (mm)	91.40	13.70	99.00	10.20	85.63	6.50	85.30	9.10	-6.31	-13.84
LER (mm)	16.00	4.24	17.90	0.60	2.00	0.00	4.20	3.50	-87.50	-76.54
Adaxial cell traits										
Cell area (μm^2)	592.80	99.80	503.00	364.80	399.61	80.34	361.80	40.00	-32.59	-28.07
Cell number (mm^{-2})	239.90	38.70	376.70	273.25	359.09	72.20	389.20	43.90	49.68	3.32
Total cell number per leaf (10^6)	7.02		10.77		6.89		5.46		-1.76	-49.28
Stomatal density (mm^{-2})	95.50	14.90	53.70	35.50	164.75	44.70	140.90	23.00	72.51	162.38
Stomatal Index	5.20	0.06	3.18	3.50	7.44	0.27	1.14	0.95	43.08	-64.15
Trichome density (mm^{-2})	4.77	4.13	7.16	10.13	2.38	4.13	4.77	8.27	-50.10	-33.38
Trichome index	0.25	0.35	0.25	0.22	0.09	0.16	0.19	0.33	-64.00	-24.00
Abaxial cell traits										
Cell area (μm^2)	781.14	92.30	820.45	0.00	419.00	127.90	310.70	42.30	-46.36	-62.13
Cell number (mm^{-2})	180.44	21.00	170.22	0.00	355.06	108.70	453.70	61.76	96.77	166.54
Cell number per leaf (10^6)	5.27		4.86		6.81		6.36		29.15	30.85
Stomatal density (mm^{-2})	204.15	5.00	232.80	76.00	343.80	101.30	393.90	82.60	68.41	69.20
Stomatal Index	14.58	18.00	12.80	0.00	10.80	2.18	11.65	2.30	-25.93	-8.98
Trichome density (mm^{-2})	0.00	0.00	7.16	0.00	0.00	0.00	2.38	4.14		-66.76
Trichome index	0.00	0.00	0.58	0.00	0.00	0.00	0.00	0.00		-100.00

Table A6.3 Percentage variation in plants growing in Italy compared to those growing in UK

	P. deltoides	*P. trichocarpa*	Clone 242	Clone 246	F_2
Leaf					
Leaf area (mm^2)	-31.35	-67.01	-34.37	-50.91	-54.73
Leaf length (mm)	-19.18	-38.05	-19.63	-28.72	-32.51
Leaf width (mm)	-11.48	-46.34	-19.03	-30.82	-35.10
Width/length ratio	9.34	-13.60	1.30	-3.03	-4.41
Dry weight (g)	30.60	-56.57	12.66	-20.81	-18.56
SLA ($mm^2\ g^{-1}$)	-49.73	-46.44	-41.41	-37.35	-40.33
Petiole width (mm)	18.75	-6.99	41.25	30.61	-9.47
Petiole height (mm)	52.54	-42.37	128.62	-22.70	-15.46
Petiole length (mm)	-14.55	-46.49	-6.31	-13.84	-28.20
Leaf extension rate (mm)	-68.20	-78.23	-87.50	-76.54	-85.05
Adaxial Cell Traits					
Cell area (μm^2)	-24.65	-40.41	-32.59	-28.07	-44.39
Cell number (mm^{-2})	42.36	75.15	49.68	3.32	90.91
Stomatal density (mm^{-2})	109.08	124.92	72.51	162.38	48.40
Stomatal Index	10.69	483.13	43.08	-64.15	-17.56
Trichome density (mm^{-2})		620.54	-50.10	-33.38	307.01
Trichome index		300.00	-64.00	-24.00	130.00
Abaxial Cell Traits					
Cell area (μm^2)	-17.80	-18.48	-46.36	-62.13	-44.91
Cell number (mm^{-2})	26.41	26.72	96.77	166.54	92.59
Stomatal density (mm^{-2})	56.82	108.13	49.84	30.15	42.97
Stomatal Index	18.75	-6.72	-25.93	-8.98	-14.16
Trichome density (mm^{-2})		60.10		-66.76	415.38
Trichome index		25.41		-100.00	177.78

Table A7.1 Total QTL mapped in the UK, Italy and the response Italy/UK.

