UNIVERSITY OF SOUTHAMPTON
School of Biological Sciences

PHYSIOLOGY, GENETICS AND GENOMICS OF DROUGHT ADAPTATION IN *POPULUS*

In one volume

by
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Thesis for the degree of Doctor of Philosophy

March 2011
UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDECINE, HEALTH AND LIFE SCIENCES
BIOLOGICAL SCIENCES

Doctor in Philosophy

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As the demand for energy rises, *Populus* species are increasingly grown as bioenergy crops. Meanwhile, due to global change, predictions indicate that summer droughts will increase in frequency and intensity over Europe. This study was carried out to evaluate the adaptation to drought in *Populus*, at different levels: genetic, genomics and physiology. Forests trees such as poplar are very important ecologically and economically but the *Populus* genus is known to be drought sensitive. Consequently, it is essential to understand drought response and tolerance for those trees. Two populations of poplar were used for this study, a mapping population (Family 331) and a natural population of *Populus nigra*.

The F_2 mapping population obtained from a cross of *Populus deltoides* and *Populus trichocarpa,* showed differences in stomatal conductance and carbon isotope composition in both clones and the F_2 progeny. It was also used to discover QTL related to water use efficiency highlighting interesting areas of the genome. Combining QTL discovery and microarray analysis of the two clones in response to drought, a list of candidate genes was defined for water use efficiency.

The natural population of *Populus nigra* consisting of 500 genotypes of wild black poplar showed variation in numerous physiological measurements such as leaf development and carbon isotope discrimination in well-watered conditions depending on their latitude of origin. The drier genotypes (from Spain and South France) had the smallest leaf area which could be linked to an adaptation to drought.
Physiological measurements of extreme genotypes in leaf size of this population revealed differences in response to water depending on their latitude of origin. Stomatal conductance rapidly decreased and water use efficiency improved for Spanish genotypes after a slow and moderate drought stress. Direct comparisons between the transcriptome of extreme genotypes from Spain and North Italy in well watered and drought conditions provided an insight into the genomic pathways induced during water deficit. Six candidate genes were selecting for further analysis using real-time PCR: two stomatal development genes (*ERECTA* and *SPEECHLESS*), two ABA related genes (*ATHVA22A* and *CCD1*), a second messenger (*IP3*) and a NAC transcription factor (*RD26*).

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Author's declaration

I Maud Viger declare that the thesis entitled 'Physiology, Genetics and Genomics of drought adaptation in *Populus*' and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that: This work was done wholly or mainly while in candidature for a research degree at this university.

- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institute, this has been clearly stated.
- Where I have consulted the published work of others, this has clearly been attributed.
- Where I have quoted from the work of others, the source has always been given. With the exception of such quotations, this thesis is entirely my own work.
- I have acknowledged all main sources of help.
- Where this thesis is based on work done by myself jointly with others, I have made clear what was done by others and what I have contributed myself.
- None of this work has been published before submission.

Signed

Date:

Acknowledgements

I would like to thank Professor Gail Taylor for the supervision, support and advice that I received since I started my PhD and also for securing the studentship funding my PhD. I thank Evoltree, which provided the majority of the financial support, and EnergyPoplar.

I thank Harriet Trewin for her advice and help. I also thank Suzanne Milner for helping recording leaf area during the greenhouse experiment.

I thank Harriet Trewin, Suzanne Milner, Nathaniel Street, Rebecca Rowe, Matthew Nelson, Gaia Biggi, Yunan Lin, Jennifer DeWoody, Caroline Dixon, Adrienne Payne, Giacomo Prete and Amanda Thomas for their help in the various filling pots with soil, planting, re-potting, cutting and labelling trees. I also thank Mike Cotton for the watering and maintenance of the plants in the greenhouse.

I thank Suzanne Milner, Nathaniel Street, Matthew Aylott and Matthew Tallis for collecting the plant material in Belgium for wood carbon isotope discrimination.

I would like to thank Robert Styles for helping me record soil water content and collect stomatal conductance data during the drought experiment of *P. nigra*. Thanks to Rebecca Rowe, Suzanne Milner, Adrienne Payne, Yunan Lin, Jennifer DeWoody and Matthew Nelson for their help with the watering during the drought experiment.

Thank you to Dr Alan Marchant for advice in primer designing, and Patrick Stephenson, Enrico Freschet and Mickael Blackney for their help with real-time qPCR.

A special thank to Jennifer DeWoody for her support and discussions related to this PhD and her help with the DNA extraction and SSR markers work.

Thanks to Adrienne Payne, Yunan Lin, Hazel Smith, Patrick Stephenson, Suzanne Milner, Stacey Travers, Matthew Tallis, Mark Totten and Jennifer DeWoody for proof-reading parts of this thesis.

Un grand merci à ma famille et particulièrement à mes parents qui m'ont toujours soutenue et encouragée tout au long de mon doctorat.

List of abbreviations

A	Assimilation rate of CO_2
ABA	Abscisic acid
aba	ABA-deficient
ABF	ABRE-binding proteins
abi	ABA-insensitive
ABRE	ABA-responsive element
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of variance
AP2	APETALA 2
ATP	Adenosine triphosphate
bp	Base pair
bZIP	Basic leucine zipper
CBF	C-repeat binding factor
CBL	Calcineurin B-like proteins
CDPK	Calcium-dependent protein kinase
CE1	Coupling element 1
CE3	Coupling element 3
cM	Centi-morgan
CRT	C-repeat or `low-temperature-responsive`
CSP1	CDPK substrate protein 1
DAD	Day after drought
DRE	Dehydration-responsive element
DREB	DRE binding
DSP	Deletion insertion polymorphisms
DSP	Chloroplast-localized stress protein
E	Transpiration rate
ERD	Early response to dehydration
EREBP	Ethylene-responsive-element-binding proteins
ERF	Early responsive factor
GB	Glycine betain
G-box	Ubiquitous regulatory elements
GLG	Germin-like genes
GLM	General linear model
g'	Leaf relative conductance
g_s	Stomatal conductance

IP3	Inositol (1,4,5)-triphosphate
LD	Linkage disequilibrium
LEA	Late embryogenesis abundant
LG	Linkage Group
LPI	Leaf plastochron index
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MIP	Membrane intrinsic proteins
MYBRS	MYB recognition sequence
MYC	MYC recognition sequence
NAC	Nitrogen assimilation control
NCED	9-*cis*-epoxycarotenoid dioxygenase
PLC	Percentage loss of xylem conductance
PK	Protein kinase
QTL	Quantitative trait loci
RAPD	Random Amplification of Polymorphism DNA
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive oxygen species
SFP	Single Feature Polymorphism
sHSP	Small heat shock protein
SLA	Specific leaf area
SNP	Single nucleotide polymorphism
SRC	Short rotation coppice
SSR	Single sequence repeat
STRP	Short tandem repeat polymorphism
TF	Transcription factor
WUE	Water use efficiency
XTH	Xyloglucan endotranglycosylase
ZFHD	Zinc-finger homeodomain
$\delta^{13}C$	Carbon isotope composition
$\Delta^{13}C$	Carbon isotope discrimination
$\delta^{18}O$	Oxygen isotope composition
$\Delta^{18}O$	Oxygen isotope enrichment
Ψ_W	Water potential

Chapter 1: Introduction

1.1. Introduction

In a world where climate is changing it is essential to understand how plant physiological, genetic and genomic processes are adapted to abiotic stress. It is considered that various regions of Europe will be subjected to increased drought periods in the future, especially during the summer, which will be more frequent and more severe (IPCC, 2001; Broadmeadow, 2002; Kundzewicz *et al.*, 2007). As water is a fundamental resource for the development of crops, plants adapted to low precipitation in order to survive and to maintain growth and biomass should be studied.

Forest trees have a significant ecological as well as economic role, with their use for timber, heating (Committee on Managing Global Genetic Resources, 1991), medicine (Mills *et al.*, 1996; Klein-Galczinshy, 1999) and more recently for intensive production for bioenergy (Kauter *et al.*, 2003; Oliver *et al.*, 2009; Sannigrahi *et al.*, 2010). Among forest trees, poplar species have been particularly used in science as a model tree because of their rapid growth properties and the small size of the genome which is fully sequenced (Tuskan *et al.*, 2006). However, they are very sensitive to drought conditions, being from a riparian habitat (Monclus *et al.*, 2006; Aylott *et al.*, 2008). It is thus important to understand the features of drought tolerance for this genus.

Responses to drought are numerous and occur at different levels in the plant. From the perception of drought to gene expression, a series of events occur progressively. In order to tolerate drought environments, various procedures happen physiologically from the roots to the leaves.

In this chapter, drought response and adaptation will be studied in plants with numerous examples of *Populus* studies. The different genetic and genomic tools used to study drought adaptation will also be presented.

1.2 Poplar: A model tree

Forest trees are economically and ecologically important (Bradshaw *et al.*, 2000). They provide materials, preserve biodiversity and reduce the effects of climate change (Harfouche *et al.*, 2011). It is thus crucial to understand their biology. *Arabidopsis* and rice are widely used model plants in research but it is also essential to answer questions for diverse biological and physiological traits related specific to trees. Although *Arabidopsis* has proved to be a valid model to study for example the formation of wood (see review Nieminen *et al.*, 2004; Zhang *et al.*, 2010), using trees rather than annual models for tree related traits is important, such as the formation of secondary xylem (Bradshaw *et al.*, 2000; Taylor, 2002), leaf and flower phenology, seasonality, cold hardiness, crown formation, juvenile-mature phase change (Bradshaw *et al.*, 2000), long-term perennial growth (Bradshaw *et al.*, 2000; Jansson & Douglas, 2007) or evolution of adaptive traits (Jansson & Douglas, 2007). In the light of these traits, the need for a model tree seems to be clear.

Among other trees, such as pine and eucalyptus, *Populus* has been proposed to represent the first model for trees, although with the onset of second generation sequencing it is likely that in the future many tree species will have their full genome sequences available. *Populus* has an economic importance in many countries with numerous uses, such as timber, pulp, plywood, paper and recently as a source of renewable energy (Altman, 1999; Taylor, 2002; Brunner *et al.*, 2004). It also has an ecological role as a forest tree (Sbay & Taroq, 2003) with many ecological processes, like carbon sequestration (Taylor, 2002; Brunner *et al.*, 2004), bioremediation, nutrient cycling, biofiltration and as well as its presence in diverse habitats (Brunner *et al.*, 2004). The genus contains at least 30 species (Taylor, 2002; Sbay & Taroq, 2003) and is a member of the *Salicaceae* family (Sbay & Taroq, 2003; Bradshaw *et al.*, 2000).
As a model tree for molecular biology, *Populus* has many advantages. Its genome has been completely sequenced and it was the first woody plant sequenced (Tuskan *et al.*, 2006). It was sequenced on a single female genotype, "Nisqually 1" of *Populus trichocarpa*. It is composed of 19 chromosomes and 45,555 protein-coding genes. Its genome has a relative small size (Bradshaw *et al.*, 2000; Taylor, 2002; Brunner *et al.*, 2004) of 485 ± 10 megabases (Tuskan *et al.*, 2006) which is about 4 times bigger than *Arabidopsis* but 40 times smaller than conifers (Bradshaw *et al.*, 2000). Genetics tools

to study poplar are numerous, with genomic resources such as GenBank and PopulusDB (Brunner *et al.*, 2004), genetic maps (Taylor, 2002; Jansson & Douglas, 2007) or extensive expressed sequence tags (Jansson & Douglas, 2007). Another advantage of using *Populus* is that it is easy to transform genetically (Jansson & Douglas, 2007; Taylor, 2002), which is not the case with many other trees (Bradshaw *et al.*, 2000; Brunner *et al.*, 2004). Genetical transformation is a useful tool to study genes expression (Taylor, 2002). The *Populus* genus is also easy to clone (Bradshaw *et al.*, 2000). An important characteristic of *Populus* trees is that they grow relatively fast (Altman, 1999; Taylor, 2002; Cronk, 2004), which permits the study of their functions and their responses to biotic and abiotic stress in a reasonable period of time (Bradshaw *et al.*, 2000; Brunner *et al.*, 2004).

Concerning the reproductive biology, *Populus* species are prolific, wind pollinated, dioeceous and long lived trees (Brunner *et al.*, 2004; Bradshaw *et al.*, 2000; Jansson & Douglas, 2007). There is also a close relation between biomass productivity and physiological traits in poplar (Bradshaw *et al.*, 2000).

Finally, *Populus* is widely distributed in the northern hemisphere and its genetic variation in natural populations is high (Bradshaw *et al.*, 2000; Cronk, 2004). Indeed, poplar clones have a high individuality which is due to small changes in their sequence (SFPs: Single Feature Polymorphisms; (Cronk, 2004)). SFPs can be single nucleotide polymorphisms (SNPs), deletion insertion polymorphisms (DSPs) or short tandem repeat polymorphisms (STRPs; (Cronk, 2004)). For example, in *Populus trichocarpa*, about every 100 base pairs, a SFP can be found in its genome (Cronk, 2004). Polymorphisms were also observed 2 to 10 fold higher in *P. tremula* than other trees such as *Pinus* and *Cryptomeria* (Ingvarsson, 2005).

However, *Populus* also has disadvantages and limitations as a model tree. It has a modest commercial importance compared to pine or eucalypus (Bradshaw *et al.*, 2000). The flowering induction is long, not earlier than four years old (Bradshaw *et al.*, 2000; Taylor, 2002). Because they are dioeceous trees, they cannot self-pollinate (Bradshaw *et al.*, 2000). They also are large trees, thus designing an experiment can be complex (Bradshaw *et al.*, 2000).

1.3 Molecular biology under drought stress in plants

Under water deficit, gene expression in plants is altered and new genes are expressed to respond to this stress. The pathway from a stress to biological and physiological responses and later to drought tolerance is complex and not yet completely solved. It includes biochemical pathways and protein formation expressed by numerous genes (Neill & Burnett, 1999).

1.3.1 Signal perception

For any stress applied to a plant, signal perception is the first step for altered gene expression and consequential stress tolerance. This mechanism is not yet fully understood and depends on several factors, such as developmental stage of the plant or level of drought.

However, several events are recurrent. In the plant as a whole, consequence of water deficit is an accumulation of ABA in the roots and its transport through the stem. Water deficit is sensed by leaves when the loss of water by transpiration exceeds the uptake (Bray, 1997). When a cell is dehydrated, the turgor pressure decreases, as a result the osmotic potential changes in the plasma membrane (Shinozaki & Yamaguchi-Shinozaki, 1997). By losing water, the cell and plasma membrane are modified in volume and area, respectively (Bray, 1997). Then, the solute content is altered, including the proteins connected to wall and plasma membrane, ion channels or protein kinases (Bray, 1997; Neill & Burnett, 1999).

Possible mechanisms have been described to explain how a cell senses an osmotic stress: oxidative burst, physical tension, two components system such as SLN1 and SHO1-like protein (Shinozaki & Yamaguchi-Shinozaki, 1997). The two components system is a widely studied mechanism, particularly using yeast and microorganisms (Maeda *et al.*, 1995; Reiser *et al.*, 2003; Clotet & Posas, 2007). Although the research in this domain is intensive and scientists have suggested components as osmosensors in higher plants such as the gene *NtC7* which encodes a receptor-like protein (Tamura *et al.*, 2003) or *Cre1* a cytokinin receptor in *Arabidopsis*, their role still needs be demonstrated (Bartels & Sunkar, 2005).

After sensing the water stress, a signal is created to initiate molecular cascades (signal transduction) in order to activate gene expression.
Different pathways exist but the ABA-dependent pathway is the main one. Abscisic acid is an anti-stress hormone and is synthesized from a carotenoid intermediate. The ABA biosynthesis is well known and during drought stress, NCED (9-cis-epoxycarotenoid dioxygenase) is activated which is a key step in the production of ABA (Guerrini *et al.*, 2005; Han *et al.*, 2004). More than one type of receptors is present when cells undergo water-stress (Neill & Burnett, 1999). ABA receptors have been proposed (see review: Muschietti & McCormick, 2010) but yet need to be confirmed: *GPCR-*type *G* proteins (*GTG1* and *GTG2*) (Pandey *et al.*, 2009; Christmann & Grill, 2009), *ABAR/CHLH* protein (Shen *et al.*, 2006), *RCAR1* protein (Ma *et al.*, 2009b), *PYR* proteins (Nishimura *et al.*, 2009). *FCA* protein was also proposed by Razem *et al.* (2006) as an abscisic acid receptor but the authors later retracted the paper (Razem *et al.* 2008) as another group showed that *FCA* did not bind to ABA (Risk *et al.*, 2008).

1.3.2 Signal transduction

A signal transduction is a biochemical cascade that transfers, in the cell, information though enzymes and second messengers in order to create a response to the signal. In drought stress, four pathways are active, two ABA-dependent and two ABA-independent pathways.

The transduction pathways in drought are not fully understood in details but they include protein phosphorylation and dephosphorylation carried out by protein kinases and protein phosphatases, linked with second messengers (Chaves *et al.*, 2003).

1.3.2.1. Second messengers

Second messengers are intracellular molecules that are activated or produced after binding primary messengers to their specific receptor. Under stress, their role is to diffuse a signal and amplify responses to this stress (Taiz & Zeiger, 2002). In dehydration, Ca^{2+} and IP_3 are the second messengers known as the most suitable for stress signalling transduction.

In the ABA-dependent transduction pathways, Ca^{2+} plays an important role as a second messenger (Hong-Bo *et al.*, 2008). As the ABA level increases in dehydrated cells, the concentration of Ca^{2+} rises (Campalans *et al.*, 1999). This increase helps to stimulate ion transport and regulate ion channels such as K^+ channels in guard cells. Three classes of Ca^{2+} sensors exist, which are all involved in drought transduction pathways: calmodulin, CDPKs (calcium-dependent protein kinase) and CBLs (calcineurin B-like proteins) (Yang & Poovaiah, 2003). Stress-induced CDPKs are very important in stress responses. They were induced earlier after a stress and sustained longer in the salt-tolerant variety than in the salt-sensitive variety (Kawasaki *et al.*, 2001).
IP_3 (Inositol (1,4,5)-triphosphate) is also increased after cells have been exposed to an osmotic stress (Shinozaki & Yamaguchi-Shinozaki, 1997). Cyclic ADP-ribose and IP_3, both induced by a ABA-binding to receptor, release Ca^{2+} into the cytoplasm through calcium-sensitive channels on the tonoplast and generate stomatal closure (Gilroy *et al.*, 1990).

1.3.2.2. Protein kinases and phosphatases

Second messengers bind and activate enzymes such as protein kinases and protein phosphatases (Fig. 1.1). Protein kinases (PK) are involved in protein phosphorylation, while phosphatases are involved in dephosphorylation.

Many PK are induced by environmental stresses and possibly involved in signal transduction pathway such as MAPK and CDPK.
MAPK cascades consist of Mitogen-Activated Protein (MAP) kinases which are serine/threonine protein kinases that respond to many environmental stimuli, including water-stress by several cellular actions, such as transcription factor activation (Neill & Burnett, 1999).
In order to activate MAPKs, phosphorylation of conserved threonine and tyrosine residues is necessary by a MAPK kinase (MAPKK). The latter is activated by a MAPKK kinase by phosphorylation of conserved threonine and/or serine residues (Bartels & Sunkar, 2005). These events are called the MAPK cascades. The real output of the MAP kinase cascade to regulate cellular osmotic stress is not yet completely clear (Zhu, 2002). Two of the consequences of MAP kinase cascade are the activation of pre-existing proteins and phosphorylation of TF (Transcription Factors) by translocating

MAPK into the nucleus (Treisman, 1996) or to other sites in the cytoplasm to target enzymes or cytoskeletal components (Robinson & Cobb, 1997).
Several MAP kinases are induced by water-stress and in alfalfa cells, they are different depending on the level of the stress. Activation of the MAPK is rapid following a drought stress. Jonak *et al.* (1996) showed that in alfalfa plants MMK4 kinase kinase was activated within five minutes in drought condition. They also discovered that $p44^{MMK4}$ kinase was activated independently of ABA.