Trait	LG	Position	%VA	CI	p	Trait	LG	Position	%VA	CI	p
Cell area (ab)						Cell area (ab)	II	0cM	6.90	0 - 7	0.010
Cell area (ab)	III	0	5.77	0 - 12	0.009						
Cell area (ab)	IV	80	5.35	64 - 85	0.014						
Cell area (ab)	VIII (a)	1	6.6	12 - 0	0.001						
Cell area (ab)	VIII (a)	15	3.8	22 - 7	0.039						
Cell area (ab)	XII	24	3.76	24 - 11	0.027						
Cell area (ad)						Cell area (ad)	II	0cM	4.78	0 - 8	0.047
Cell area (ad)	VIII (b)	3	4.6	25 - 0	0.017						
Cell area (ad)						Cell area (ad)	XI	45cM	5.59	29 - 55	0.017
Cell area (ad)						Cell area (ad)	XIV	0cM	5.46	0 - 9	0.011
Cell area (ad)	XII	8	5.67	17 - 0	0.001	Cell area (ad)	XII	8cM	5.89	0 - 16	0.007
Cell number ab						Cell number ab	I	23cM	5.71	1-36	0.038
Cell number ab						Cell number ab	II	1cM	8.81	0-9	0.001
Cell number (ab)	III	0	6.04	0 - 34	0.002						
Cell number (ab)	IV	77	7.72	63 - 85	0.001						
Cell number (ab)	VIII (a)	0	3.64	9 - 0	0.01	Cell number ab	VIII(a)	0cM	3.66	0-6	0.040
Cell number (ab)	VIII (b)	15	4.26	21 - 8	0.01	Cell number ab	VIII(b)	2cM	4.36	0-11	0.024
Cell number (ab)	XII	24	3.19	24 - 14	0.047						
Cell number ad						Cell number ad	II	0cM	5.06	0-8	0.037
Cell number ad						Cell number ad	V	10cM	5.83	0-38	0.008
Cell number (ad)	VI	0	5.17	17 - 0	0.029						
Cell number (ad)	VIII (a)	5	3.95	24 - 0	0.017						
Cell number ad						Cell number ad	XI	42cM	5.62	28-55	0.008
Cell number (ad)	XII	9	6.45	16 - 1	0.003	Cell number ad	XII	6cM	6.28	0-15	0.003
Cell number ad						Cell number ad	XIV	0cM	5.40	0-8	0.007
Cell number (ad)	XVIII	53	4.05	44 - 67	0.31						
Dry weight	I	12	4.89	24 - 9	0.045						

Trait	LG	Position	%VA	CI	p	Trait	LG	Position	%VA	CI	p
Dry weight	IV	66	7.45	54 - 78	0.001						
Dry weight	IX	7	2.56	10 - 0	0.046						
Dry weight	V (b)	79	4.17	82 - 65	0.032						
Dry weight	VIII (a)	9	7.13	20 - 1	0.001	Dry weight	VIII (a)	8cM	3.53	1 - 26	0.043
Dry weight	XIII	57	5.21	46 - 66	0.017						
Dry weigh						Dry weight	XIV	0cM	3.82	0 - 15	0.037
Dry weight	XVII	52	3.69	78 - 20	0.05						
Leaf area	IV	59	9.08	51 - 78	<0.001						
Leaf area	IX	7	2.59	9 - 0	0.043	Leaf area	IX	9cM	3.55	0 - 9	0.026
Leaf area	V (b)	81	3.95	82 - 67	0.001						
Leaf area	VIII (a)	9	5.38	23 - 0	0.007						
Leaf area	XI	12	3.67	1 - 25	0.036						
Leaf area						Leaf area	XII	13cM	3.30	0 - 9	0.049
Leaf area	XIII	52	3.96	44 - 68	0.036						
Leaf area	XVII	51	4.67	73 - 23	0.018						
Leaf extension rate	IV	53	10.2	44 - 79	0.036						
Leaf extension rate						Leaf extension rate	XIV	28cM	4.42	12 - 28	0.019
Leaf length	I	15	4.93	29 - 9	0.03						
Leaf length	I	153	5.47	165 - 140	0.016						
Leaf length	IV	60	13.7	54 - 79	<0.001						
Leaf length	VIII (a)	11	5.1	25 - 0	0.004	Leaf length	VIII (a)	11cM	3.84	1 - 27	0.027
Leaf length						Leaf length	VIII (a)	8cM	3.67	0 - 25	0.03
Leaf length	XII	19	3.41	24 - 3	0.036	Leaf length	XII	17cM	5.52	2 - 24	0.011
Leaf length	XV	10	5.45	0 - 29	0.004						
Leaf length	XVII	47	4.63	65 - 22	0.013						
Leaf width	IV	59	5.35	49 - 79	0.008						
Leaf width	V (b)	80	4.43	64 - 28	0.015						
Leaf width	VI	64	6.23	71 - 56	0.009						
Leaf width	VIII (a)	10	4.74	25 - 0	0.012						