CDPK cascades are Ca^{2+} dependent protein kinase cascades for stress signalling. They are serine/threonine protein kinases (Patharkar & Cushman, 2000). In *Arabidopsis*, CDPK are induced by two genes, ATCDPK1 and ATCDPK2, quickly after a drought stress (Urao *et al.*, 1994).

SNF1/AMP-activated protein kinases are other protein kinases that are expressed by drought or by ABA and as a consequence could be involved in osmotic stress and ABA signalling (Bartels & Sunkar, 2005). They are activated by serine or threonine phosphorylation.

The main role of phosphatases is to dephosphorylate its substrate. They are activated by second messengers during a stress. As protein kinases, protein phosphatases may activate transcription, either by translocating to the nucleus or by activating transcription-factors located in the cytoplasm, which would be transferred into the nucleus (Neill & Burnett, 1999). Phosphatases can play a role in stomatal closure in response to ABA (see review Luan, 1998). They might also be involved in stress response as it was observed in *Mesembryanthemum crystallinum* an increase of several protein phosphatases by drought and salt stress (Miyazaki *et al.*, 1999).
Phosphatases can be divided into two different groups, the phosphoproteins (serine/threonine) phosphatases (PPases) and the phosphotyrosine (protein tyrosine) phosphatases (PTPases) (Bartels & Sunkar, 2005).

1.3.2.3. ABA-dependent and –independent transduction pathway

Under drought stress, different pathways work in order to regulate gene expression in plants: ABA-dependent and –independent pathways. Signal transduction can differ

between the pathways and it has been suggested that their action is in parallel so that gene expression is regulated to respond to osmotic stress (Yamaguchi-Shinozaki & Shinozaki, 1994). Other studies showed that instead of acting in a parallel manner, the pathways were interacting and converging (Ishitani *et al.*, 1997; Bonetta & McCourt, 1998).

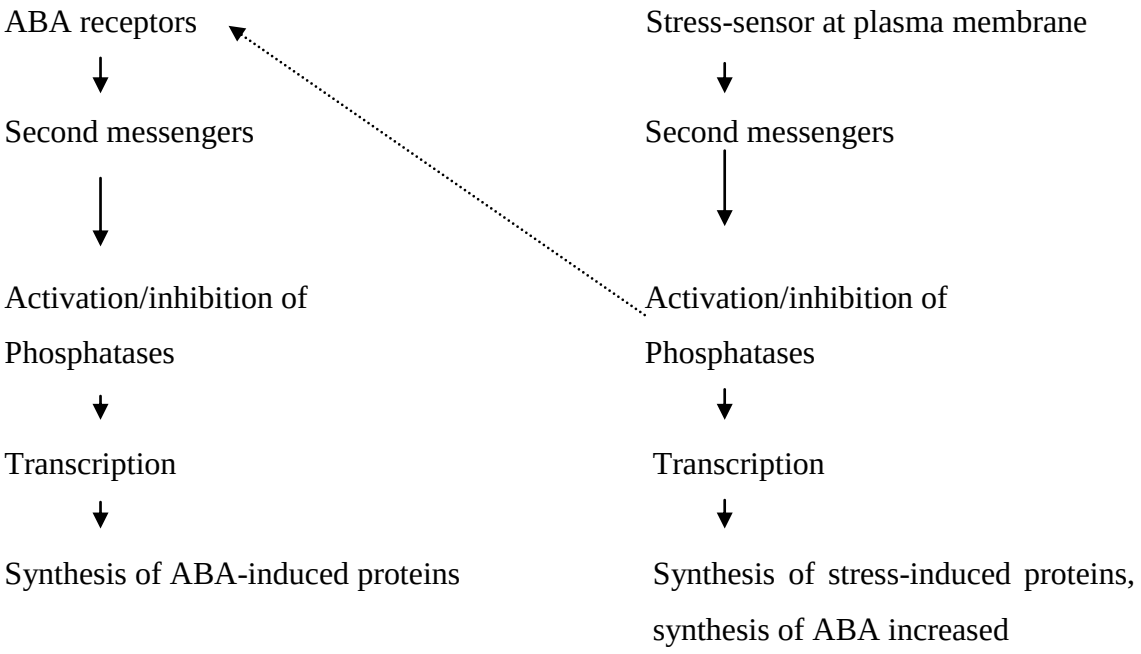


Figure 1.1: Summary of signalling and transduction pathway in dehydrated cells

1.3.3 Gene induction

Gene induction in response to drought stress is necessary to activate the expression of stress inducible-genes (Table 1.1). It is possible by cooperation and interaction between *cis*-acting regulatory elements and transcription factors (Fig.1.2). The amount and activity of both these elements related to water-stress responsive genes are modified by water deficit (Neill & Burnett, 1999).

Cis-acting elements play a role in responding to environmental signals, such as drought stress (Yamaguchi-Shinozaki & Shinozaki, 2005). They regulate the expression of genes which are on the same strand and they are located in their promoter region. Transcription factors control the expression of related genes. Many transcription factors are involved in the drought stress response (Umezawa *et al.*, 2006). The initiation of

transcriptional factors permits to activate the transcription of their genes (Yamaguchi-Shinozaki & Shinozaki, 2005).

Table 1.1: *Cis*-acting elements and corresponding transcription factors for drought stress gene induction modified from Yamaguchi-Shinozaki & Shinozaki (2005).

***Cis*-element**	**Sequence**	**Transcription factor**	**Binding protein**
ABRE	10bp with an ACGT core	AREB	bZIP
CE3	ACGCGTGTCCTC	?	?
CE1	TGCCACCGG	?	ERF/AP2
MYCR	CANNTG	MYC	myc
MYBR	YAACPyPu	MYB	myb
DRE	TACCGACAT	DREB	AP2
CRT	GGCCGACAT	CBF	ERF
NACR	ACACGCATGT	NAC	NAC
ZFHDR	Not yet reported	ZFHD	ZFHD

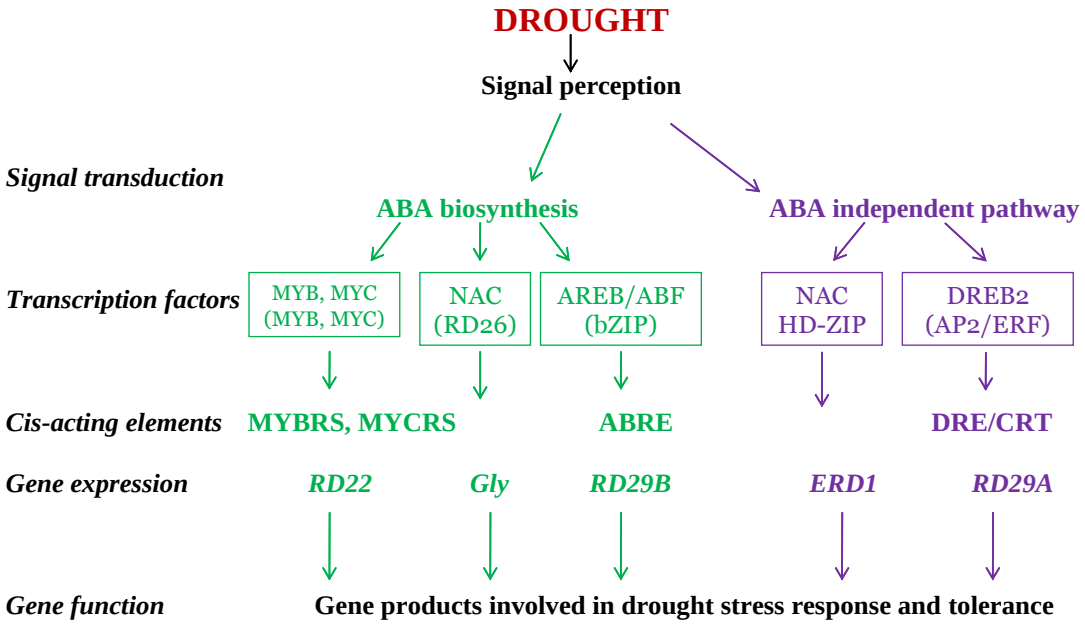


Figure 1.2: Transcriptional regulatory networks of *cis*-acting elements and transcription factors involved in drought stress-responsive genes, modified from Shinozaki & Yamaguchi-Shinozaki (2007).

1.3.3.1. ABA-dependent pathways

As mentioned in the previous section, ABA is known to accumulate in a period of drought, thus there is a hypothesis that it plays a role in regulating water-stress inducible gene expression. Moreover, exogenous application of ABA has the same consequences on plants as osmotic stress (Zhu, 2002). Because these pathways involve ABA biosynthesis, it may mean long-distance signalling, slow and adaptive responses to drought (Shinozaki & Yamaguchi-Shinozaki, 2000; Goh *et al.*, 2003). They are divided into two routes, one with new protein synthesis and one without.

The first ABA-dependent pathway described involves the transcription factors MYC and MYB. This pathway requires the synthesis of new proteins (Yamaguchi-Shinozaki & Shinozaki, 2006; Zhang *et al.*, 2006). In this route, after drought stress signalling, endogenous ABA is accumulated which results in the synthesis of the two transcription factors MYC and MYB, proving their late role in drought responses (Yamaguchi-Shinozaki & Shinozaki, 2005). The *cis*-acting elements involved are MYCRS and MYBRS. The genes related to MYB-like motif can have many different functions (Meissner *et al.*, 1999).
An example for this gene expression pathway is the gene *RD22* which is regulated by ABA and might play a role in stress memory in plants (Goh *et al.*, 2003). It requires protein biosynthesis to be expressed (Bray, 1997). The promoter of this gene has a region which contains conserved motifs of DNA binding proteins such as MYC and MYB (Iwasaki *et al.*, 1995).

The second ABA-dependent pathway does not require new protein biosynthesis and involves the *cis*-acting element ABRE and the transcription factors AREB/ABF (Shinozaki & Yamaguchi-Shinozaki, 1997; Campalans *et al.*, 1999). The cDNAs, AREB (ABRE-binding proteins) and ABFs (ABRE-binding factors), encode bZIP (basic leucine zipper) transcription factors. They can bind to their corresponding ABRE (ABA-responsive *cis*-acting element), which might result in the expression of the related gene (Ramanjulu & Bartels, 2002; Zhang *et al.*, 2006). Genes comprising ABREs element in their promoter play a role in the protection and stabilisation of protein complexes and membranes (Chaves *et al.*, 2003).

But it has been shown that one ABRE element is not enough to induce transcription and either two or more ABRE copies are needed or one copy with coupling elements. For example, in order to activate the gene *RD29*, two ABRE copies are necessary (Uno *et al.*, 2000). In barley, the gene *HVA22* contains a coupling element CE1 with an ABRE element (Shen & Ho, 1995) and the ABA-inducible gene *HA1* (group 3 LEA) has another coupling element CE3 to activate its expression (Shen *et al.*, 1996).
G-box is similar to ABRE and is contained in many genes responding to environmental and external elements (Yamaguchi-Shinozaki & Shinozaki, 2006). bZIP proteins bind to G-box sequences (Menkens *et al.*, 1995).

1.3.3.2. ABA-independent pathways

The importance of ABA is obvious in drought stress response but it has been proved that there are other pathways that do not require ABA biosynthesis. Indeed, the *Arabidopsis* null mutants *aba* (ABA-deficient) and *abi* (ABA-insensitive) can activate many genes in response to drought (Shinozaki & Yamaguchi-Shinozaki, 1997). This shows that these genes do not need ABA biosynthesis to induce their expression.

The main ABA-independent pathway is the transcription factors DREB/CBF (AP2/ERF) and *cis*-element DRE/CRT. This pathway plays a role in stress signalling converging and integrating multiple stresses (Knight & Knight, 2001). DRE motif is a dehydration-responsive element and CRT, similar to DRE, is called C-repeat or "low-temperature-responsive" element. DREBs and CBF proteins contain a conserved DNA-binding motif that is also contained in EREBP (ethylene-responsive-element-binding protein) and in AP2 (APETALA2) (Shinozaki & Yamaguchi-Shinozaki, 1997). This family of transcription factors is unique to plants (Singh *et al.*, 2002). It has been reported that the *Arabidopsis* genome contains 124 ERF proteins (Singh *et al.*, 2002) and 145 DREB/ERF-related proteins (Sakuma *et al.*, 2002). Different DREBs (dehydration responsive element binding) proteins exist but it is known that DREB2s are involved in dehydration genes responsive expression (Seki *et al.*, 2001; Liu *et al.*, 1998). For example, the gene *RD29* (dehydrated-regulated) contains in its promoter a CRT and DRE motif.

ABA does not activate the DRE and CRT transcription factors but Zhu (2002) said that ABA might be necessary for DRE elements in order to be fully activated in osmotic stress.

The second ABA-independent pathway activates many genes, including early response to dehydration genes (*ERD*), with two *cis*-acting elements that are both necessary for gene expression and two transcription factors. *ERD1* is an example of a gene activated during this ABA-independent pathway. The transcription factor NAC (Nitrogen Assimilation Control) works in coordination with the transcription factor ZFHD (zinc-finger homeodomain). The *cis*-acting elements, NACRS (a MYC-like element) and ZFHDRS are located separately in the promoter of the gene *ERD1* (Yamaguchi-Shinozaki & Shinozaki, 2006; Yamaguchi-Shinozaki & Shinozaki, 2005). The *cis*-acting element ZFHDRS (or called 14 base pair rsp1 site 1-like sequence) encodes ZFHD proteins (Yamaguchi-Shinozaki & Shinozaki, 2005).

1.3.3.3. ABA-dependent and ABA-independent pathway

Some genes have also been shown using both ABA-dependent and -independent pathways with two transcription factors in their promoter, one requires ABA biosynthesis while the other one does not. There is convergence between the two pathways, but scientists do not know exactly how (Ishitani *et al.*, 1997). The gene *RD29A* is a good example, activated by both *cis*-elements DRE/CRT and ABRE (Fig. 1.3) under drought (Liu *et al.*, 1998). It is known to be regulated by both pathways because in *aba* and *abi* null mutants, the transcription of this gene is partially blocked (Zhu, 2002).

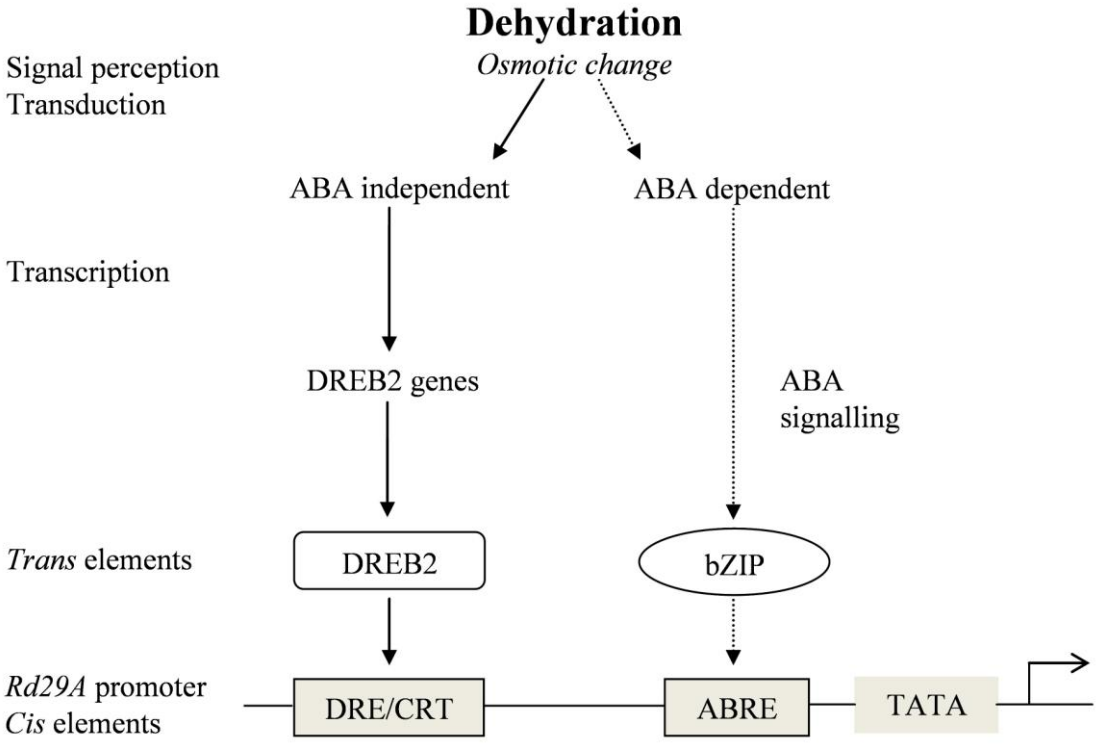


Figure 1.3: Induction of the gene *rd29A* including the *cis*- and *trans*-elements involved in the drought-responsive gene expression, modified from Shinozaki & Yamaguchi-Shinozaki (2000)

1.3.4 Gene expression

Gene expression under drought stress can be divided into two groups: functional and regulatory genes. Regulatory proteins formed by the gene expression for stress response are diverse, such as transcription factors and protein kinases. These gene products have already been described in the previous sections (signal transduction and gene induction). In the other group, gene products expressed in stress response and adaptation are functional proteins such as water and ion channel proteins, chaperones, LEA proteins, detoxicating enzymes and many more (Shinozaki & Yamaguchi-Shinozaki, 1997).

1.3.4.1. Transport protein and aquaporins

Under drought conditions, plants have to regulate the transport of molecules, such as water, ions or proteins, in order to avoid dehydration in cells. For example, Seki *et al.* (2002) have reported the up regulation of many transporters by drought, cold and high-salinity (ERD6, ABA, oligopeptide, potassium, sodium sulphate, amino acid, mitochondrial dicarboxylate, Na^+-dependent inorganic phosphate, chloroplast proteins). In another paper, Bray (2004) compared the genes expressed under water-deficit stress in three different experiments, using filter paper, mannitol or soil water deficit. Two genes for transporters were expressed for the three experiments: mitochondrial substrate carrier proteins and amino acid/polyamine transporters.

Water channel proteins are very important in drought stress because they are involved in the control of the water status in cells (Assmann & Haubrick, 1996; Bray, 1997). Aquaporin proteins are from a large protein family and are transmembrane channel proteins regulating water flux (Caramelo & Iusem, 2009). They play a major role in the permeability of the membrane (Maurel & Chrispeels, 2001; Bartels & Sunkar, 2005). Depending on the gene controlling the different aquaporins, these proteins can be up regulated or down regulated by drought stress. Indeed, when their gene expression is up regulated, aquaporins can control the water movement through membranes (Sarda *et al.*, 1999; Bartels & Sunkar, 2005), while when it is down regulated the water is preserved in the cells so that water loss is limited (Johansson *et al.*, 1998; Bartels & Sunkar, 2005). The gene *RD28* is an example described in literature as being up regulated by drought

and forms plasma membrane water channel proteins (Seki *et al.*, 2002). *RD28* is involved in the supply of water to cells from xylem (Chrispeels & Maurel, 1994).