Trait	LG	Position	%VA	CI	p	Trait	LG	Position	%VA	CI	p
Leaf width						Leaf width	IX	2cM	4.63	0 - 9	0.009
Leaf width	XI	12	4.82	2 - 25	0.033						
Leaf width	XIII	52	4.39	45 - 66	0.032						
Leaf width	XIV	29	4.19	18 - 29	0.011						
Leaf width	XVII	49	3.85	70 - 19	0.038						
Leaf width to length ratio						Leaf width to length ratio	III	81cM	4.30	66 - 95	0.040
Leaf width to length ratio	V (b)	73	6.06	82 - 56	0.016						
Leaf width to length ratio	VI	63	9.09	69 - 55	<0.001						
Leaf width to length ratio						Leaf width to length ratio	IX	0cM	6.45	0 - 3	0.001
Leaf width to length ratio	XI	18	6.06	1 - 29	0.004						
Leaf width to length ratio	XIII	45	6.06	23 - 53	0.017						
Leaf width to length ratio	XIV	29	9.09	23 - 29	0.001	Leaf width to length ratio	XIV	0cM	4.30	0 - 7	0.025
Leaf width to length ratio	XVIII	48	6.06	36 - 55	0.009	Leaf width to length ratio	XVIII	33cM	6.45	22 - 47	0.009
Petiole diameter 1	I	89	5.69	103 - 67	0.023						
Petiole diameter 1						Petiole diameter 1	II	58cM	4.58	43 - 80	0.049
Petiole diameter 1	VIII(a)	21	3.48	27 - 4	0.03	Petiole diameter 1	VIII (a)	14cM	3.69	0 - 27	0.038
Petiole diameter 1						Petiole diameter 1	IX	4cM	5.32	0 - 9	0.008
Petiole diameter 1						Petiole diameter 1	XII	11cM	6.11	2 - 24	0.005
Petiole diameter 1	XVI	28	6.16	52 - 22	0.001						
Petiole diameter 1	XVII	78	3.95	78 - 69	0.037						
Petiole diameter 2	I	74	5.31	94 - 62	0.022						
Petiole diameter 2	VIII (a)	20	2.93	0 - 27	0.046						
Petiole diameter 2	XI	18	4.8	1 - 30	0.012	Petiole diameter 2	IX	9cM	3.58	0 - 9	0.023
Petiole diameter 2	XIII	57	4.66	43 - 66	0.026						
Petiole diameter 2	XVII	53	4.29	73 - 32	0.036						
Petiole length	V (b)	82	4.53	84 - 67	0.048						
Petiole length	XIII	58	6.58	51 - 61	0.006						
Petiole length	VIII (a)	13	7.3	82 - 70	0.024	Petiole length	VIII (a)	9cM	11.8	4 - 18	<0.001
Petiole length						Petiole length	VIII (b)	12cM	4.65	0 - 25	0.019

Trait	LG	Position	%VA	CI	p	Trait	LG	Position	%VA	CI	p
Petiole length						Petiole length	IX	9cM	2.86	0 - 9	0.049
SLA	VII	4	8.89	13 - 0	0.001						
SLA	IV	43	5.11	35 - 52	0.013						
SLA						SLA	V (b)	49cM	4.05	19 - 73	0.049
SLA	VI	18	5.03	29 - 0	0.033						
SLA	VIII (a)	0	3.55	17 - 0	0.027						
SLA						SLA	X (a)	11cM	5.64	0 - 25	0.005
SLA	XII	7	3.6	23 - 0	0.019						
SLA	XIII	58	5.12	40 - 70	0.019						
Stomatal density (ab)	III	9	8.78	0 - 21	<0.001						
Stomatal density (ab)	IV	83	7.06	65 - 85	0.003						
Stomatal density (ab)						Stomatal density (ab)	V (b)	0cM	6.97	0 - 6	0.006
Stomatal density (ab)	VI	108	4.74	133 - 95	0.034	Stomatal density (ab)	VI	25cM	6.04	2 - 39	0.028
Stomatal density (ab)	XIII	63	4.72	56 - 83	0.019	Stomatal density (ab)	XIII	11cM	5.57	5 - 31	0.016
Stomatal density (ad)	III	14	5.8	0 - 32	0.009						
Stomatal density (ad)	V (b)	73	3.84	82 - 58	0.046						
Stomatal density (ad)	XII	0	14	3 - 0	<0.001	Stomatal density (ad)	XII	0cM	12.82	0 - 4	<0.001
Stomatal density (ad)	XIII	63	5.57	47 - 89	0.01						
Stomatal density (ad)						Stomatal density (ad)	XIV	0cM	5.53	0 - 12	0.005
Stomatal index (ab)						Stomatal index (ab)	I	51cM	5.10	28 - 81	0.037
Stomatal index (ab)	V (b)	0	7.53	6 - 0	0.003						
Stomatal index (ab)	XII	24	3.86	7 - 0	0.019						
Stomatal index (ad)						Stomatal index (ad)	I	38cM	6.75	26 - 48	0.001
Stomatal index (ad)	III	90	4.77	49 - 95	0.024	Stomatal index (ad)	III	24cM	6.37	6 - 41	0.012
Stomatal index (ad)	V (b)	3	3.83	19 - 0	0.016						
Stomatal index (ad)	XII	0	6.71	3 - 0	0.001	Stomatal index (ad)	XII	0cM	3.68	6 - 35	0.013
Stomatal index (ad)	XIII	48	4.88	37 - 64	0.019						
Stomatal number (ab)	III	36	6.21	23 - 48	0.001						