1.3.4.2. Osmoprotectants

Under drought conditions, plants accumulate in their cells many different solutes. These solutes are small and non toxic, they do not usually affect the metabolism of cells under normal conditions (Mahajan & Tuteja, 2005). They are located in the cytoplasm at high concentrations in cells undergoing osmotic stress (Chaves *et al.*, 2003). In *Populus*, it has been demonstrated that osmoprotectant related-genes are up regulated in response to drought (Street *et al.*, 2006). Their role can vary, depending on each one. One of the major roles of osmolytes is the osmotic adjustment (Mahajan & Tuteja, 2005), this process maybe an important part of drought tolerance (Morgan, 1984). They maintain an osmotic balance in dehydrated cells, by decreasing the osmotic potential in the cells and this process has for consequence to maintain cell turgor (Hsiao & Xu, 2000; Chaves *et al.*, 2003; Valliyodan & Nguyen, 2006). Other reports show that their role is not only on osmotic adjustment but many others, such as scavenging reactive oxygen species (ROS) (Chaves *et al.*, 2003), increasing desiccation tolerance (Pelah *et al.*, 1997), preventing detrimental changes caused by osmotic stress (Vinocur & Altman, 2005; Valliyodan & Nguyen, 2006) or protecting macromolecules and membranes (Shinozaki & Yamaguchi-Shinozaki, 1997).

Proline, aspartic acid and glutamic acid are examples of amino acids accumulated by plants in cells under drought stress (Hamilton & Heckathorn, 2001). Proline is a major amino acid when plants are dehydrated and it is the compatible solute most studied (Bray, 1997). When the level of proline is high, it plays many important roles in defence against drought stress, such as a stabilizer of subcellular structure, energy sink, scavenger of ROS, prevention of protein denaturation (Smirnoff & Cumbes, 1989; Rajendrakumar *et al.*, 1994; Verbruggen *et al.*, 1996; Hamilton & Heckathorn, 2001; Seki *et al.*, 2007). These processes are thought to be more crucial for drought tolerance than osmotic adjustment (Hare *et al.*, 1999). In a recently published study, Cocozza *et al.* (2010) compared two clones of *P. nigra* from Italy (Poli which is adapted to hot and dry environments in the South and 58–861, which prefers the cooler and moister conditions

of the North). Under severe stress conditions, the drought adapted clone showed an accumulation of proline in old leaves to preserve plants from drought damage (Cocozza *et al.*, 2010).

Sugars and sugar alcohols are non structural carbohydrates that accumulate in drought conditions. A strong correlation has been shown between their accumulation and dehydration tolerance (Ramanjulu *et al.*, 1994; Streeter *et al.*, 2001; Taji *et al.*, 2002). Simple sugars, such as glucose and fructose, play many roles: osmotic adjustment, protection of macromolecules, stabilization of membrane structure (Carpenter *et al.*, 1990), prevention of membrane fusion (Bartels & Sunkar, 2005).
Sucrose and sucrose synthetase play a role in the adaptation to drought in plants (Pelah *et al.*, 1997). The role of dehydration tolerance has been shown in Pelah *et al.* (1997) by comparing the accumulation of sucrose synthetase in two species of *Populus*. *P. popularis* is more drought tolerant than *P. tomentosa* and accumulates highly in leaves a sucrose synthase under drought (15- and 24-h water stressed), while in *P. tomentosa* stressed and unstressed leaves no sucrose synthase was detected.
Oligosaccharides such as raffinose, galactose and fructans are carbohydrates that are thought to reflect stress adaptation and can be found at high levels in cells. They have an osmoprotectant role rather than osmotic adjusment in response to dehydration (Taji *et al.*, 2002). The role of fructans, which are found in the vacuole, in water deficit tolerance is significant through osmotic adjustment and protection of membranes (Valliyodan & Nguyen, 2006). Transgenic plants introducing the synthesis of fructan showed improvement in crop productivity under drought stress (Pilon-Smits *et al.*, 1999).
Mannitol and D-ononitol are the main sugar alcohols synthesised during water-stress (Seki *et al.*, 2007). Mannitol is a photosynthetic product and functions in ROS scavenging (Shen *et al.*, 1997b; Shen *et al.*, 1997a) and also stabilizing macromolecules (Seki *et al.*, 2007). D-ononitol is accumulated during drought and plays a role in the protection of enzymes and membranes from dehydration effects (Seki *et al.*, 2007).
Upregulated genes found using microarray analysis of drought stressed *Arabidopsis* included those encoding for the synthesis of nearly all the sugars and sugar alcohols such as galactinol synthases, raffinose synthases, sucrose synthases and trehalose-6-phosphate synthase (Seki *et al.*, 2002).

Amine is another compound that plays a role in drought stress response. Glycine betaine (GB) is the main amine in water-stress and is an osmoprotectant. GB is thought to maintain water balance in cells, stabilize the structure and activity of macromolecules and protect membranes from lipid peroxidation (Sakamoto & Murata, 2002).

1.3.4.3. LEA proteins

LEA (Late-Embryogenesis-Abundant) proteins were first discovered in maturing cotton seeds (Dure & Croudh, 1981) but are thought to be present in many types of tissues, such as roots and seedlings (Hong-Bo *et al.*, 2005; Chen *et al.*, 2002b). They respond to abiotic stress, such as drought, osmotic stress and ABA (Galau *et al.*, 1986; Gómez *et al.*, 1988; Hong-Bo *et al.*, 2005). They are rich in hydrophilic amino acids, like lysine and glutamic acid, and they lack the hydrophobic amino acids, cysteine and tryptophan (Ingram & Bartels, 1996; Altman, 1999). This molecular property shows the possible function of the LEA proteins linked with water stress in cells. The roles played by these proteins are numerous but still remain uncertain and unclear (Bray, 2004). The main possible role of LEA proteins in response to drought is to protect the cells from water stress damage in several ways. They stabilize enzymes and membranes structure (Campalans *et al.*, 1999; Close, 2006; Hundertmark *et al.*, 2011) by retention of water (Wise, 2003), sequestration of ions and binding to water (Bray, 1997). They also prevent the aggregation of proteins (Goyal *et al.*, 2005). All these functions prove the role of LEA proteins in drought tolerance (Chandler & Robertson, 1994; Wise, 2003). In addition to that, many LEA genes are up regulated during drought stress, five LEA genes were up regulated in common from the review by Bray (2004), nine LEA proteins in Seki *et al.* (2002) research in abiotic stress and also genes in the *Populus* genome such as LEA4, LEA5-D, LEA14-A genes (Street *et al.*, 2006).

1.3.4.4. Detoxification enzymes

During drought stress, products considered as toxic (activated oxygen species) can be present in cells. For example superoxide radicals and H_2O_2 are accumulated due to the increase of photorespiration (Sgherri *et al.*, 1993). ROS (Reactive Oxygen Species) are dangerous for cells because they are responsible of the breakdown of membrane lipids, proteins, nucleic acids and they can damage DNA (Hajheidari *et al.*, 2005). This is why

under drought stress, genes are activated to generate antioxidants that eliminate them: glutathione S-transferase, soluble epoxide hydrolase, catalase, superoxide dismutase and ascorbate peroxidase (Shinozaki & Yamaguchi-Shinozaki, 1997). Detoxification enzymes are up regulated during drought, such as glutathione S-transferases and peroxidises (Seki *et al.*, 2002). In peas, the transcript level of cytosolic isozymes of ascorbate peroxidises and Cu/Zn-superoxide dismutases increased during drought stress as well as during the recovery from drought (Mittler & Zilinskas, 1994).

1.3.4.5. Proteins involved in repair and degradation

Under drought stress, proteins in cells undergo damage such as the disruption of their native conformation and have difficulties to maintain their activity. In order to survive cells need to activate processes of proteins repair and avoid non-native proteins aggregation (Wang *et al.*, 2004). This involves the formation of small heat-shock proteins (e.g. chaperones), ubiquinin proteins or L-isoaspartyl methyltransferases. When the damage is irreversible, then the degradation of the cell would take place (Campalans *et al.*, 1999; Bartels & Sunkar, 2005).

L-isoaspartyl methyltransferases are enzymes involved in protein repair, damaged during drought stress (Ingram & Bartels, 1996; Ramanjulu & Bartels, 2002). In seedlings of wheat, drought and ABA induced the transcription and the enzymatic activity of L-isoaspartyl methyltransferases (Mudgett & Clarke, 1994)

During dehydration, many heat-shock-like proteins are found, including chaperones and small heat shock proteins (sHSPs). Their abundance under drought stress might prove their role in water stress tolerance as drought sensitive-mutant seeds had their HSP expression reduced compared to wild type (Wehmeyer & Vierling, 2000). The role of chaperones is diverse in repairing mechanisms but the major functions are to prevent protein aggregation, to assist refolding (Wang *et al.*, 2004; Arslan *et al.*, 2006), to help denaturated proteins to recover their native structure (Campalans *et al.*, 1999), to facilitate protein translocation, to target damaged proteins to be degraded, as well as sequestering damaged proteins to aggregate (Arslan *et al.*, 2006), all in order to re-establish cellular homeostasis (Wang *et al.*, 2004). Small heat shock proteins are

induced by water stress, found in many vegetative tissues and they play a role in drought tolerance (Hajheidari *et al.*, 2005).
Numerous papers show the up regulation of HSPs and chaperones. For example, in poplar species, chaperones and HSPs have been found induced after water deficit and involved in protein repair (Street *et al.*, 2006; Bogeat-Triboulot *et al.*, 2007). Hajheidari *et al.* (2005) discovered that in sugar beet leaves two small heat shock proteins were activated following a progressive water deficit.

It has been noticed that the mRNA contents encoding the protein ubiquinin increase after drought stress in *Arabidopsis* (Kiyosue *et al.*, 1994c). Its role is to tag damaged proteins for destruction (Ingram & Bartels, 1996).

Finally, proteases are enzymes that are responsible for proteolysis of damaged proteins in response to water stress, which corresponds to the degradation of denaturated polypeptides (Campalans *et al.*, 1999). They can either destroy damaged proteins or mobilize nitrogen (Vierstra, 1996). They have been found induced in different plants, like pea (Guerrero *et al.*, 1990), *Arabidopsis* (Koizumi *et al.*, 1993; Kiyosue *et al.*, 1994a), bean and cowpea (de Carvalho *et al.*, 2001).

1.3.4.6. Cell wall alterations

Drought stress is responsible for altering the chemical composition and physical properties in the cell wall, such as wall extensibility (Campalans *et al.*, 1999). This can result in stopping cell expansion (Nonami & Boyer, 1990) and later in the reduction of leaf area (Bray, 2004). Many genes in order to answer the need of low extensibility are expressed during water deficit. S-adenosylmethionine synthetases are encoded (Ingram & Bartels, 1996) and proline-rich-proteins are accumulated (Colmenero-Flores *et al.*, 1997). Wall extensibility is controlled by the structure of the cell wall and the activity of cell-wall-modifying proteins, including xyloglucan endotransglycolsylase (XTH), expansins and glucanases. The role of XTHs is in the cleavage and reformation of bonds between xyloglucan chains (Campos *et al.*, 2004). In *Arabidopsis,* Bray (2004) reviewed three experiments on gene expression under drought, one XTH was common to the three studies and was down regulated for all. This would result in the loss of cell wall extensibility and a decrease of cell expansion. They also found two germin-like

genes (GLG) down regulated under dehydration. The functions of these genes are not clear but they are probably involved in cell wall extensibility. The expansion of the cell wall is also controlled by the genes involved in the wall synthesis. If these genes are down regulated during drought then the expansion of the cell wall will decrease and eventually stop (Bray, 2004). Expansins are a family of extracellular proteins that play a role in cell and tissue growth. In a study conducted by Pien *et al.* (2001), expansin expression was induced in meristem and had for consequence changes in leaf development and modification of leaf shape by increasing lamina formation.

1.4. Acclimation and physiological mechanisms in response to drought

Plants can be subjected to water stress in two ways: a slow water shortage or a short water deficit. Physiologically, the responses to these stresses can then be observed at two levels depending on the intensity of drought: short-term and long-term responses (Fig. 1.4). In term of short-term responses, plants react quickly by minimising water loss (for example stomatal closure) or protect themselves against damages (osmotic adjustment). Long-term responses are considered as acclimatory responses including shoot growth inhibition, reduced leaf area and increased root growth (Chaves *et al.*, 2003). In this sub-chapter, both responses will be reviewed focusing on above-ground responses.

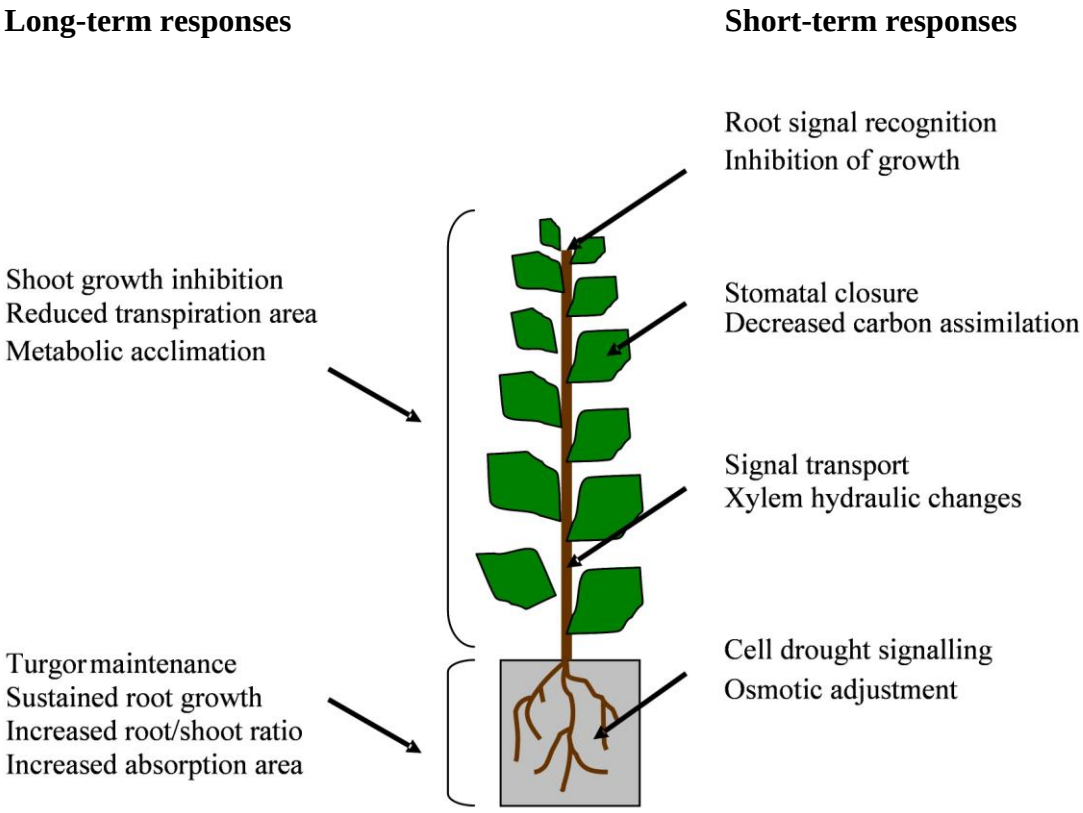


Figure 1.4: Whole-plant responses to drought: long-term (left) and short-term (right) responses, adapted from Chaves *et al.* (2003)

1.4.1 Modification in plant growth

As shown previously, cell wall extensibility is modified under drought and accompanied by other physiological changes, which can result in the inhibition of cell enlargement, reduced production of cells and thus in leaf growth inhibition (Lu & Neumann, 1998; Bray, 2004). The inhibition of cell expansion is due to several factors, such as changes in water potential gradients and metabolic changes (Lu & Neumann, 1998). The relation between cell expansion and leaf growth has already been shown and reduced cell expansion can explain inhibition of leaf growth in dehydrated plants (Lu & Neumann, 1998). Indeed, under drought, leaves reduce or eventually stop growing, resulting in small leaf size compared with well-watered plants (Lu & Neumann, 1998; Chaves *et al.*, 2003). Small leaf size is considered to be an adaptive mechanism to drought. Indeed Radin *et al.* (1994) observed a negative correlation between leaf size and water potential (Ψ_W) suggesting small leaves would present higher Ψ_W. This phenomenon improves photosynthetic rate and consequently heat or drought resistance (Radin et al., 1994; Levi et al., 2009; Ashraf, 2010).

ABA plays a major role in drought, particularly in signalling. Yin *et al.* (2004) demonstrated a role of ABA in growth by observing changes in many growth parameters after exogenous ABA application to leaves (decreased height growth, total biomass, total leaf area, specific leaf area).

1.4.2 Stomata closure

One of the first responses, including leaf growth inhibition, to drought as a whole plant is stomatal closure (Chaves *et al.*, 2003). Under dehydration, through aerial pores, leaves lose water (transpiration) more than they gain water. As a consequence, stomata are closed in order to reduce this loss. Stomata closure is controlled in two different manners: hydroactive closure and hydropassive closure (Mahajan & Tuteja, 2005). The latter does not require metabolic production but guard cells sense water loss and respond directly, while hydroactive closure results in changes in turgor of guards cells accompanied with metabolic formation and ABA signal (Chaves *et al.*, 2003; Bartels & Sunkar, 2005; Mahajan & Tuteja, 2005). Hydroactive closure is the most studied process. With roots sensing soil drying, ABA is produced and chemical signals such as ABA are transported to the shoots and leaves (Stoll *et al.*, 2000). ABA promotes efflux of K^+ and Cl^-, sucrose is removed and malate is converted into osmotically inactive starch (Talbott & Zeiger, 1996; MacRobbie, 2000). All these mechanisms cause a decrease of turgor pressure in guard cells which respond by stomatal closure (Schroeder & Hedrich, 1989; Schroeder *et al.*, 2001).

1.4.3 Photosynthetic inhibition

With stomatal closure preventing water loss, CO_2 uptake is then reduced during water stress which results in lower CO_2 availability. In consequence of this process, the photosynthetic rate declines (Lawlor, 2002; Flexas *et al.*, 2004) and photosynthesis is strongly reduced (Bogeat-Triboulot *et al.*, 2007; Galmes *et al.*, 2007). Photosynthesis can also be reduced directly, through limitation of ribulose bisphosphate synthesis and inhibition of ATP synthesis (Lawlor, 2002). Molecularly, photosynthesis decline can be also observed with the down regulation of many genes in plants undergoing drought (Street *et al.*, 2006).

Plants tolerating drought develop mechanisms to protect photosynthetic structures (Ramanjulu & Bartels, 2002). For example, the desiccation tolerant plant, *Craterostigma plantagineum* up regulates three genes under drought that encode for chloroplast-localized stress proteins (DSP) (Schneider *et al.*, 1993) which role is important in protecting the chloroplast against stress damage (Lee *et al.*, 2000).

1.4.4 Water use efficiency and carbon isotope discrimination

Water use efficiency can be defined in three ways, as a whole plant (WUE_p) or at the leaf level (WUE_t or WUE_g) (Ponton *et al.*, 2001). WUE_p is the ratio between biomass production and water consumption. Instantaneous water use efficiency WUE_t is the ratio between the net carbon assimilation and the water loss while intrinsic water use efficiency WUE_g is the ratio between the net carbon assimilation and stomatal conductance (Ponton *et al.*, 2001; Masle *et al.*, 2005; Monclus *et al.*, 2005; Seibt *et al.*, 2008). Under drought, stomata closure results in a decline of CO_2 uptake, thus plants that develop good water use efficiency are likely to be advantaged (Ponton *et al.*, 2001). WUE is difficult to measure but it can be indirectly calculated with carbon isotope discrimination ($\Delta^{13}C$) because WUE and $\Delta^{13}C$ are linear and negatively correlated (Farquhar & Richard, 1984; Farquhar *et al.*, 1989; Yin *et al.*, 2004; Monclus *et al.*, 2005). During the fixation of carbon by photosynthesis, plants discriminate ^{13}C for ^{12}C as the ratio of ^{13}C to ^{12}C in plant tissues is lower than the ratio of ^{13}C to ^{12}C of the atmosphere. When stomata stay open, $\Delta^{13}C$ is higher as the plant uses more ^{12}C than ^{13}C and WUE is lower as water loss increases (Fig. 1.5). On the other hand, if stomata are closed, water loss is decreased thus WUE is high and $\Delta^{13}C$ is low (Fig. 1.5). $\Delta^{13}C$ can be measured in many parts of a plant (e.g. roots, leaf, wood…) and corresponds to a life time or seasonal measurement for the plants. It is calculated from the carbon isotope composition ($\delta^{13}C$) in ^{13}C and ^{12}C of a sample:

$$\delta^{13}C\ (‰) = [(R_{sample} / R_{reference}) / R_{reference}] \times 1000$$

where R_{sample} and $R_{reference}$ are the $^{13}C/^{12}C$ ratios of the sample and the reference respectively (Farquhar *et al.*, 1989). Carbon isotope discrimination is then calculated from this formula:

$$\Delta^{13}C = (\delta_a - \delta_p)/[1 + (\delta_p/1000)]$$

where δ_a and δ_p are a refers to air and p to plant, δ_a is approximately -8‰ used in Monclus *et al.* (2005) from Farquhar & Richard (1984).

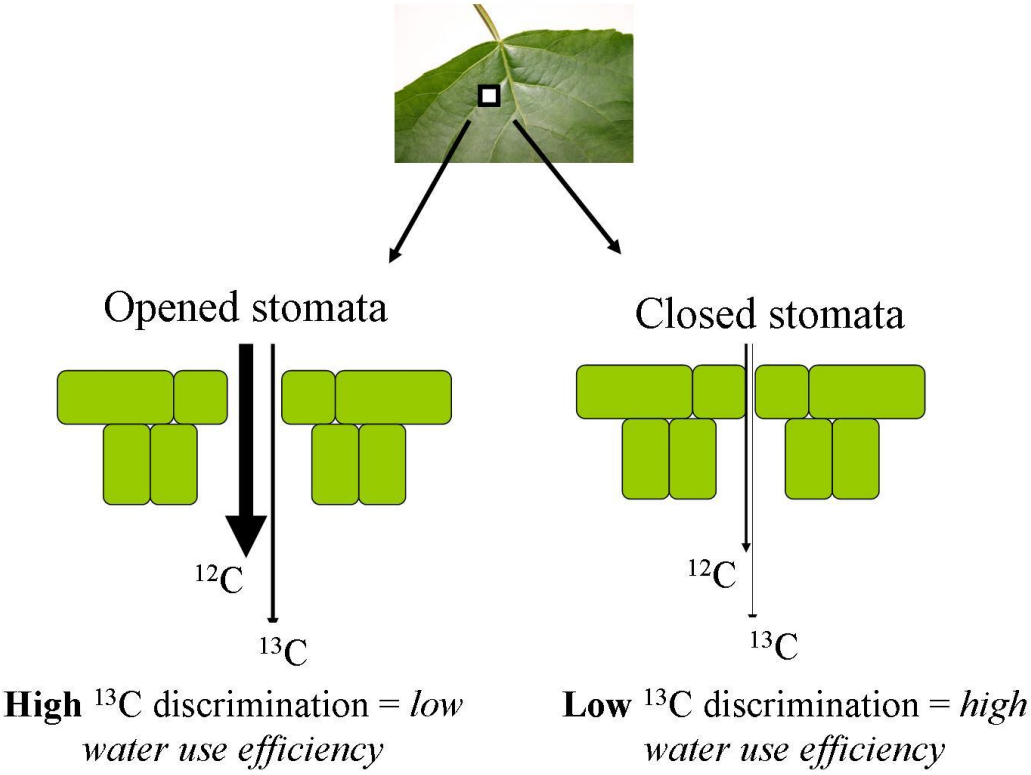


Figure 1.5: Simple schematic diagram representing the correlation between water use efficiency (WUE) and carbon isotope discrimination ($\Delta^{13}C$)

$\Delta^{13}C$ is a reliable and sensitive marker that can be used to detect plants with good transpiration efficiency (Farquhar & Richard, 1984; Masle *et al.*, 2005). In *Arabidopsis,* a gene called *ERECTA* has been discovered to be involved in plant transpiration efficiency. The *ERECTA* gene is a putative leucine rich repeat receptor-like kinase (Masle *et al.*, 2005) and thus plants activating this gene would have improved their transpiration efficiency. ERECTA can modify transpiration efficiency in different ways: epidermal and mesophyll development, stomatal density and leaf porosity (Masle *et al.*, 2005). Transgenic plants also experienced a decrease in carbon isotope discrimination with minimal variations in plant growth, implying the possibilities of manipulation of this gene for breeding programs (Masle *et al.*, 2005). Breeding selection of genotypes with high water use efficiency is useful for maintenance of growth under drought (Cregg & Zhang, 2000). In *Eucalyptus globulus*, a strong correlation (r^2=0.95, $p<0.02$) between survival and carbon isotope discrimination was observed (Pita *et al.*, 2001).

Carbon isotope discrimination has been measured previously in different species,

including studies done on trees. For example, in beech trees, Peuke *et al.* (2006) noticed an increase in $\delta^{13}C$ values under drought. $\Delta^{13}C$ was also measured in *Populus deltoides* × *P. nigra* clones by Monclus et al. (2005, 2006) and it was noticed that this trait is highly heritable (H^2=0.71). These clones were also subjected to a moderate drought treatment and for the drought tolerant individuals carbon isotope discrimination decreased while the ones that had their value increased were all intolerant to drought (Monclus *et al.*, 2006). Carbon isotope discrimination was also studied in four Euramerican hybrid poplars and had their values decreased under low irrigation (Voltas *et al.*, 2006). Leaf $\delta^{13}C$ was also measured recently in a F_1 family of oak (*Quercus robur*) and the genetic variation of this trait within the family was used to map ten quantitative trait loci (Brendel *et al.*, 2008).

1.4.5 Waxy or reflective cuticle

Plant cells synthesise wax and cuticle in aerial organs, particularly in leaves. This process can increase in periods of drought or application of exogenous ABA and reduces water loss during transpiration by impermealizing the epidermis of leaf surface (Bohnert, 2000; Macková *et al.*, 2010). Plants tend to adapt to dehydration with this mechanism (Ramanjulu & Bartels, 2002) and it proves the biochemical adaptation in the level of the leaf structure (Street *et al.*, 2006). Indeed under drought, genes in wax and cuticle biosynthesis are up regulated (Street *et al.*, 2006). For example, lipid transfer proteins are produced in response to drought. They are involved in cuticle biosynthesis and are synthesised mostly in aerial organs (Treviño & O'Connell, 1998).

1.4.6 Xylem cavitation

Xylem cavitation is the block of xylem vessels by air bubbles or embolisms (Harvey & van den Driessche, 1997). Xylem conduits then become non-functional (Willson & Jackson, 2006). This can result in hydraulic conductivity reduction, decrease in productivity and growth and thus limit survival (Tyree *et al.*, 1992; Willson & Jackson, 2006). Poplars are very vulnerable to xylem cavitation (Hukin *et al.*, 2005). In consequence, trees showing no or little xylem cavitation would have an advantage.

A study done on hybrid poplar, with two drought resistant (*P. deltoides*) and two drought-sensitive (*P. trichocarpa*), found that the drought-sensitive poplars were more prone to xylem cavitation. The drought-resistance clones were less vulnerable to membrane damage than the drought-sensitive clones and had increased pit membrane strength (Harvey & van den Driessche, 1997). Another study on *P. trichocarpa* showed that vulnerable clones to xylem cavitation with limited stomatal control were more sensitive to drought treatment and were dehydrated more rapidly than the resistant clones to xylem cavitation (Sparks & Black, 1999).

1.4.7. Root development

Water, like inorganic nutrients, is mainly absorbed by roots. During water deficit, root development is modified and assimilates are distributed to the roots (Wilson, 1988) which is believed to improve tree water balance and survival (Tschaplinski *et al.,* 1994; Marron *et al.*, 2003). Root to shoot ratio is controlled by the balance between water uptake from the roots and photosynthesis by the shoots. When water is in short supply root:shoot ratio increases (Wilson, 1988). Elongation of root system could increase the capacity to withdraw water from deeper soil.

Root development and carbon allocation to the roots under water stress has been widely studied in *Populus.* In cuttings of Balsam Spire poplar, dry matter allocation to roots increased in response to drought (Ibrahim *et al.*, 1997). Genotypes of a population of *P. davidiana* originally from drier environments showed a higher root:shoot ratio in water limited treatments (Zhang *et al.*, 2004). Root density and root density to stem volume was also studied in two poplar clones by Tschaplinski *et al.* (1997) and showed an increase under moderate water stress in the clone showing better resistance to drought.

1.5. Resistance to drought

Three main strategies exist in resistance to drought for plants: escape, avoidance and tolerance (Levitt, 1972; Chaves *et al.*, 2003). Plants might combine the different strategies in response to water stress.

Escaping drought would require the plants to rapidly complete their life cycle before the effects of water stress (Chaves *et al.*, 2003). This strategy is developed by annual plants in arid regions. In the *Arabidopsis* ecotype Landsberg *erecta* (L*er*), Meyre *et al.* (2001) observed an escape strategy during a progressive drought stress with early flowering. Another annual plant (*Brassica rapa*) showed drought escape by flowering early (Franks, 2011). *Populus* being a perennial plant, drought escape is not a relevant strategy for this species.

During drought, another strategy is to avoid tissue dehydration by keeping high tissue water potential (Chaves *et al.*, 2003). Avoidance can be made with a reduction of water loss by stomatal closure, leaf rolling which reduces light absorbance, reduction of leaf area and older leaf senescence (Chaves *et al.*, 2003). *Populus deltoides* exhibited typical drought avoidance in response to soil drying with a rapid stomatal closure, reduction of leaf area and leaf senescence (Street, 2005). Another strategy in drought avoidance is to increase water uptake with a deep rooting system (Levitt, 1972; Jackson *et al.*, 2000) and an efficient system in water transfer from roots to leaves combined with a lack of xylem cavitation (Dreyer *et al.*, 2004). Moderate drought tolerant potato clones showed an increase in root growth under drought (Schafleitner *et al.*, 2007) in order to obtain moisture available in deeper soil layers (Tuberosa & Salvi, 2006). *Populus euphratica* is common in arid regions and survive in deserts with deep roots constantly accessing water stored underground (Dreyer *et al.*, 2004).

Tolerant plants maintain high biomass under drought by osmotic adjustment (Wilson *et al.*, 1980; Morgan, 1984), by developing rigid cell walls or smaller cells and/or by entering a dormant phase during drier seasons (Chaves *et al.*, 2003). High water use efficiency (WUE) is also a strategy allowing drought tolerance by maintaining biomass with less water (Dreyer *et al.*, 2004). Recent successes with wheat adapted for arid environments in Australia, showed the use of WUE as a target trait for breeding for

future drier climates, with the release of new cultivars (Condon *et al.*, 2004; Richards, 2006; Hochman *et al.*, 2009). In *Arabidopsis,* Columbia ecotype showed a drought tolerance with high biomass allocation to vegetative organs, high RWC (Relative Water Content) and high WUE (Meyre *et al.*, 2001).

1.6. Genetic technology for the adaptation to drought stress

1.6.1. Genetic markers

Markers are widely used in genetic studies for various reasons such as creating a genetic linkage map (Chang *et al.*, 1988; Bradshaw *et al.*, 1994; Wullschleger *et al.*, 2005; Gaudet *et al.*, 2008), finding genetic variation between individuals and populations (Gonzalez-Martinez *et al.*, 2006; Smulders *et al.*, 2008), studying association genetics (Neale & Savolainen, 2004; Gonzalez-Martinez *et al.*, 2007), population history and migration (Brumfield *et al.*, 2003; Keller *et al.*, 2010).

Genetic markers are numerous, for example AFLP (amplified fragment length polymorphism), RAPD (random amplification of polymorphism DNA), SSR (Simple Sequence Repeats), SNP (single nucleotide polymorphism) and RFLP (restriction fragment length polymorphism). Each has advantages and disadvantages. They are used to reveal polymorphism at the DNA level (Vignal *et al.*, 2002).
RFLP are bi-allelic co-dominant markers (Vignal *et al.*, 2002) which means both alleles are studied in an individual (Liu & Cordes, 2004) and uses restriction enzymes digesting DNA (Kumar, 1999). RAPD and AFLP are bi-allelic dominant markers (Vignal *et al.*, 2002). RAPD using primers of 8-10 bp amplify random sequences of DNA (Liu & Cordes, 2004). AFLP combines RFLP and RAPD methods by digestion of the whole DNA with restriction enzymes and ligation of the fragments and amplification by PCR using primers (Liu & Cordes, 2004). AFLP can reveal a high level of polymorphism at a low cost and it is also reproducible because the annealing temperatures are high (Liu & Cordes, 2004). Microsatellites (SSR) are multi-allelic co-dominant makers (Vignal *et al.*, 2002). SSR are multiple repetitions of sequence of 1-6 bp and amplified at a specified locus using primers (Liu & Cordes, 2004).
Alternatively, a SNP is a single nucleotide polymorphism and corresponds to a single base change in DNA (A, T, C or G). Two main types of mutation creating a SNP are transitions (A ↔ G or C ↔ T) or transversions (A ↔ C, A ↔ T, G ↔ C, G ↔ T) (Vignal *et al.*, 2002). The frequency of SNPs within the genome is high and they represent an important type of variation (Brookes, 1999). They can be identified by comparing regions of the genomes between individuals (Brookes, 1999). Recently high-thoughput resequencing tools such as 454 and Illumina enable to discover SNP rapidly

in various plants (Barbazuk *et al.*, 2007; Lijavetzky *et al.*, 2007; Pavy *et al.*, 2008; Bundock *et al.*, 2009).

A linkage map has also been built in *Populus nigra* using various genetic markers (Gaudet *et al.*, 2008). From a mapping population containing 165 individuals, they found 40 AFLP primer combinations, 130 SSR segregating markers, 7 SNPs and a locus for the sex trait. This map shows the possibility to use markers on *P. nigra* to obtain genetic information on this species. Smulders *et al.* (2008) studied the genetic diversity of a population of European black poplars using AFLPs and microsatellites. Genetic diversity was also studied in a population of european *P. nigra* from nine different countries using AFLP and SSR markers (Storme *et al.*, 2004).
A genetic linkage map of the mapping pedigree Family 331 was produced by G.A. Tuskan *et al.* (personal communication), and consisted of 91 SSR markers genotyped on 350 of the full-sib progeny, and 92 fully informative amplified fragment length polymorphisms (AFLP) genotyped on 165 genotypes of the progeny. This map has been widely used to discover QTL on adaptive traits such as leaf development (Wu *et al.*, 1997; Rae *et al.*, 2006; Street *et al.*, 2006; Street *et al.*, 2010) and biomass (Bradshaw & Stettler, 1995; Rae *et al.*, 2008; Rae *et al.*, 2009). QTL related to drought or under a water stress were also mapped for this pedigree (Street *et al.*, 2006; Tschaplinski *et al.*, 2006). For this thesis, drought adaptive traits were studied using the genetic linkage map provided by G.A. Tuskan: carbon isotope composition and stomatal conductance.

1.6.2. QTL

Genetic markers in a mapping population enable the creation of map which can be used for the discovery of Quantitative trait loci. Also called QTL, they have been widely used for several decades and are regions of the genome linked to phenotypic traits which are controlled by several genes (Abdurakhmonov & Abdukarimov, 2008). The effect of these complex traits depends on the interaction between the genes and the environment (Abdurakhmonov & Abdukarimov, 2008). QTLs are discovered using a mapping population (Price, 2006). A phenotypic trait is measured on a high number of individuals from a mapping population which has been genotyped with genetic markers, then by performing statistical analysis the loci that control the trait are discovered (Asíns, 2002; Ashraf, 2010). When QTLs are revealed, they can be linked to the sequence map using the genetic markers and used to find candidate genes within the loci of interest. This method is utilised to discover genes responsible for the variation in the phenotypic traits (Hansen *et al.*, 2008). For example, in *Populus*, candidate genes were listed within QTL related to cell wall traits (Ranjan *et al.*, 2010). A single gene can sometimes be related to a QTL such as in tomato an invertase gene within a QTL hotspot explained variation for sugar content (Fridman *et al.*, 2000). Again in tomato, a single gene, *OVATE*, is associated with fruit shape discovered within QTL (Liu *et al.*, 2002). In rice, a QTL for salt tolerance revealed a gene *SKC1* involved in sodium transport and K^+/Na^+ homeostasis under salt stress (Ren *et al.*, 2005).

In drought stress, various crops have been used to measure QTL related to water tolerance (see review: Tuberosa & Salvi, (2006)) and improved yield under stress: rice (Lilley *et al.*, 1996; Courtois *et al.*, 2000; Zhang *et al.*, 2001; Ren *et al.*, 2005; Yue *et al.*, 2008), *Arabidopsis thaliana* (Masle *et al.*, 2005), maize (Tuberosa *et al.*, 2002), soybean (Tuberosa *et al.*, 2002), wheat (Quarrie *et al.*, 2005), sunflower (Poormohammad Kiani *et al.*, 2008), tomato (Martin *et al.*, 1989), *Stylosanthes scabra* (Thumma *et al.*, 2000), but also in trees such as a salix hybrid (Rönnberg-Wästljung *et al.*, 2005) and poplar (Street *et al.*, 2006; Tschaplinski *et al.*, 2006).

Candidate genes from QTL studies can be confirmed by using transgenic methods and mutants by reintroducing an alternate allele or by gene replacement (Borevitz & Chory, 2004). Using transgenics, genotypes can thus be improved for drought tolerance (Vinocur & Altman, 2005; Tuberosa & Salvi, 2006).

1.6.3. Microarrays

In the past, to identify the genes involved in a phenotypic trait, the genes needed to be studied one after the other. Knowing that a complex trait, such as drought tolerance, can be multigenic (Polle *et al.*, 2006), the task was then difficult and time-consuming. However, the advances in microarray technology permit now to study thousands of genes at once (Jansen & Nap, 2001).

Microarrays techniques used to require RNA from treatment and control samples to observe differences in gene expression (e.g. drought vs. well-watered plants, healthy vs. infected cells). Using Affymetrix GeneChips, gene expression is done per sample with one colour (phycorerythrin, PE, red) and not paired (Staal *et al.*, 2003). Transcripts are isolated as mRNA, reverse transcripted into cDNA. It is labeled using biotin into labeled cRNA which is then fragmented (Fig. 1.6). A microarray contains a high number of DNA fragments which are each placed in spots or probes. The cRNA are then hybridised with these DNA fragments on the array and the labelled probe is excited by light (Kehoe *et al.*, 1999). After washing and staining, a laser scan is used to analyse the chips and gene expression is quantified for each probe (Staal *et al.*, 2003).
A GeneChip array is available specifically for *Populus* species with Affymetrix (Santa Clara, USA) and contains over 61000 probes which represent more than 56000 transcripts and gene predictions (http://media.affymetrix.com/support/technical/datasheets/poplar_datasheet.pdf). It is a single-channel oligonucleotide microarray.

Using this tool, thousands of genes are studied at the same time and microarrays can thus be used relatively quickly to find interesting candidate genes without a priori knowledge of which genes may be affected (Umezawa *et al.*, 2006). Recently several papers were published using the GeneChip Poplar Genome Array (Affymetrix, Santa Clara, USA), studying different stresses, such as pathogen infections (Azalez *et al.*, 2009), root hypoxia (Kreuzwieser *et al.*, 2009) and salt stress (Janz *et al.*, 2010).