Trait	LG	Position	%VA	CI	p	Trait	LG	Position	%VA	CI	p
Stomatal number (ab)	IV	84	5.31	63 - 85	0.012						
Stomatal number (ab)						Stomatal number (ab)	V (b)	0cM	6.97	0 - 6	0.006
Stomatal density (ab)	VI	108	4.74	133 - 95	0.034	Stomatal density (ab)	VI	25cM	6.04	1 - 38	0.020
Stomatal number (ab)						Stomatal number (ab)	XIII	11cM	5.57	5 - 30	0.018
Stomatal number (ab)	XIV	29	5.85	17 - 29	0.002						
Stomatal number (ad)	III	14	5.8	0 - 33	0.007						
Stomatal number (ad)	V (b)	73	3.84	82 - 58	0.047						
Stomatal number (ad)	XII	0	14	3 - 0	<0.001	Stomatal number (ad)	XII	0cM	13.05	0 - 5	<0.001
Stomatal number (ad)	XIII	63	5.57	46 - 89	0.007						
Stomatal number (ad)						Stomatal number (ad)	XIV	0cM	5.31	0 - 12	0.006
Trichome number (ab)	IV	10	7.73	1 - 24	<0.001	Trichome number (ab)	IV	11cM	4.59	0 - 25	0.038
Trichome number (ab)						Trichome number (ab)	XII	24cM	4.13	1 - 24	0.022
Trichome number (ab)						Trichome number (ab)	V (a)	0cM	3.49	0 - 19	0.033
Trichome density (ab)						Trichome density (ab)	V (b)	0cM	3.52	0 - 19	0.036
Trichome density (ab)	IV	10	7.69	1 - 24	0.001	Trichome density (ab)	IV	11cM	4.65	0 - 21	0.031
Trichome density (ab)						Trichome density (ab)	XII	24cM	4.15	2 - 25	0.034
Trichome density (ad)						Trichome density (ad)	XIX	11cM	5.78	0 - 21	0.008
Trichome index (ab)						Trichome index (ab)	II	0cM	4.39	0 - 15	0.049
Trichome index (ab)	IV	13	5.18	1 - 30	0.012	Trichome index (ab)	IV	8cM	5.17	0 - 24	0.021
Trichome index (ab)	VIII (a)	0	3.19	21 - 0	0.049	Trichome index (ab)	VIII	27cM	3.76	21 - 27	0.035
Trichome index (ab)						Trichome index (ab)	XII	24cM	4.08	4 - 24	0.021
Trichome index (ab)						Trichome index (ab)	VIII	17cM	3.45	0 - 25	0.042
Trichome index (ad)						Trichome index (ad)	XIII	88cM	6.07	66 - 89	0.013
Trichome index (ad)						Trichome index (ad)	XII	2cM	3.85	0 - 24	0.038
Cell, stomata & Trichome number (ab)						Cell, stomata & Trichome number (ab)	I	23	4.98	2 - 45	0.045
Cell, stomata & Trichome number (ab)						Cell, stomata & Trichome number (ab)	II	1	10.25	0 - 7	<0.001
Cell, stomata & Trichome number (ab)	III	0	6.55	0 - 35	0.007						
Cell, stomata & Trichome number (ab)	IV	78	8.39	64 - 84	<0.001						
Cell, stomata & Trichome number (ad)						Cell, stomata & Trichome number (ad)	V	10	4.04	0 - 39	0.015