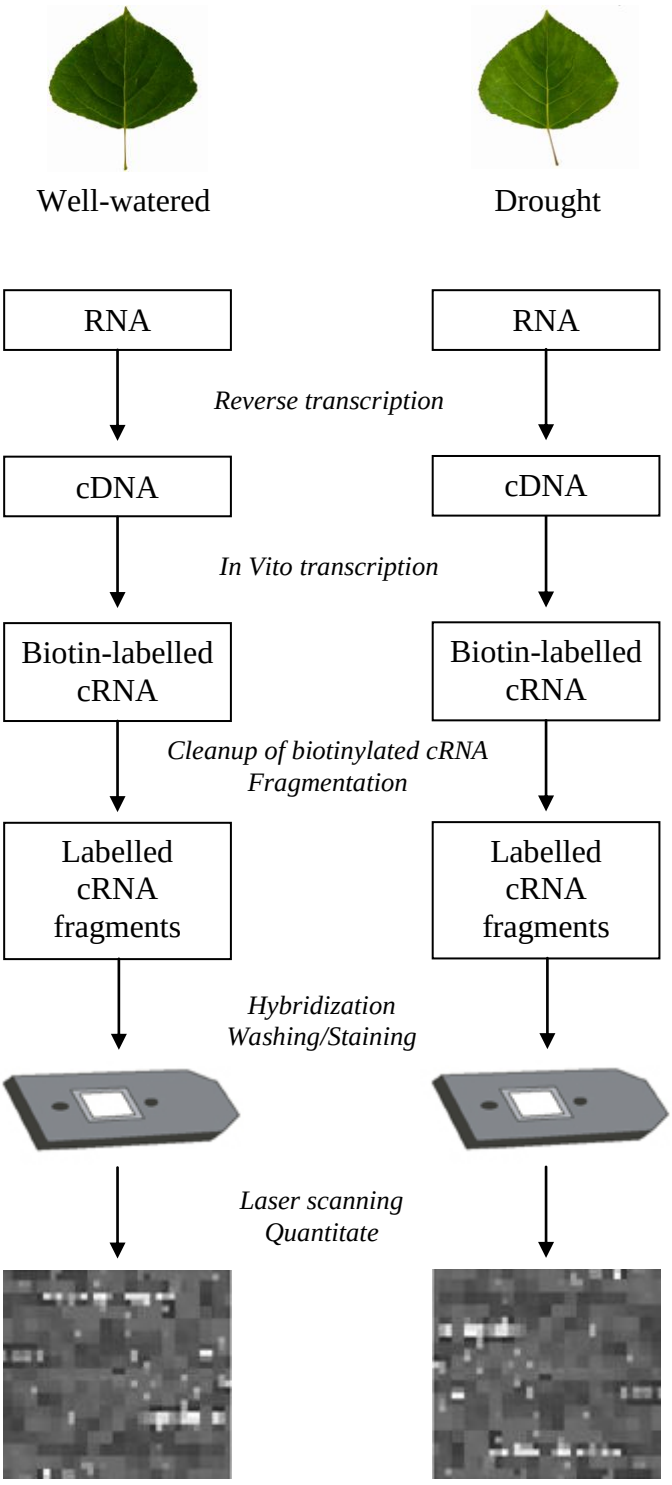


Figure 1.6: Affymetrix GeneChip® labeling assays for expression analysis, modified from (Affymetrix, 2009)

1.7. Aims of the project

The aim of this study is to understand the genetics, genomics and physiology of drought tolerance in *Populus*. Two types of population were used to study natural variation in response to water deficit in order to unravel the genetic basis of drought adaptation in this genus (Fig. 1.7). A F_2 mapping population, created from *P. deltoides* and *P. trichocarpa* originally from contrasting environments in the United States of America, was used for QTL analysis. Natural variation in leaf size and carbon isotope discrimination was studied in a population of *P. nigra* collected from five European countries, from Spain to Germany, reflecting a wide range of rainfall and temperatures. Extreme genotypes were selected to study their physiological and genetic responses to water deficit.
Carbon isotope discrimination and leaf size changes were the traits which were focused on because of their relation to drought tolerance.
Direct comparisons between the transcriptome of extreme genotypes in well watered and drought conditions provided insight into the genomic pathways induced during water deficit.

Although using similar techniques, the aim of these projects was different depending on which population was used for the study of responses to water deficit. The aim using a mapping population was to develop a better crop able to maintain growth under water shortage and to exploit the results of the study for breeding purposes. *P. nigra* was used to understand natural variation in an association population in order to conserve and manage a species in threat of extinction in future climates.

The main objectives of this PhD were to:

- Unravel the genetic basis of Water Use Efficiency in *Populus* using a F_2 mapping population (Family 331), identify QTL in stomatal conductance and carbon isotope composition, and define a list of candidate genes for Water Use Efficiency combining QTL and microarray analysis.
- Investigate natural variation in leaf growth and carbon isotope discrimination in a natural population of black poplar (*P. nigra* L.) under well-watered conditions.

- Study morphological and physiological variation in response to water deficit between genotypes of *P. nigra* showing extreme differences in leaf size and carbon isotope discrimination.
- Define a list of candidate genes related to drought adaptation by performing transcriptomic analysis using microarrays and real-time qPCR on extreme genotypes of *P. nigra* in response to drought and under well-watered conditions.

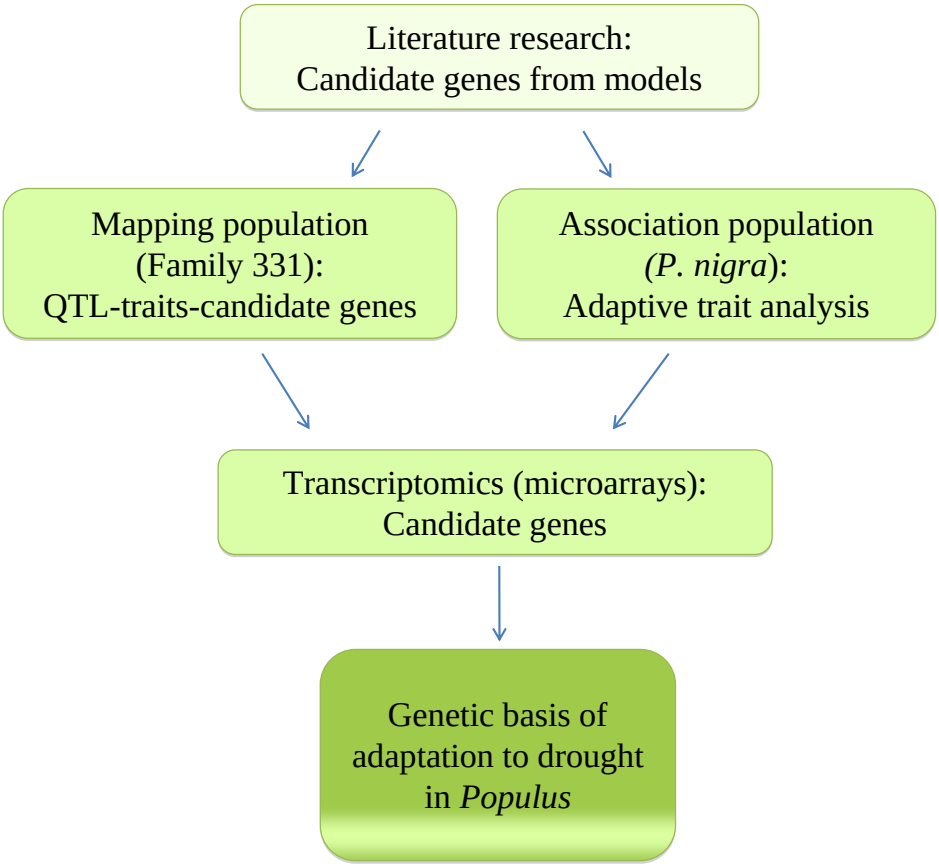


Figure 1.7: Approach used in this study to unravel the genetic basis of adaptation to drought in *Populus*

Chapter 2: Material and Methods

2.1. Poplar populations

Two types of population of *Populus* were used for this PhD: a mapping population and an association population. Each was utilised for diverse measurements and assessments (biomass, $\delta^{13}C$, g_s, QTL, microarrays, qPCR).

2.1.1. Mapping population, Family 331

The mapping population, Family 331 is a hybrid population originating from different species, *Populus trichocarpa* (93-968) which is native from a humid environment in the North West of America, and *Populus deltoides* (ILL-129) from a drier area in the North East of America (Fig. 2.1).

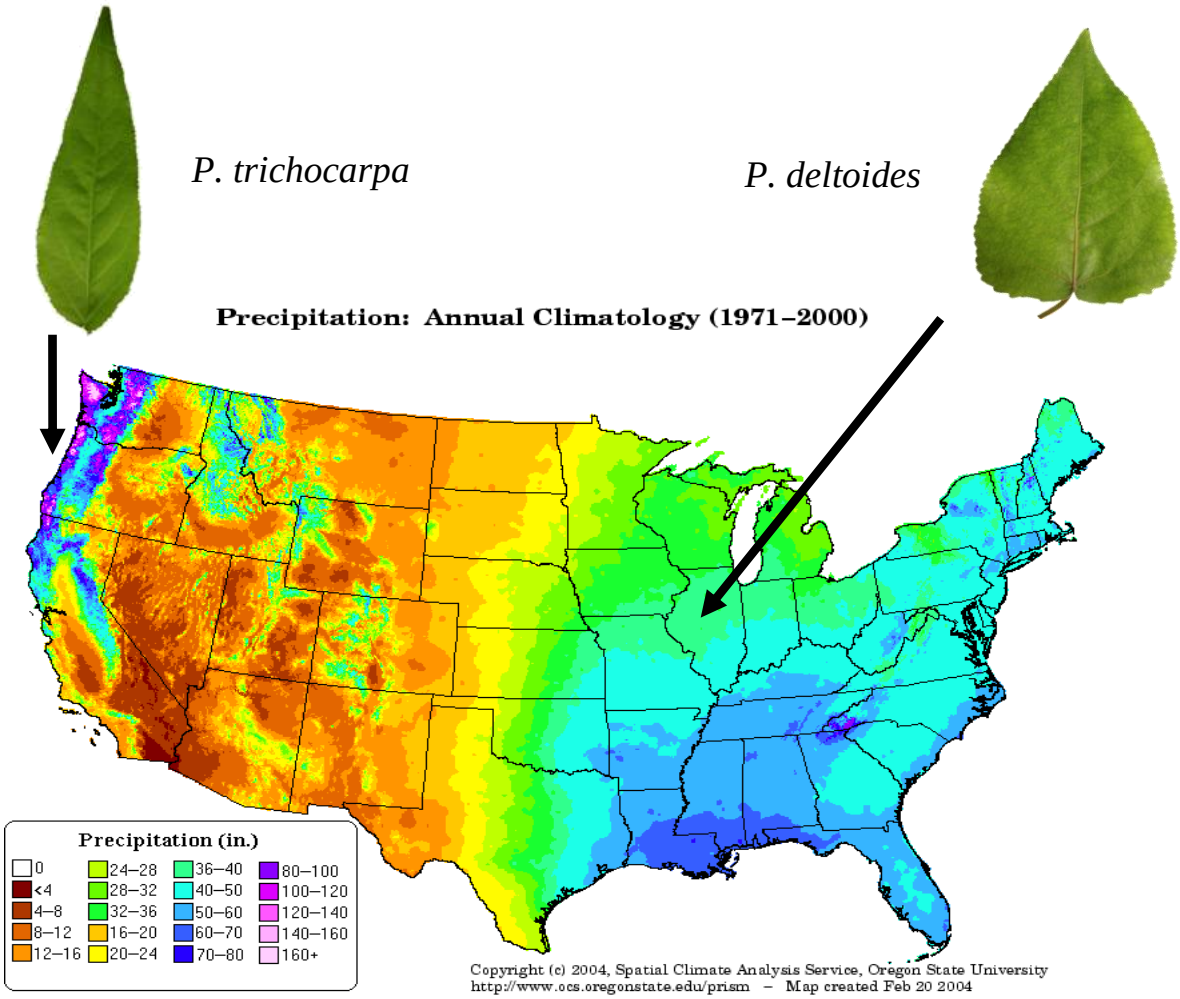


Figure 2.1: Map of the original location of *P. deltoides* and *P. trichocarpa*, with a representative leaf.

A male *P. deltoides* (ILL-129) and a female *P. trichocarpa* (93-968) were crossed resulting in a F_1 family called Family 53 (Bradshaw & Stettler, 1993) and two siblings (53-242 and 53-246) were crossed to obtain an inbred F_2 population, Family 331 (Wu *et al.*, 1997; Rae *et al.*, 2009).

This population has previously been used by other members of the laboratory to determine QTL (Table 2.1). The various QTL were then used and they helped to define regions in the genome in order to find candidate genes related to drought tolerance.

Table 2.1: Review of previous studies of QTL using Family 331

QTL traits	Conditions	References
Growth, form and phenology	-	(Bradshaw & Stettler, 1995)
Leaf traits	-	(Wu *et al.*, 1997)
Leaf growth and delayed senescence	Elevated CO_2	(Rae *et al.*, 2006)
Cell and stomatal behaviour	Drought	(Rodriguez-Acosta, 2006)
Leaf traits	Drought	(Street *et al.*, 2006)
Osmotic potential	Wet and dry sites	(Tschaplinski *et al.*, 2006)
Biomass	Sites in the UK, France and Italy	(Rae *et al.*, 2008)
Biomass	Short Rotation Coppice	(Rae *et al.*, 2009)
Leaf traits	Ozone	(Street *et al.*, 2010)

2.1.2. Association population of Populus nigra

The second population is an association population of 500 genotypes of black poplar (*Populus nigra* L.), collected in five European countries (Spain, France, Italy, Germany and The Netherlands). Genotypes were grouped in 11 populations depending on their location and river systems (Fig. 2.2). They are wild trees originating from various environments representing a range of temperature and precipitation. The Spanish and South French genotypes are from dry regions, while the German, the Dutch and Italian trees come from wetter regions (Table 2.2). Spanish and South French populations were also the warmest (Table 2.2).

This population was collected from previous European projects: European Forest Genetic Resources Programme EUROGEN (Lefèvre *et al.*, 1998; Frison *et al.*, 1994) and EUROPOP (Haska *et al.*, 2004). The common garden experiment was conducted within the POPYOMICS project (Rohde *et al.*, 2010; Trewin *et al.*, 2011). The population is now available for research within the EVOLTREE Network of Excellence (www.evoltree.soton.ac.uk/popmap).

All the genotypes were planted in a common garden experiment at Geraardsbergen in Belgium (Rohde *et al.*, 2010). Using a study from Harriet Trewin, 16 genotypes from the 500 genotypes were used and described as the "extremes", eight genotypes with the largest leaves and eight with the smallest leaves (Trewin, 2008). The "large leaves" genotypes were from The Netherlands, North Italy and Germany and the "small leaves" genotypes from Spain and Southern France.

Table 2.2: Details of the populations of *Populus nigra*, their location and climates. Temperature and precipitation data were collected by Jennifer DeWoody from the website http://www.worldclim.org/. Range of temperatures and precipitations are given for the population collected at different locations (e.g. along a river system).

Population name	**Latitude**	**Longitude**	**Country**	**River system**	**Collection**	**Average annual temperature (°C)**	**Maximum temperature of warmest month (°C)**	**Minimum temperature of coolest month (°C)**	**Average annual precipitation (mm)**	**Precipitation of wettest month (mm)**	**Precipitation of driest month (mm)**
Loire Est	47°28'N	02°54'E	France	Loire	Along the river	10.8 - 11.1	24.7 - 24.9	-0.4 - 0	657 - 744	66 - 76	47 - 49
Loire W	47°28'N	00°33'W	France	Loire	Along the river	11.3 - 11.7	23.7 - 25	1 - 2.3	650 - 779	70 - 87	43 - 45
Drôme1	44°41'N	05°24'E	France	Drôme	Exact location	10.3	25.7	-2.4	890	83	48
Drôme6	44°45'N	04°55'E	France	Drôme	Exact location	12.4	28.1	0	840	95	41
Durance	43°42'N	05°22'E	France	Durance	Along the river	12.2 - 13.6	26.7 - 28.7	0 - 1.1	639 - 732	84 - 90	21 - 28
Ebro1	41°56'N	01°23'W	Spain	Ebro	Exact location	14.1	29.7	1.8	439	56	20
Ebro2	41°35'N	01°00'W	Spain	Ebro	Exact location	13.7	29.5	1.3	365	53	17
Rhine	49°49'N	08°30'E	Germany	Rhine	Exact location	9.8	24.5	-2	605	67	37
Ticino W	45°16'N	08°59'E	Italy	Ticino	Exact location	13	29	-1	982	122	55
Ticino Est	45°12'N	09°04'E	Italy	Ticino	Exact location	13	29	-0.9	966	121	55
Netherlands	52°02'N	05°13'E	Netherlands		Around the location	8.6 - 10.2	20.6 - 21.8	-1.3 - 1.3	731 - 1021	73 - 100	43 - 73

All the graphs in this report used the same colour code for each country as in this map (red = Spain, yellow = France, Purple = Italy, blue = Germany, Green = Netherlands) for Chapters 4, 5 and 6.