Trait	LG	Position	%VA	CI	p	Trait	LG	Position	%VA	CI	p
Cell, stomata & Trichome number (ab)	VIII(a)	0	3.62	0 - 11	0.023	Cell, stomata & Trichome number (ab)	VIII(a)	0	4.15	0 - 6	0.032
Cell, stomata & Trichome number (ab)	VIII(b)	15	3.89	8 - 22	0.028	Cell, stomata & Trichome number (ab)	VIII(b)	2	3.66	0 - 20	0.036
Cell, stomata & Trichome number (ad)						Cell, stomata & Trichome number (ad)	II	0	4.88	0 - 8	0.050
Cell, stomata & Trichome number (ad)	VI	0 cM	4.49	0 - 16	0.012						
Cell, stomata & Trichome number (ad)	VIII	6	4.09	0 - 25	0.023						
Cell, stomata & Trichome number (ad						Cell, stomata & Trichome number (ad	XI	14	5.61	0 - 32	0.023
Cell, stomata & Trichome number (ad)	XII	8	7.58	1 - 16	0.001	Cell, stomata & Trichome number (ad)	XII	6	6.69	0 - 15	<0.001
Cell, stomata & Trichome number (ad)						Cell, stomata & Trichome number (ad)	XIV	28	5.41	20 - 28	0.010
Cell, stomata & Trichome number (ad)	XVIII	52	4.57	45 - 63	0.026						

Table A7.2 Variation and heritability in Family 331 per trait evaluated. Experimental sites in the UK and Italy and the response Ita/UK are presented.

Trait	UK				Italy				Ita/UK	
	QTL	%Va	h^2	h^2_c	QTL	%Va	h^2	h^2_c	QTL	%Va
Leaf										
Leaf area	7	33.3	0.68	0.86	2	6.85	0.38	0.58	2	11.04
Leaf length	7	38.7	0.71	0.87	3	13.03	0.38	0.58	2	10.69
Leaf width	8	38	0.69	0.86	1	4.63	0.49	0.68	2	12.64
Leaf width/length ratio	6	44.85	0.71	0.87	4	21.5	0.60	0.77	1	5.56
Leaf extension rate	1	10.17	0.07	0.11	1	4.41	0.12	0.17		
Dry weight	7	35.1	0.64	0.83	2	7.35	0.36	0.55	2	10.85
SLA	6	31.3	0.30	0.53	2	9.69	0.50	0.69	1	9.09
Petiole width	4	29.8	0.10	0.35	4	19.70	0.14	0.26	3	11.53
Petiole height	5	21.99	0.09	0.35	1	3.58	0.30	0.48	2	7.14
Petiole length	3	18.41	0.22	0.59	3	29.31	0.45	0.64	3	13.87
Total QTL number	**54**				**23**				**18**	
Cell adaxial										
Cell area (ad)	2	10.67	0.44	0.65	4	21.72	0.36	0.55	2	10.71
Cell number (ad)	4	19.62	0.32	0.52	5	17.30	0.30	0.49	1	6.05
Cell, trichome and stomata number (ab)	4	20.73	0.33	0.54	5	26.63	0.21	0.37	2	10.23
QTL number	10				14				5	
Stomatal density (ad)	4	29.21	0.43	0.63	2	18.35	0.39	0.59	2	3.15
Stomatal index (ad)	5	29.09	0.42	0.63	4	16.8	0.39	0.59	1	1.71
QTL number	9				6				3	
Trichome density (ad)			0.13	0.25	1	5.78	0.27	0.45	3	13.60
Trichome index (ad)			0.10	0.21	2	6.97	0.48	0.67		
QTL number	0				3				3	
Total QTL number (ad)	**19**				**23**				**11**	

Trait	UK				Italy				Ita/UK	
	QTL	%Va	h^2	h^2_c	QTL	%Va	h^2	h^2_c	QTL	%Va
Cell abaxial										
Cell area (ab)	5	25.28	0.33	0.53	1	6.9	0.33	0.51	3	13.89
Cell number (ab)	5	18.81	0.30	0.50	4	22.54	0.26	0.43		
Cell, trichome and stomata number (ab)	4	22.45	0.32	0.53	4	23.04	0.25	0.42		
QTL number (ab)	14				9				3	
Stomatal density (ab)	5	20.56	0.32	0.53	3	18.58	0.32	0.51	2	11.81
Stomatal index (ab)	2	11.34	0.21	0.38	1	5.10	0.32	0.51		
QTL number	7				4				2	
Trichome density (ab)	1	7.69	0.04	0.09	3	4.15	0.14	0.26	1	3.09
Trichome index (ab)	2	8.37	0.13	0.26	5	20.85	0.25	0.43	1	4.37
QTL number	3				8				2	
Total QTL number (ab)	**24**				**21**				**7**	

Table A7.3 Common QTL mapped in the same LG for leaf traits in both experimental sites and in the response Ita/UK.