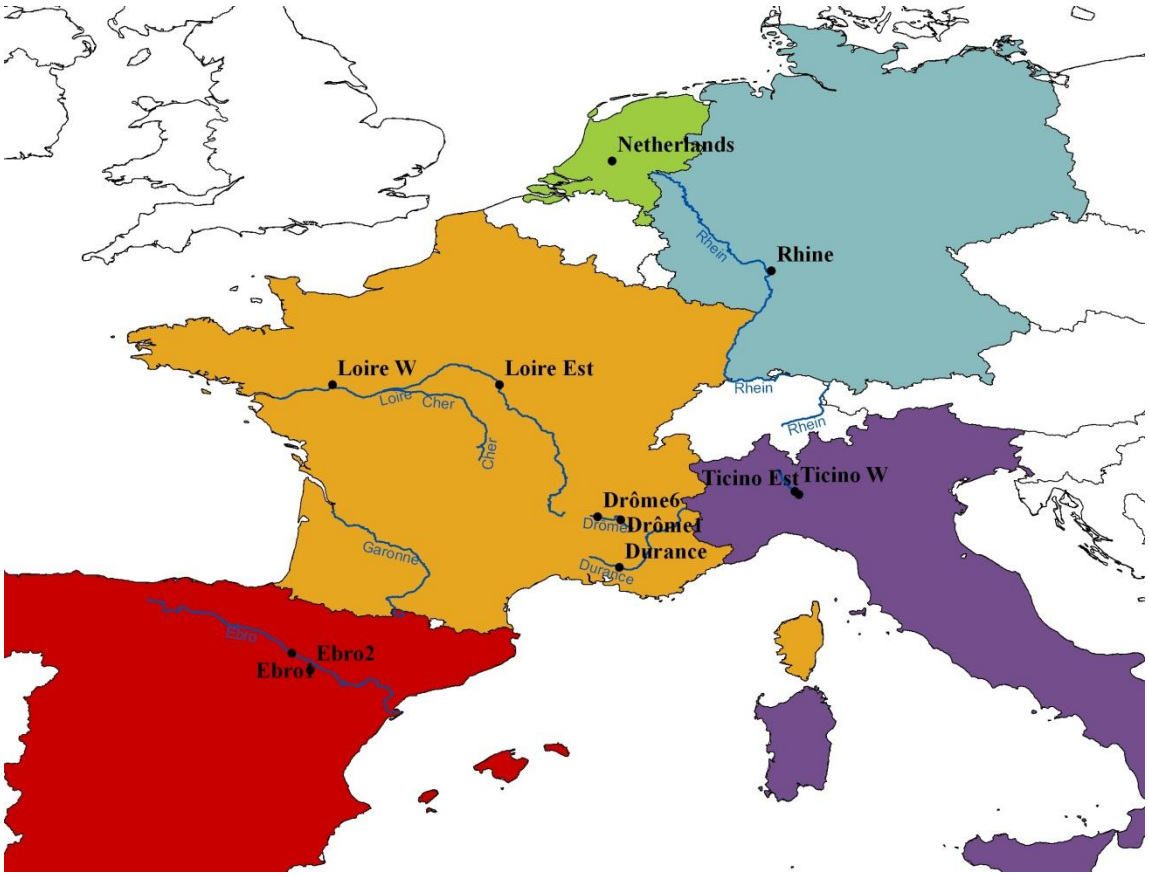


Figure 2.2: Map of the 11 river populations of *Populus nigra* collected in five European countries (coordinate conversion provided by Jennifer DeWoody)

2.2. Experimental design

2.2.1. F2 population P. deltoides x P. trichocarpa

2.2.1.1. Field experiment at Headley (UK) and Cavallermaggiore (Italy)

Two hundred and ten genotypes of Family 331 were planted in two contrasting environments, in North Italy (Cavallermaggiore, 44°21'N, 8°17'E) and Southeast UK (Headley, 51°07', 0°50'W). The climates of each site have been described previously (Rae *et al.*, 2008) and summarized in Table 2.3. Unrooted cuttings were planted in April 2003 at both sites. Each field was divided into 6 blocks, which contained a single replicate of *P. deltoides* (ILL-129), *P. trichocarpa* (93-968), the F1 parents (53-242 - male and 53-246 - female) and 206 genotypes of the F2 population. All genotypes were present in each block and the hardwood cuttings were planted randomly within blocks. A double row of commercial varieties were planted in order to reduce the edge effect (Rae *et al.*, 2008). Plants in the UK received water three times a week during the night and in Italy the site was irrigated by flooding on four occasions (June 24th, July 16th, July 30th and August 17th 2003). Plants at both sites were grown as single stem trees (Fig. 2.3).

Table 2.3: Information about the sites where the mapping population Family 331 was planted – location and climates

Site	Latitude	Longitude	Elevation (m)	Annual average tempera-ture (°C)	Annual rainfall (mm)	Solar radiation (MJ m^{-2} d^{-1})
Headley (UK)	51°07′N	0°50′W	60	10.9	470.9	10.7
Cavallermaggiore (Italy)	44°42′N	7°40′E	285	12.9	729.3	13.7

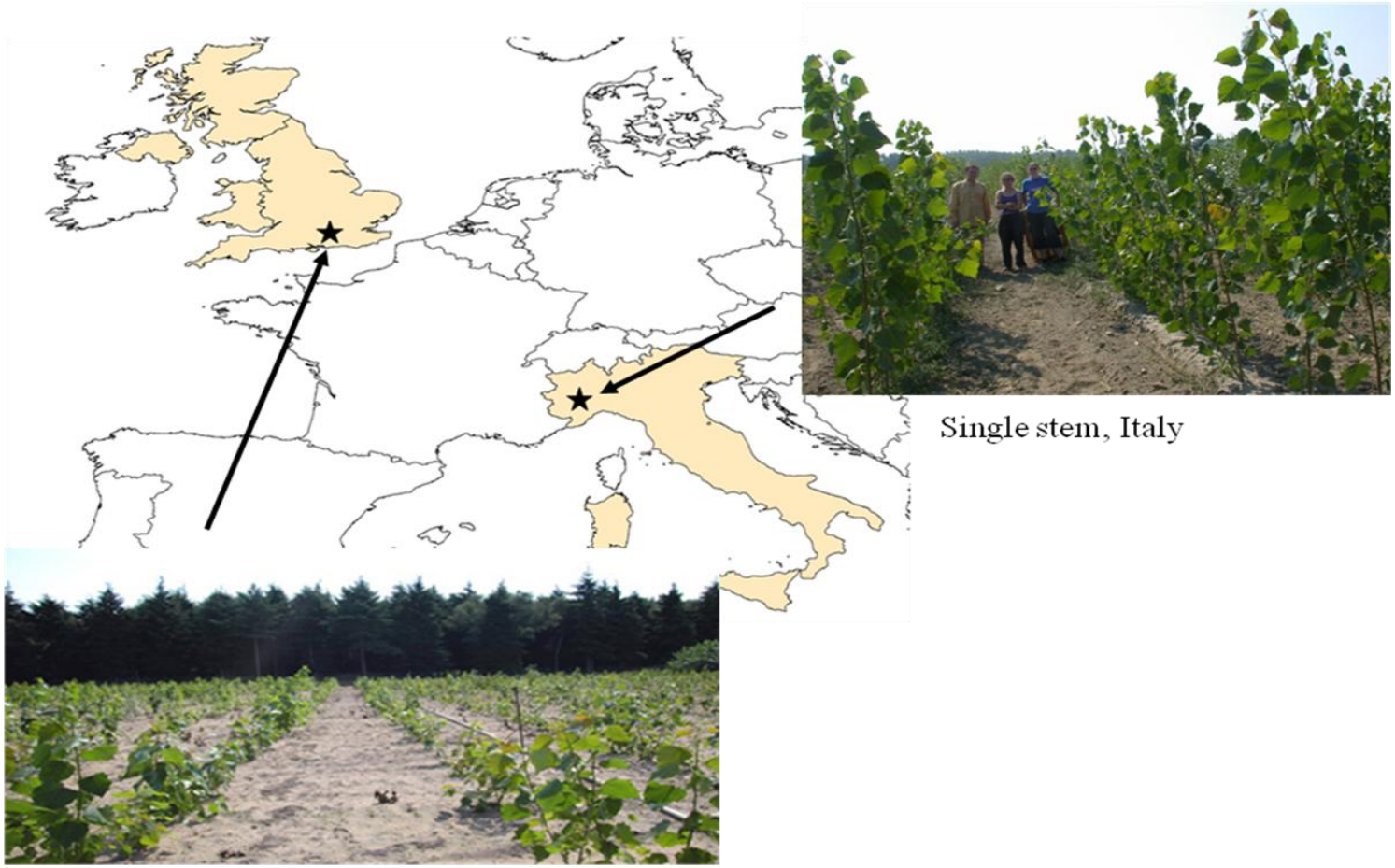


Figure 2.3: Photos of field sites in the UK and in Italy

2.2.1.2. Drought experiment at Chillworth (UK)

As previously described (Street *et al.*, 2006), hardwood cuttings were obtained for *P. deltoides* (ILL-129) and *P. trichocarpa* (93-968) from the field site in the south-east of the UK at Headley and were grown in a greenhouse at Chilworth (Fig. 2.4). They were watered daily until establishment and the drought treatment was initiated 131 days after planting by Nathaniel Street and continued for 17 days. At 0DAD (Days After Drought), droughted trees were given 0.5L of water then no more water was given and soil drying continued, while control trees were watered daily to reach field capacity. Details of the method are given in Street *et al*. (2006).

Figure 2.4: Photos of the experiment conducted at Chilworth greenhouse a day before planting and 60 days after planting (Street, 2005)

2.2.2. European population of Populus nigra

2.2.2.1. Common garden in Belgium

Cuttings from wild trees of *Populus nigra* were collected to generate an association population of 479 genotypes from five different European countries: Spain, France, Italy, Germany and The Netherlands, representing a wide range of precipitation and temperature (Table 2.2). Through the POPYOMICS project, cuttings were planted in a common garden in Belgium, Geraardsbergen (50º 46'N 3°E) in the spring 2004, cut at the base in the spring 2005 and in June 2005 side cuttings were cut so the trees grew as single stem. The field was divided into 6 blocks each containing a replicate of each genotype with a double row of *Populus* 'Muur' planted around the 6 blocks in order to reduce the edge effect (Fig. 2.5). The trees were planted at 0.75 m x 2 m spacing. The site was not irrigated or fertilized but it was weed controlled and treated with fungicides every three weeks between March and September (Trewin, 2008).

Figure 2.5: Common garden in Belgium of *Populus nigra* (summer 2006)

2.2.2.2. Leaf development experiment in a greenhouse at Southampton University

From the measurements done in the field in Belgium (Trewin, 2011), 16 extreme genotypes were selected from the size of their mature leaves (Fig. 2.7), eight with the smallest leaves and eight with the largest (Table 2.4).

Table 2.4: Summary of the extreme genotypes of *P. nigra* and their origin

Genotype	River population	Country of origin	Leaf size
B7	Ebro1	Spain	Small
C15	Ebro1	Spain	Small
C7	Ebro1	Spain	Small
CART2	Ebro2	Spain	Small
FR7	Ebro2	Spain	Small
RIN2	Ebro2	Spain	Small
71092-36	Durance	France	Small
71095-1	Durance	France	Small
N30	Ticino W	Italy	Large
N38	Ticino W	Italy	Large
N53	Ticino W	Italy	Large
N56	Ticino W	Italy	Large
N66	Ticino W	Italy	Large
SN19	Ticino Est	Italy	Large
NL1682	Netherlands	Netherlands	Large
NVHOF5-16	Rhine	Germany	Large

A greenhouse experiment using these extreme genotypes was undertaken in partnership with Harriet Trewin. Settings in the greenhouse were kept at 16h:8h, light:dark, 22.5ºC:21ºC. Unrooted cuttings were planted in pots on January 17th 2007 with 5 replicates for each genotype and watered daily. The experimental design consisted of 5 blocks each containing a replicate of each genotype randomly allocated in the block (Fig. 2.6). Two rows of *P. nigra* guard trees surrounded the experimental trees on two sides (left and right) and five rows on the other sides (top and bottom) reducing the edge effect. The experiment started on February 13th 2007 until March 30th 2007.

Row \ Column	1	2	3	4	5	6	7	8	9
1			Guard trees						
2			Guard trees						
3			Guard trees						
4			Guard trees						
5			Guard trees						
6									
7									
8									
9									
10									
11	Guard trees	Guard trees	Block 1	Block 2	Block 3	Block 4	Block 5	Guard trees	Guard trees
12									
13									
14									
15									
16									
17									
18			Guard trees						
19			Guard trees						
20			Guard trees						
21			Guard trees						
22			Guard trees						

Figure 2.6: Layout of the experiment for the leaf development at the University of Southampton

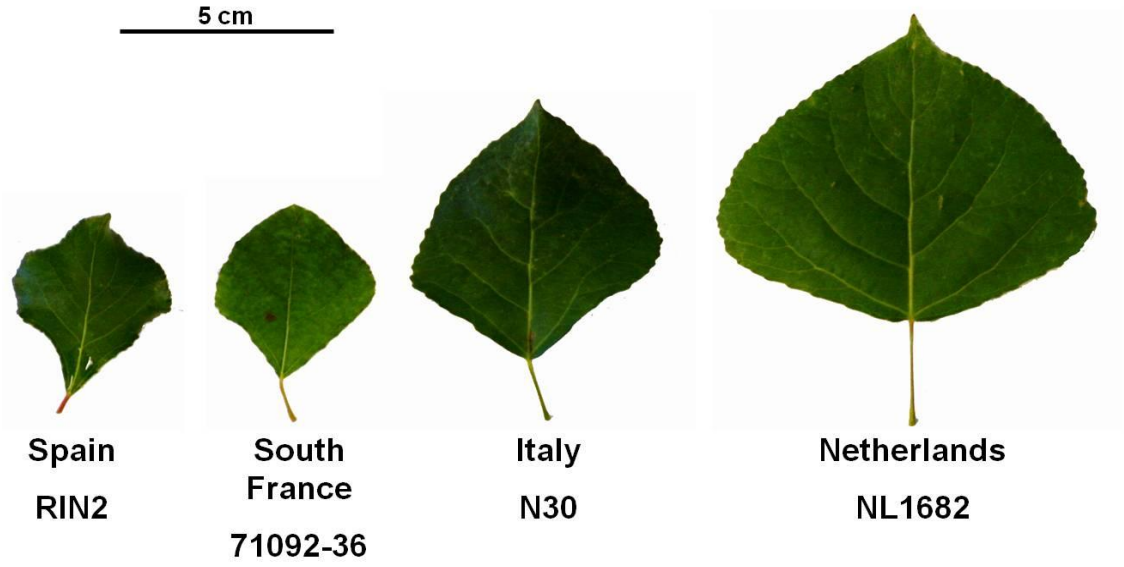


Figure 2.7: Representative leaves of the extreme genotypes showing variation in leaf size and shapes.

2.2.2.3. Drought experiment in a greenhouse at Southampton University

Six genotypes were selected from the *P. nigra* population (Table 2.5), two from the Drôme population in France and four from the extreme "leaf size" genotypes (two Spanish, one Italian and one from the Netherlands).

Table 2.5: Provenance of the six *P. nigra* genotypes used in the drought experiment

Genotype	Genotype code	River population	Country
C7	Sp1	Ebro1	Spain
RIN2	Sp2	Ebro2	Spain
6A03	Fr1	Drôme6	France
6A05	Fr2	Drôme6	France
N38	It	Ticino (left side)	Italy
NL1682	NL	Netherlands	Netherlands

Cuttings were planted in January 2007 for the leaf experiment (Chapter 4) and cut back on April 24th 2007 and November 24th 2007 at 10 cm from the base. They were watered daily and put in dormancy conditions (natural light, 15ºC:13ºC day:night). In May 2008, the trees started to grow and the temperature in the greenhouse was set at 22ºC:16ºC, day:night. During the time of the experiment, photoperiod was maintained 16h:8h, light:dark with an average photosynthetic active radiation at the top of the plants of 150 μmol m^{-2} s^{-1}. Day and night temperature varied between 19 and 22ºC, and 15 and 17ºC respectively.

Figure 2.8: Photo of the experimental trees taken on September 7th 2008 in the greenhouse at the University of Southampton

Each genotype had up to 10 replicates in control treatment and 10 in drought treatment, except for Fr1 which had nine replicates under control treatment. Replication number reduced after SSR marker analysis in the Spanish genotypes (details of the protocol in Appendix D) and few replicates were found belonging to other genotypes. Sp1 and Sp2 had eight and five replicates respectively under control and nine and eight replicates respectively under drought stress. The trees were positioned in the middle bench in Boldrewood greenhouse at the University of Southampton in 10 blocks containing one replicate per genotype in each treatment (Fig. 2.8). On September 1st 2008, 200mL of water was added to each tree and the pots were then covered in aluminium foil to prevent water evaporation. The first mature leaf and the first emerging young leaf were tagged with cotton string.

Over the next month (31 days), soil moisture content was measured every morning with a Delta-T ML2x ThetaProbe connected to an HH2 moisture meter (Delta-T Devices, Cambridge, UK). Well-watered trees (control treatment) were watered up to field capacity and drought stressed trees (drought treatment) were kept between 15-20% soil moisture (Fig. 2.9). Using a repeated measurements test over time, soil moisture content showed significant differences between treatment ($F_{1,50}$=363.17, $p<0.001$) but no

significant differences were found between genotypes ($F_{5,50}$=1.06, p=0.392) and no genotype x treatment interaction effect ($F_{5,50}$=0.82, p=0.543), which means that all the genotypes had their soil moisture decreased under water stress treatment..

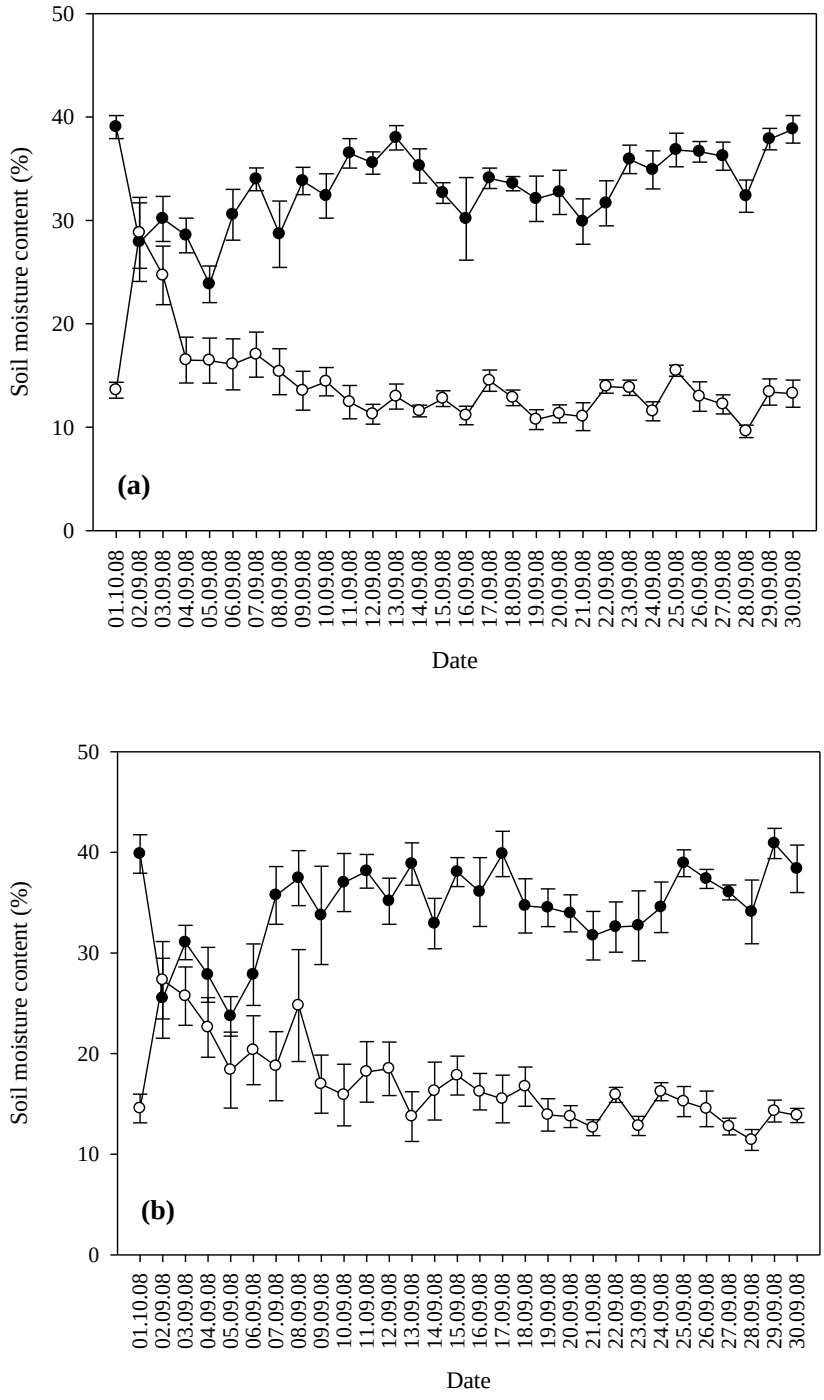
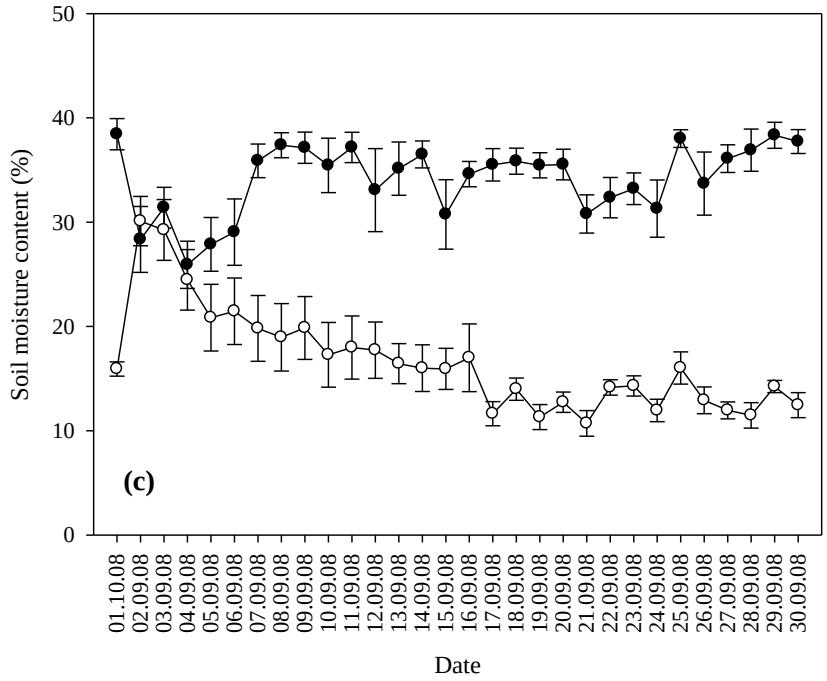


Figure 2.9: Soil moisture content (%) over time (days after drought) for each genotype: Sp1 **(a)**, Sp2 **(b)**, Fr1 **(c)**, Fr2 **(d)**, It **(e)** and NL **(f)**. Black symbols represents well-watered (control) and white symbols for drought treatment. Each value with bars represents the average ± standard error.