Location	UK					Ita				Ita/UK			
Trait	LG	cM	%VA	CI	p	cM	%VA	CI	P	cM	%VA	CI	P
Cell area (ad)	XII	8	5.67	17 - 0	0.001	8	5.89	0 - 16	0.007				
Cell number (ab)	VIII (a)	0	3.64	9 - 0	0.01	0	3.66	0-6	0.040				
	VIII (b)	15	4.26	21 - 8	0.01	2	4.36	0-11	0.024				
Cell number (ad)	XII	9	6.45	16 - I	0.003	6	6.28	0-15	0.003				
Dry weight	VIII (a)	9	7.13	20 - 1	0.001	8	3.53	1 - 26	0.043				
Leaf area	IX	7	2.59	9 - 0	0.043	9	3.55	0 - 9	0.026				
Leaf length	VIII (a)	11	5.1	25 - 0	0.004	11	3.84	1 - 27	0.027				
	VIII (a)					8	3.67	0 - 25	0.03				
	XII	19	3.41	24 - 3	0.036	17	5.52	2 - 24	0.011				
Leaf width to length ratio	XVIII	48	6.06	36 - 55	0.009	33	6.45	22 - 47	0.009				
Petiole width	VIII(a)	21	3.48	27 - 4	0.03	14	3.69	0 - 27	0.038				
Petiole length	VIII (a)	13	7.3	82 - 70	0.024	9	11.8	4 - 18	<0.001	10	5.45	2 - 27	0.013
Stomatal index (ad)	XII	0	6.71	3 - 0	0.001	0	3.68	6 - 35	0.013				
Stomatal density (ad)	XII	0	14	3 - 0	<0.001	0	13.05	0 - 5	<0.001	7	1.46	0 - 24	0.01
Trichome density (ab)	IV	10	7.73	1 - 24	<0.001	11	4.59	0 - 25	0.038				
Trichome index (ab)	IV	13	5.18	1 - 30	0.012	8	5.17	0 - 24	0.021				
	VIII (a)	0	3.19	21 - 0	0.049	27	3.76	21 - 27	0.035				
Cell, stomata & Trichome number (ab)	VIII(a)	0	3.62	0 - 11	0.023	0	4.15	0 - 6	0.032				
	VIII(b)	15	3.89	8 - 22	0.028	2	3.66	0 - 20	0.036				
Cell, stomata & Trichome number (ad)	XII	8	7.58	1 - 16	0.001	6	6.69	0 - 15	<0.001				

Table A7.4 Candidate genes identified in coincident positions. The number in brackets indicates the position in cM in the UK and Italy respectively.

Trait	LG (cM)	Candidate genes (cM)	Identifier	**Full Name** and Function
Leaf traits				
Leaf area	IX (7- 9)	*CDK-putative* (9.3)	AT1G73690	***CYCLIN-DEPENDENT KINASE PUTATIVE***. CDK, putative, similar to Cks1 protein (*A. thaliana*)
		CKS1 (9.3)	AT2G27960	***CYCLIN-DEPENDENT KINASES (CDKS)***. Essential, role in the regulation of the cell cycle
		EXP6 (6.8)	AT2G28950	***EXPANSIN PUTATIVE (EXP6).*** Cell wall loosening.
		REV (8.13)	AT5G0690	***REVOLUTA***. Regulates meristem initiation at lateral positions.
Leaf length	VIII (a) (11 - 8, 11)	*AS2* (5.6)	AT1G65620	***ASYMMETRIC LEAVES 2.*** Determination of symmetry. Involved in *KNOX* gene regulation
		XTH9 (17.3)	AT4G03210	***XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9.*** Catalyze the cleavage and molecular grafting of xyloglucan chains. Loosening/rearrangement cell wall
	XII (19 - 17)	*LOB* (19.7)	AT1G63090	***LATERAL ORGAN BOUNDARIES***. Involved in lateral organ development
		VFK1 (17)	AJ310523	***VICIA FABA K^+ CHANNEL***. Involved in phloem unloading. mRNA for P-type H+-ATPase (vha4 gene)
Leaf width to length ratio	XVIII (48 - 33)	*LEU* (28.8)	AT4G32551	***LEUNIG.*** Regulates floral organ identity. Encodes a glutamine-rich protein
Dry weight	VIII (a) (9 - 8)	*AS2* (5.6)	AT1G65620	Described above
		XTH9 (17.3)	AT4GO3210	Described above
Petiole width	VIII (a) (21 – 14)	*XTH9* (17.3)	AT4GO3210	Described Above
Petiole length	VIII (a) (13 - 9)	*XTH9* (17.3)	AT4GO3210	Described Above

Trait	LG (cM)	Candidate genes (cM)	Identifier	**Full Name** and Function
Cell traits				
Adaxial side				
Cell number (ad)	XII (9 - 6)	*TTG1* (6.1)	AT5G24520	***TRANSPARENT TESTA GLABRA 1***.Involved in trichome and root hair development. Epidermal cell fate specification
Cell area (ad)	XII (8,8)	*AGO1* (1.2)	AT1G48410	***ARONAUTE_1*** *OR* ***ARGONAUTE.*** Mutants are defective in post-transcriptional gene silencing and have pleiotropic developmental and morphological defects
		TTG1 (6.1)	AT5G24520	Described Above
		VFK1 (17)	AJ310523	Described above
Stomatal density (ad)	XII (0,0)	*AGO1* (1.2)	AT1G48410	Described above
		TTG1 (6.1)	AT5G24520	Described above
Stomatal index (ad)	XII (0, 0)	*AGO1* (1.2)	AT1G48410	described above
		TTG1 (6.1)	AT5G24520	Described above
Cell, stomata & trichome number (ad)	XII (8 - 6)	*TTG1* (6.1)	AT5G24520	Described above
Abaxial side				
Cell number (ab)	VIII (a) (0, 0)	*AS2* (5.6)	AT1G65620	Described above
	VIII (b) (2 – 15)	No candidate gene		
Trichome density (ab)	IV (10 – 11)	No candidate gene		
Trichome index (ab)	IV (13 – 8)	No candidate gene		
	VIII (a) (0- 27)	*AS2* (5.6)	AT1G65620	Described above
		XTH9 (17.3)	AT4GO3210	Described above
Cell, stomata & trichome number (ab)	VIII (a) (0 - 0)	*AS2* (5.6)	AT1G65620	Described above
	VIII (b) (15 - 2)	No candidate gene		

. **Table A7.5** Candidate genes in correlated cell and leaf traits QTL and Distinctive groups. Two subgroups are included in the first.

Trait	LG (cM)	Candidate genes (cM)	Identifier	**Full Name** and Function
Correlated cell QTL				
Cell number (ad) UK	**VIII(a)** (5)	*AS2* (5.6)	AT1G65620	***ASYMMETRIC LEAVES 2.*** Determination of symmetry. Involved in *KNOX* gene regulation
	XII (9)	*TTG1* (6.1)	AT5G24520	***TRANSPARENT TESTA GLABRA 1.***Involved in trichome and root hair development. Epidermal cell fate specification
Cell number (ab) UK	**VIII(a)** (0)	*AS2* (5.6)	AT1G65620	Described above
	XII (24)	*IRE* (25.7)	AT5G62310	***INCOMPLETE ROOT HAIR ELONGATION***. Serine /threonine kinase activity leaf, root, root hair, cell growth.
		PLE (26.3)	AT5G51600	***PLEIADE*** .Cytokinesis by cell plate formation.
Cell area (ad/ab) Ita	**II** (0,0)	*CDKB2;2* (1.1)	AT1G20930	***CYCLIN-DEPENDENT KINASE B2;2***. Cyclin-dependent protein kinase activity, M phase of mitotic cell cycle.
		CDKB2;1 (1.1)	AT1G76540	***CYCLIN-DEPENDENT PROTEIN KINASE B2;1***. Regulation of the G2/M transition of the mitotic cell cycle.
		ACCI (1.4)	AT1G36160	***ACETYL-COENZYME A CARBOXYLASE 1***. Mutant displays uncoordinated cell divisions.
		XTH15 (2.3)	AT1G01480	***XYLOGLUCAN: XYLOGLUCOSYL TRANSFERASE (XTR7)***. Cell wall, hydrolase activity, acting on glycosyl bonds
Stomata index (ad) Ita	**I** (38)	*ACC synthase* (37.4)	AT4G11280	1-***AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) SYNTHASE 6.*** Ethylene biosynthesis, induction of apoptosis by oxidative stress.
		GL3 (39.9)	AT5G41315	***GLABRA 3***. Encodes protein that interacts with GL1 in trichome development.
		EGL3 (39.41)	AT1G63650	***ENHANCER OF GLABRA 3***. Mutant with less trichomes, abnormal stomata. Interacts with *TTG1 GL3*.
Stomata index (ab) Ita	**I** (51)	*CER6* (58.8)	AT1G6853	***ECERIFERUM 6***. Involved in wax biosynthesis.
Trichome index (ad) Ita	**XII** (2)	*ARONAUTE_1*	AT1G48410	***ARONAUTE_1 OR ARGONAUTE***. 1. Developmental and morphological defects,gene silencing
Trichome index (ab) Ita	**XII** (24)	*PLE*	AT5G51600	Described above
		IRE	AT5G62310	Described above
Trichome density (ab) Ita	**XII** (24)	*PLE*	AT5G51600	Described above
		IRE	AT5G62310	Described above