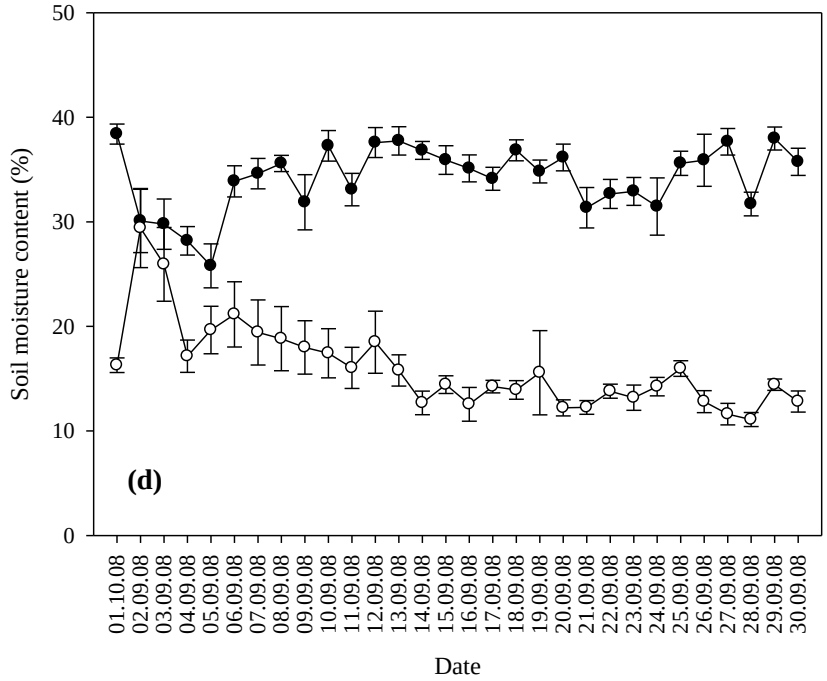


Figure 2.9: Soil moisture content (%) over time (days after drought) for each genotype: Sp1 **(a)**, Sp2 **(b)**, Fr1 **(c)**, Fr2 **(d)**, It **(e)** and NL **(f)**. Black symbols represents well-watered (control) and white symbols for drought treatment. Each value with bars represents the average ± standard error.

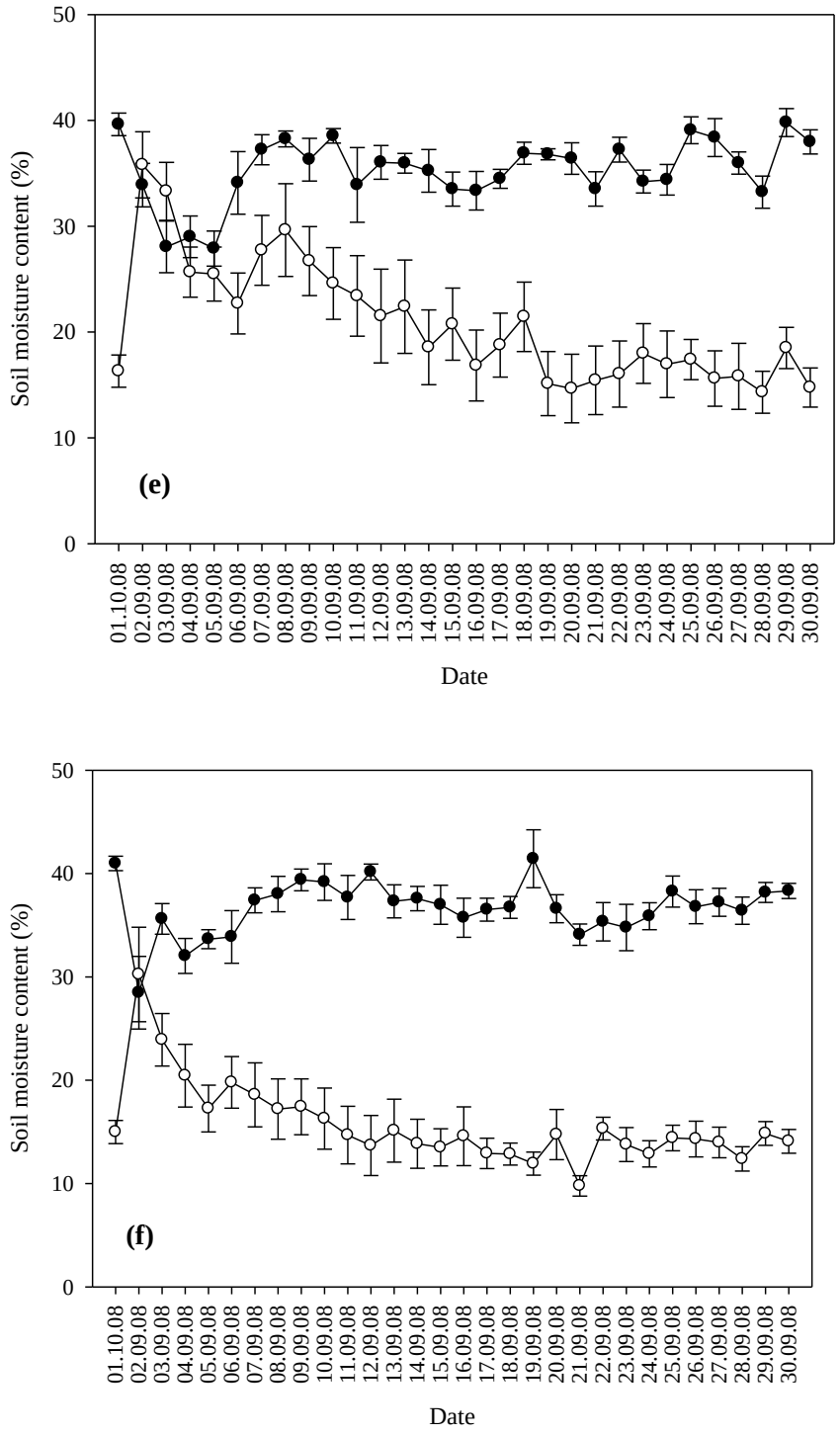


Figure 2.9: Soil moisture content (%) over time (days after drought) for each genotype: Sp1 **(a)**, Sp2 **(b)**, Fr1 **(c)**, Fr2 **(d)**, It **(e)** and NL **(f)**. Black symbols represents well-watered (control) and white symbols for drought treatment. Each value with bars represents the average ± standard error.

2.3 Physiological Measurements

2.3.1. Carbon isotope discrimination and oxygen isotope composition

Wood and leaves collected to measure carbon isotope discrimination ($\Delta^{13}C$) and oxygen isotope composition ($\delta^{18}O$) were dried in the oven for 48-50h at 80ºC. Samples were ground using a ball grinder (Glen Creston ball, Retsch MM300, London, UK) and stored in a glass container. For carbon isotope discrimination, 1mg of material was weighed and placed into a 6 x 4 mm tin capsule (Ultra-clean pressed tin capsules, Elemental Microanalysis, Devon, UK). Samples were analysed using a SerCon 20-20 Stable Isotope Analyzer with ANCA-GSL Solid/Liquid Preparation Module (SerCon, Crewe, UK). Carbon isotope composition was determined by $\delta^{13}C$ (‰) = δ_{plant} = [(R_{sample} - $R_{reference}$) / $R_{reference}$] × 1000, where R_{sample} and $R_{reference}$ are the $^{13}C/^{12}C$ ratios of the sample and the reference respectively, in VPDB (Vienna Pee Dee Belemnite) units (Scrimgeour & Robinson, 2004). Carbon isotope discrimination was calculated as $\Delta^{13}C$ (‰) = [(δ_{air} - δ_{plant})/(1+(δ_{plant} /1000)] with δ_{air} assumed to be close to -8‰ (Farquhar & Richard, 1984; Monclus *et al.*, 2006).

For oxygen isotope composition, 0.2mg was weighed and placed in a 4 x 6 mm silver capsule (Pressed silver capsules, SerCon, UK). Oxygen isotope composition was measured as $\delta^{18}O_{plant}$ (‰) = (R_{plant} / $R_{reference}$) – 1, where R_{sample} and $R_{reference}$ are the $^{18}O/^{16}O$ ratios of the sample and the reference respectively, in VSMOW (Vienna Standard Mean Ocean Water) units (Farquhar *et al.*, 2007). Oxygen isotope enrichment $\Delta^{18}O$ (‰) = [($\delta^{18}O_{plant}$ - $\delta^{18}O_{source\ water}$)/(1+ $\delta^{18}O_{source\ water}$) (Cernusak *et al.*, 2003) was not calculated as $\delta^{18}O_{source\ water}$ was unknown but was considered identical with $\delta^{18}O$ as values are positive compared to $\delta^{13}C$ values.

All the isotopes samples were analysed by the Scottish Crop Research Institute (Dundee, UK).

2.3.2. Stomatal conductance

Stomatal conductance (g_s) was measured using a portable steady-state diffusion porometer (LI-1600, LI-COR inc. Lincoln, Nebraska), with manual data recording. g_s was calculated directly using relative humidity, temperature of the leaf and the air and flow rate. Values were in $mmol.m^{-2}.s^{-1}$.

The LI-1600 was constituted of two parts: a readout with control console and a sensor head with cuvette (Fig. 2.10). A leaf is placed in the cuvette which causes the relative humidity in the cuvette to increase. A flow rate of dry air is injected to balance the water transpired by the leaf until the cuvette relative humidity returns to a set point determined by the user (LI-COR, 1989). The leaf is maintained in the cuvette between 20 and 30 seconds until the readings are stable.

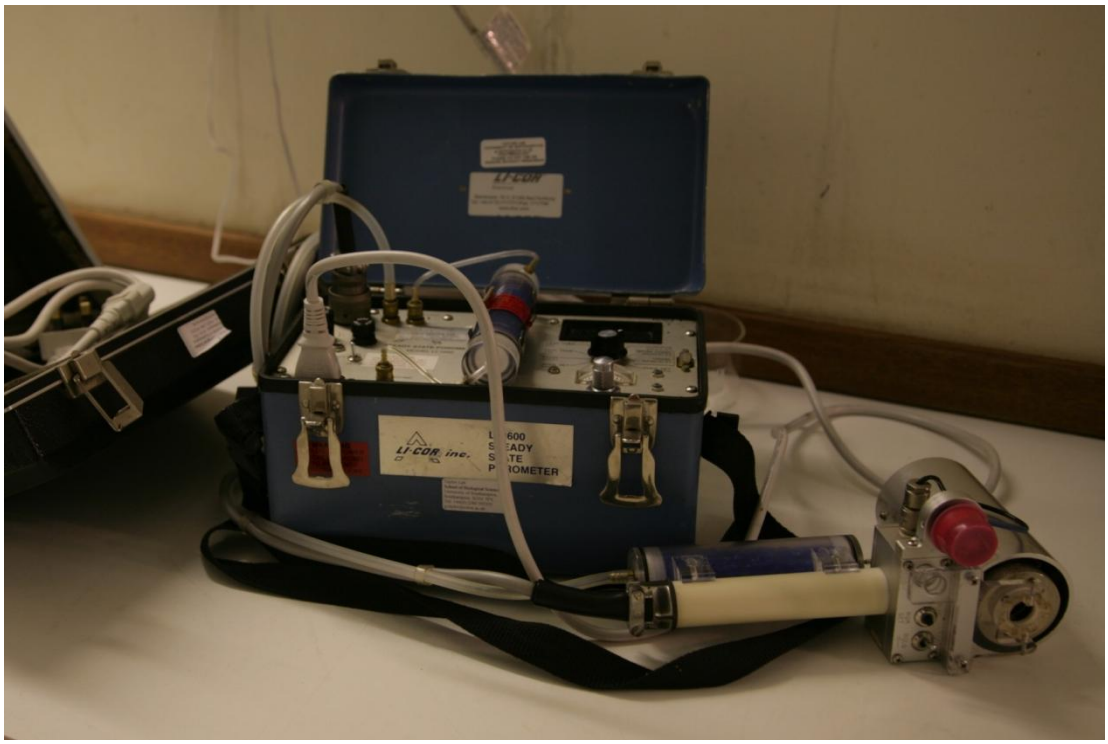


Figure 2.10: Portable steady-state diffusion porometer, LI-1600

This technique applying the LI-1600 is commonly used to measure stomatal conductance in various plants (Radin *et al.*, 1994; Lu *et al.*, 1998; Wang *et al.*, 1999; Lemoine *et al.*, 2002; Lambs *et al.*, 2006; Benlloch-González *et al.*, 2008; Lee *et al.*, 2009; Kim & van Iersel, 2010).

2.3.3. Leaf development

In order to measure leaf area, the contour of the leaves were drawn on a white paper or brown paper bag and labeled accordingly. The images were scanned (Umax Astra 6700 scanner) and processed using ImageJ (Abramoff *et al.*, 2004). Comparison was possible using the same scale for all the images.

Dried leaves at 80°C for 48h were used to measure Specific Leaf Area. SLA is the ratio of leaf area in cm^2 (prior drying) to leaf dry mass in grams (Macfarlane *et al.*, 2004; Marron *et al.*, 2005).

2.3.4. Cell measurements

Cell measurements were done on the abaxial and adaxial side of the leaf using nail vanish. Nail vanish was applied in the same area of the leaf for each replicate, left to dry, carefully removed and place on a microscope slide (Gardner *et al.*, 1995). Areas with large veins were avoided. Imprints were collected with a Zeiss microscope at x40 magnification attached with a camera capturing the images. Cell number (CN), cell area and stomata number were measured using ImageJ (Abramoff *et al.*, 2004). From these values, stomatal density (SD) and stomatal index (SI) were calculated (Ferris & Taylor, 1994). Cell area (CA) was the average of 10 cell area (μm^2) in the field of view.

SD = Σ stomata number / Field of view

SI = 100 x [Σ stomata number / (Σ cell number + Σ stomata number)]

Cell number per leaf on the abaxial and adaxial side was measured using the cell number in the field of view converted in mm^2 and mature leaf area (mm^2)

CN per leaf = CN / leaf of view * leaf area

2.4. Genetic and Genomic measurements

2.4.1. RNA extraction

Leaf sampling for microarrays and real-time PCR was done at mid-day. Each sample was placed in an individual pre-labeled foil bag and flash frozen in liquid nitrogen. Samples were then stored at -80°C until further analysis. Leaf grinding was performed in liquid nitrogen using mortar and pestle and the ground material of fine powder was stored in an eppendorf tube.

RNA was extracted following the CTAB protocol, modified from Chang *et al.* (1993) and revised by Street, Tucker and Stephenson (Personal communication). CTAB extraction buffer was prepared prior RNA extraction and consisted of 2% CTAB (hexadecyltrimethylammoniumbromide (w/v)), 2% PVP (polyvinylpyrrolidone (w/v)), 100mM Tris-HCl (v/v, pH 8.0), 25mM EDTA (ethylenediaminetetraacetic acid (v/v)) and 2M NaCl (sodium chloride (w/v)). 900 µL of pre-warmed CTAB extraction buffer in a water bath at 65°C and 50 µL of 2-ME (2% β-mercaptoethanol) were added into the eppendorf tube containing up to 300 mg of ground material. After 5-10 minutes of incubation at 65°C in a water bath, 600 µL of CHISAM (Chloroform : Isoamyl alcohol, 24:1 (v/v)) was added and vortexed vigorously. The tubes were centrifuged for 10 minutes at 12,000g at room temperature. The aqueous phase was transferred to a fresh eppendorf tube and 180 mL of 10M LiCl (lithium chloride) added. The mixture was left to precipitate at 4°C for 30 minutes. Samples were centrifuged at 12,000g for 15 minutes at 4°C. The supernatant was removed and the pellet dissolved in 700 µL of pre-warmed SSTE (60°C). SSTE is composed of 1M NaCl (w/v), 0.5% SDS (sodium dodecyl sulphate (v/v)), 10mM Tris-HCl (Tris-Hydrochloride (v/v)) and 1mM EDTA (v/v). The samples were incubated for 5 minutes at 60°C. 600 µL of CHISAM was added and, after vortexing, centrifuged at 12,000g for 10 minutes at room temperature. The aqueous phase was transferred into a fresh eppendorf tube and 1000 µL of 100% ethanol added and left to precipitate at -20°C for 10 minutes. The samples were then centrifuged at 12,000g for 10 minutes at 4°C. The supernatant was removed, the pellet washed with 1 mL of 70% ethanol and centrifuged at 12,000g for 2 minutes at 4°C. The supernatant was removed and spin briefly to collect the residual ethanol which was

pipetted off. The pellet was air dried for 20 minutes and re-suspended in 30 µL of DEPC (Diethylpyrocarbonate) -treated H_2O. All the samples were stored at -80°C.

RNA quality and concentration were done with a spectrophotometer (NanoDrop® ND-1000 Spectrophotometer, Thermo Fisher Scientific, Wilmington, USA) with 1 µL of the re-suspended solutions of RNA in DEPC-treated water and using the DEPC-treated water as blank. The ratios of absorbance 260nm / 280nm are checked and values above 2 are considered as "pure" for RNA. Another analysis of RNA quality was done by the European Arabidopsis Stock Centre microarray service (NASC, Loughborough, UK) prior microarray hybridization using Agilent Bioanalyzer to check the integrity of RNA (http://affy.arabidopsis.info/qc.html).

2.4.2. Microarrays

Twelve RNA samples of 15 µL were sent on ice to the European Arabidopsis Stock Centre (NASC, Loughborough, UK) microarray service at the University of Nottingham for the cDNA synthesis, fragmentation and arrays hybridization and scanning using Affymetrix GeneChip Poplar Genome Arrays (Affymetrix, Santa Clara, USA). After reception of the data, Affymetrix .CELs files were imported into GeneSpring (Agilent Technologies, Santa Clara, USA) to apply a robust multi-array average (RMA) to each chip. Normalisation and data analysis were performed at the University of Southampton. Normalisation per chip was applied using three positive control genes: *UBQ11* (grail3.0064002701), *TUA5* (estExt_fgenesh4_pm.C_LG_III0736) and *ACT2* (estExt_fgenesh4_kg.C_LG_I0082). Genes were selected from Brunner *et al.* (2004). A normalisation per gene was also performed to the median. Data analysis with a Volcano Plot ($p<0.05$, 2-fold change) followed to discover genes differentially expressed for each genotype under drought and under well-watered conditions using three biological replicates per genotype and per condition.