Trait	LG (cM)	Candidate genes (cM)	Identifier	**Full Name** and Function
Correlated LG QTL				
Stomatal density (ab)	XIII (63) UK	*SPS* (60.8)	AT1G78510	***SOLANESYL DIPFOSPHATE SYNTHASE***. Involve in isoprenoid metabolism and response to heat.
	XIII (11) Ita	No candidate gene		
Stomatal index (ad)	III (90) UK	VFK1 (50.4)	AJ310523	***VICIA FABA K$^+$ CHANNEL***. Involved in phloem unloading. mRNA for P-type H+-ATPase (vha4 gene)
		ACC_synthase (69.3)	AT4G11280	Described above
		XTH5 (80.3)	AT5G13870	***ENDOXYLOGLUCAN TRANSFERASE A4***. Hydrolase activity, acting on glycosyl bonds, endomembrane system
	III (24) Ita	*MINA5.9* (8.5)	AT1G01760	***MINA5.9*** .Threonine dehydratase biosynthetic chloroplast precursor. Histone H3, identical to Zea mays
		STI(13.7)	AT2G02480	***STICHEL***. Trichome differentiation. *STICHEL* mutant shows trichomes with fewer than normal branches.
		GL2 (29.4)	AT1G79840	***GLABRA 2***. Affects epidermal cell identity including trichomes, root hairs, and seed coat.
Stomatal density (ab)	VI (108 and 113) UK	*GPA1* (121.9)	AT2626300	***G PROTEIN ALPHA SUBUNIT 1***. Involve in cell death. oxygen reactive species and stomatal movements.
	VI (25) Ita	*VFK1* (3.4)	AT5G62310	Described above
		ASD1 (14.93)	AT3610740	**Hydrolase**, acting on glycosyl bonds.
		BRU1 (37.7)	AT4G30270	***ENDO- XYLOGLUCAN TRANSFERASE MERI-5***. Involved in brassinosteroid response.
		XTH23 (37.9)	AT4G25810	***XYLOGLUCAN ENDOTRANSGLYCOSYLASE 6***. Xyloglucan: up regulated by ABA.
Leaf width to length ratio	XIV (29) UK	PROLIFERA (22.3)	AT2636985	***ROTUNDIFOLIA 4***. Controlling cell proliferation
		XTH5 (28.6)	AT5G13870	***ENDOXYLOGLUCAN TRANSFERASE A4***. Hydrolase activity, acting on glycosyl bonds, endomembrane system
		PPC1 (29.2)	PU06684	***PHOSPHOENOL PIRUVATE CARBOXILASE***. Phosphoenol carboxilase activity. Tricarboxylic acid cycle.
	XIV (0) Ita	*ANT (1.4)*		***AINTEGUMENTA***. Organ initiation and growth
Contrasting QTL				
LER				***CELL DIVISION CONTROL 2***. Cyclin-dependent kinase subunit. Mutations abolish cell division.
	IV (53) UK	*CDC2A* (55.7)	AT3G48750	
	XIV (28) Ita	*XTH5* (28.6)	AT5G13870	Described above
		PPC1 (29.2)	PU06684	Described above
		XTH16 (30.5)	AT3G23730	***XYLOGLUCAN ENDOTRANSGLYCOSYLASE, PUTATIVE***. Hydrolase activity, glucan metabolism