2.4.3. Real-time qPCR

After the analysis of the microarrays, candidate genes were selected for qPCR and forward and reverse primers were designed specifically for each gene.

RNA samples were treated with the Turbo DNA-*free* kit (Ambion, Austin, USA) to remove genomic DNA, following manufacturer's instructions. In an eppendorf tube, 0.1 volume of 10X TURBO DNase buffer and 0.5µL of TURBO DNase were added to the RNA. The samples were incubated at 37°C for 30 minutes. To stop the reaction, 0.1 volume of re-suspended DNase Inactivation Reagent was added. It was then incubated 5 minutes at room temperature and mixed occasionally. The samples were centrifuged at 10,000g for 1.5 minutes. The supernatant containing the DNA-free RNA was transferred into a fresh eppendorf tube. RNA concentration and quality were assessed again with a spectrophotometer (NanoDrop® ND-1000 Spectrophotometer, Thermo Fisher Scientific, Wilmington, USA).

Reverse transcription of 5µg of RNA to cDNA was performed using the ImProm-II Reverse Transcription kit (Promega UK, Southampton, UK) following manufacturer's instructions. 1µL of oligo dT was added to 5µg of RNA in 20µL of reverse transcription. Samples were incubated at 70°C for 5 minutes and quickly chilled at 4°C for 5 minutes. A reverse transcription mix combined 4µL of ImProm-II 5X Reaction Buffer, 8mM of $MgCl_2$, 10mM of dNTP mix and 1µL of ImProm-II reverse transcriptase. After vortexing the reverse transcription mix gently, it was added to the RNA/primer mix. The reverse transcription by PCR consisted of three steps: anneal at 25°C for 5 minutes, extend for 60 minutes at 42°C and heat-inactivate at 70°C for 15 minutes. cDNA was stored at -20°C.

cDNA was diluted 1:5 in DEPC-treated water. qPCR reaction was composed of 5µL 2X Precision-SY Master Mix (PrimerDesign Ltd, UK), 5pmol forward and reverse primers and 25ng diluted cDNA. Plates were run on a Chrom4 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, USA). Reactions were incubated at 95°C for 10 minutes and then 40 cycles of 15 seconds at 95°C, 1 minute at 60°C and a plate read. An incubation at 72°C for 10 minutes followed. A melting curve was then performed from 60°C to 95°C with a read every 0.2°C and 1 second hold, in order to check for

primer dimers, DNA contamination and secondary products. Values were exported with the software Opticon Monitor 3.1 (Bio-Rad Laboratories, Hercules, USA).

Amplification efficiency was measured following the equation from Liu & Saint (2002):
$E = (R_{n,A} / R_{n,B})$ ^ $[1/C_{T,A}-C_{T,B})] + 1$
where $R_{n,A}$ and $R_{n,B}$ are R_n at arbitrary thresholds A and B in an individual curve, respectively, and $C_{T,A}$ and $C_{T,B}$ are the threshold cycles at these arbitrary thresholds (Liu & Saint, 2002).
Ratios were calculated as $E^{(control - drought)_{target}} / E^{(control - drought)_{reference}}$

Chapter 3: Understanding the genetic basis of adaptation to drought: Water Use Efficiency QTL in bioenergy *Populus*

Abstract

Dedicated bioenergy crops including poplar and willow are needed for renewable energy in a future drier climate. Such crops should have a low water, carbon and chemical footprint, to give a favourable life cycle analysis (LCA). Capturing natural variation in traits contributing to water use efficiency (WUE) is the first step to developing trees that require less water and may be adapted to drier environments.

Here we have assessed stomatal conductance (g_s, a measure of stomatal opening) and leaf carbon isotope composition ($\delta^{13}C$, an indirect indicator of plant water use efficiency, WUE) in two *Populus* species, *P. deltoides* and *P. trichocarpa* and their F_2 progeny, grown in three contrasting conditions - one site in the UK in two different growing conditions and one site in Italy.

P. deltoides leaves showed lower $\delta^{13}C$ than *P. trichocarpa*. The stomatal conductance of *P. trichocarpa* was less responsive to soil drought, dehydration and abscisic acid treatment than *P. deltoides*. Quantitative Trait Loci (QTL, areas of the genome determining the expression of a trait) were identified for $\delta^{13}C$ on nine linkage groups (LG) and two QTL for g_s. From these QTL and gene expression from microarrays, we have identified three hotspots and twenty three novel candidate gene models on linkage groups VI, X and XVI.

We have begun to unravel the genetic basis of WUE in bioenergy *Populus*. These data are important for breeding and improvement in poplar and willow genotypes with higher WUE with a lower water footprint and a future drier climate.

3.1 Introduction

Global climate is changing and summer rainfalls are predicted to be reduced in the future across many areas of the world, leading to increased soil moisture deficits (Kundzewicz *et al.*, 2007). These changes are likely to have consequences for forests because growth and productivity will be reduced and forest ecosystem function affected. There may be increased vulnerability to pathogens, insect attack, reduced tree health and increased mortality (Hanson & Weltzin, 2000; Broadmeadow, 2002; Easterling *et al.*, 2007). Reduction of productivity due to drought is important particularly for trees cultivated intensively such as short rotation coppice (SRC) for bioenergy (Oliver *et al.*, 2009), which in temperate climates tend to be poplar and willow.

One solution is tree breeding for adaptation to drought (Grattapaglia *et al.*, 2009), where water use efficiency (WUE, the ratio between net carbon assimilation and water loss) is used as a target trait of adaptive significance. Genotypes better adapted to dry climate regimes have been identified in a range of arable crop species (recently reviewed by Morison *et al.* (2008)) and also trees such as pine (Guehl *et al.*, 1995), oak (Brendel *et al.*, 2008) and poplar (Monclus *et al.*, 2005; Monclus *et al.*, 2006). Improving WUE is particularly relevant in bioenergy crops to ensure that trees developed for biofuels can be produced under predicted future drier conditions.

Poplar is a target tree for bioenergy, but they are moderately tolerant to drought (Somerville *et al.*, 2010), since many species of this genus are usually adapted to a riparian, wet habitat (Aylott *et al.*, 2008). However, there is also evidence to suggest that enough genetic diversity exists across the genus for targeted selection and breeding for high WUE trees with wide variations reported in traits related to drought tolerance (Monclus *et al.*, 2006), in gene expression and metabolic changes in response to drought (Street *et al.*, 2006) and in the ability of certain *Populus* species to tolerate extremely droughted environments. For example, *Populus euphratica* was found in highly saline and arid environments such as the Negev desert (Brosché *et al.*, 2005). Identifying this genetic diversity and understanding the physiological traits associated with genetic variation provides the first step to identify superior plants for future breeding efforts.

WUE can be defined either at the plant scale, WUE_p (the ratio between biomass production and water consumption over a period of time, usually weeks or months) or at short-term and smaller scale of leaves, WUE_t - the 'instantaneous' ratio between the net CO_2 assimilation rate and the transpiration loss (Ponton *et al.*, 2001; Bacon *et al.*, 2004; Seibt *et al.*, 2008). High WUE_t can be achieved by reducing stomatal conductance, g_s (Leffler & Evans, 2001) and/or increasing photosynthetic rates (Condon *et al.*, 2002). Several studies have shown that WUE can be improved with stomatal closure at midday (Tenhunen *et al.*, 1982) or through stomatal opening early in the morning (Bacon *et al.*, 2004). A positive correlation between WUE_t and leaf carbon isotope composition, $\delta^{13}C$ is now established (Farquhar *et al.*, 1989; Condon *et al.*, 2002; Brendel *et al.*, 2008), enabling a rapid screen for WUE in plants in many environments and from a large number of genotypes (Farquhar *et al.*, 1989; Jones, 1993; Condon *et al.*, 2002; Bacon *et al.*, 2004; Rajabi *et al.*, 2009).

A difficulty in this type of research however, is the strong link between plant water consumption and yield, with consequent reductions in water use and higher WUE often associated with lower yield under most favourable conditions where there is no drought occurring (Collins *et al.*, 2008). Although breaking this link is difficult, there have been recent successes with wheat adapted for arid environments in Australia, that show it is a useful target trait for breeding for future drier climates, with the release of new cultivars as a result of carbon isotope research (Condon *et al.*, 2004; Richards, 2006; Hochman *et al.*, 2009).

Quantitative genetics provides a frame work that enables physiological and biochemical traits related to drought to be considered at the level of the genome and in several model and crop species. Quantitative Trait Loci (QTL) have been identified for drought traits including $\delta^{13}C$, WUE and stomatal behaviour. For example, in *Arabidopsis*, Masle *et al.* (2005) mapped a transpiration efficiency QTL, linked to the *ERECTA* gene, whilst more recently $\delta^{13}C$ QTL have been mapped and related to flowering time in *Arabidopsis* (Tisné *et al.*, 2010). These data have value for molecular breeding efforts (Tuberosa & Salvi, 2006; Collins *et al.*, 2008) but also provide an insight into plant adaptation and evolution in a changing climate. For example, in an *Arabidopsis* recombinant inbred population generated from plants selected from extremely arid or wet environments, QTL for response to drought revealed areas of the genome controlling WUE traits of

adaptive significance (McKay *et al.*, 2008). For *Arabidopsis*, it appears that one strategy of adaptive significance for drought tolerance is avoidance. QTL quantified as drought tolerance were close to those for flowering time and associated with reduced time to flower in droughted conditions in germplasms selected from more arid sites.

Clearly, this highlights a limitation in the use of *Arabidopsis* as an example for long-lived perennial tree species, where flowering may be only one part of the adaptive strategy deployed by these perennial organisms (Taylor, 2002). Genotypic variation for WUE, $\delta^{13}C$ and g_s has, however, been described previously for a limited number of tree species (Ponton *et al.*, 2001; Prasolova *et al.*, 2003; Monclus *et al.*, 2005; Voltas *et al.*, 2006; Brendel *et al.*, 2008). In *Populus*, Monclus *et al.* (2005, 2006) showed that productivity was not correlated consistently with $\delta^{13}C$, with genotypes found combining high WUE and productivity and so understanding the genetic basis of such a trait would be useful in the development of efficient crops growing under drought stress. No published data on QTL and the genes underlying QTL for WUE exist for *Populus*.

The aim of this work was to unravel the genetic basis of WUE in *Populus*, for which limited information is published, using an F_2 mapping pedigree. We investigated WUE, g_s and $\delta^{13}C$ to identify QTL and candidate genes underlying QTL hotspots and we identified three areas of the genome for further study, which will enable rapid future progress to be made in molecular tree breeding for enhanced WUE, in addition to an improved understanding of adaptation of this tree genus to contrasting soil moisture environments, likely in future climates.

3.2 Material and Methods

This chapter represents a reanalysis of morphological and QTL data collected by Maricela Rodriguez-Acosta (Rodriguez-Acosta, 2006) and is the work of a paper in preparation for a journal paper (Biotechnology for Biofuels). The other authors (Maricela Rodriguez-Acosta, Anne Rae, James Morison and Gail Taylor) contributed to the proof-reading of the drafts to improve the clarity of the work.

3.2.1. Plant material

The materials used for the experiments in this chapter were a genotype of *P. trichocarpa* and a genotype of *P. deltoides, and* the mapping population Family 331. Details of the crossing are in Chapter 2.

3.2.2. Family 331 carbon isotope composition and stomatal conductance

Two hundred and ten genotypes of Family 331 were planted in two contrasting environments, in North Italy (Cavallermaggiore, 44°21'N, 8°17'E) and south-east UK (Headley, 51°07', 0°50'W). The climates of each site and experimental design have been described previously (Rae *et al.*, 2008). Unrooted cuttings were planted in April 2003 at both sites. Each field was divided into 6 blocks which contained a single replicate of *P. deltoides* and *P. trichocarpa*, the F1 parents (53-242 - male and 53-246 - female) and 206 genotypes of the F_2 population in a randomized complete block design (RCBD). A double row of commercial varieties were planted in order to reduce the edge effect (Rae *et al.*, 2008). Plants in the UK received water three times per week during the night and in Italy the site was irrigated by flooding on four occasions (June 24th, July 16th, July 30th and August 17th 2003). Plants at both sites were grown as single stem trees.

Three replicates of 188 genotypes of Family 331 were used to determine $\delta^{13}C$ for each site with one mature leaf per tree harvested in April 2004. The collection of the leaves was carried out by Maricela Rodriguez-Acosta. Leaves were dried for 48h in an oven at 80°C and then ground using a ball grinder (Glen Creston ball, Retsch MM300). Material was weighed (1mg) and placed into a 6 x 4 mm tin capsule (Ultra-clean pressed tin capsules, Elemental Microanalysis, Devon, UK). Samples were analysed using a Sercon

20-20 Stable Isotope Analyzer with ANCA-GSL Solid/Liquid Preparation Module (Sercon, Crewe, UK). The carbon isotope composition was determined by $\delta^{13}C$ (‰) = $[(R_{sample} - R_{reference}) / R_{reference}] \times 1000$, where R_{sample} and $R_{reference}$ are the $^{13}C/^{12}C$ ratios of the sample and the reference respectively (methods fully described in Scrimgeour & Robinson, 2004).

The $\delta^{13}C$ results from this experiment were compared to data from Rae *et al*. (2009) on Family 331 trees grown under short rotation coppice (SRC) in the same field in south-east UK at Headley.

Stomatal conductance of all F_2 trees at the UK site was measured by Maricela Rodriguez-Acosta in midsummer 2004. For each genotype, leaf 7 from the top of the main stem was removed. Leaf petioles were re-cut under water (Sperry *et al.*, 1988), transferred to a tube with distilled water and transported to a controlled environment with photosynthetic active radiation (PAR) approximately 300 µmol m^{-2} s^{-1}, air temperature of 25°C and relative humidity of 50% (±10%). Measurements were made on the replicates of each genotype in a period not exceeding 4 hours after excision.

3.2.3. Differences in stomatal behaviour between P. deltoides and P. trichocarpa

3.2.3.1. Slow droughting and diurnal changes

As previously described (Street *et al.*, 2006), hardwood cuttings were obtained for *P. deltoides* (ILL-129) and *P. trichocarpa* (93-968) from the field site in the south-east of the UK at Headley and were grown in a greenhouse at Chilworth. They were watered daily until establishment and the drought treatment was initiated 131 days after planting and continued for 17 days. At 0DAD (Days After Drought), droughted trees were given 0.5 L of water then no more water was given and soil drying continued while control trees were watered daily up to field capacity. Details of the method are given in Street *et al*. (2006). Diurnal time courses of g_s were measured by Maricela Rodriguez-Acosta 15 days after drought treatment commenced using a portable steady-state diffusion porometer (LI-1600, LI-COR inc. Lincoln, Nebraska), with manual data recording. Three plants of each species and each treatment were used for the experiment. g_s on the

abaxial side of a mature leaf was determined at ambient temperature every one or two hours for a period of 19 hours beginning at 4 a.m. and finishing at 11 p.m.

3.2.3.2. Thermal imaging

In constant environmental conditions (radiation, temperature, wind speed and humidity), changes in leaf temperature will indicate changes in g_s (Jones, 1999; Grant *et al.*, 2006; Leinonen *et al.*, 2006). The dynamics of stomatal response to drying and ABA treatment across leaves were investigated using thermal imaging. Mature leaves (LPI 9) from 9 month old greenhouse-grown plants were excised by Maricela Rodriguez-Acosta under water and placed in a controlled environment room to allow stabilization of stomatal conductance. LPI is the Leaf Plastochron Index measured using the PI (Plastochron Index) which is equivalent to the interval in time between two successive leaves reaching 30 mm, as previously described in Taylor *et al.* (2003).

The temperature of the room was 25°C and the relative humidity approximately 65-75%. The petioles were then re-cut under water (Sperry *et al.*, 1988) and the leaves transferred to a tube with distilled water, exposed approximately horizontally and supported by a nylon net above a tray of cool water to provide a constant and thermally contrasting background. The temperature of the leaves was monitored at 30 seconds intervals using an infrared camera (NEC ThermoVision, TH7102MV, Metrum Information Storage, Finchampstead, Berkshire, UK) with a temperature resolution of 0.05ºC and a spatial resolution of 0.5 mm^2. A grease spot was applied to the leaf as a dry reference and pieces of wet filter paper were used as a wet reference surface. After approximately one hour of stabilization, one leaf of each genotype was transferred to a solution of ABA at 10^{-4} M and a second leaf excised from the petiole, simulating acute dehydration. The remaining leaf was kept with the petiole in water as a control.

Images were analysed by Maricela Rodriguez-Acosta using the software ImageJ (Abramoff *et al.*, 2004) to determine mean leaf temperature across the whole leaf and the temperatures of the grease spot and wet reference paper. From these temperatures, an index of conductance was calculated by Maricela Rodriguez-Acosta as $g' = ((T_{dry} - T_{leaf})/(T_{leaf} - T_{wet}))$, with T_{wet} (wet leaf temperature) taken from the filter paper and T_{dry} (dry temperature) taken from the grease spot, in a modification of the procedures of Jones (1999).

3.2.4. Data analysis

Data analysis was performed using the statistical package SPSS statistical software package (SPSS 17.0, Chicago, IL, USA). A test for normal distribution of data (Kolmogorov-Smirnov test) was performed for $\delta^{13}C$ and g_s of the mapping population, for the g_s diurnal data and for the g' values measured by thermal imaging. Transformations (log_{10}) were carried out in the case of non-normality and verified with a Kolmogorov-Smirnov test. General Linear Model test (GLM) was performed to identify significant differences between genotypes for the $\delta^{13}C$ and g_s of the mapping population. The diurnal values of g_s were tested with a GLM test for differences between treatment, time and their interaction treatment x time for each species. A paired t-test was performed between the initial and final time for each genotype and treatment for the thermal data.

3.2.5. AMMI model

Genotype x site interactions were studied for $\delta^{13}C$ data from the two sites for trees grown in single stem, for which consistent experimental design and planting were undertaken. Genetic variation of a plasticity parameter was estimated by Anne Rae through a multi-parametric approach, the additive main effect and multiplicative interaction (AMMI) analysis (Mandel, 1969; Gauch, 1992). The AMMI model was:

$$E(Y'_{jk}) = \mu + \alpha_j + \beta_k + \theta_1 \gamma_{j1} \delta_{k1},$$

where Y'_{jk} is the spatially corrected trait score of genotype j in site k, μ is the grand mean, α_j are genotype mean deviations (mean minus the grand mean), β_k are the site mean deviations, θ_1 is the singular value for SVD axis n, γ_{j1} is the genotypic interaction parameter (score) that measures sensitivity to hypothetical site factor denoted by δ_{k1}, and δ_{k1} is the site interaction parameter (score) that measures sensitivity to hypothetical genotypic factor denoted by γ_{j1}. For more details see Rae *et al.* (2008).

The greater the deviation of a principal component of a genotype from zero, the less stable is the genotype across sites. The resulting plasticity scores were mapped as QTL to identify genomic regions affected by the contrasting sites in the UK and Italy.

3.2.6. QTL mapping and discovery of candidate genes

The genetic linkage map used to map QTL was produced by G.A. Tuskan *et al.* (personal communication), and consisted of 91 SSR markers genotyped on 350 of the full-sib progeny, and 92 fully informative amplified fragment length polymorphisms (AFLP) genotyped on 165 genotypes of the progeny. Papadakis spatially corrected (Papadakis, 1984) averages for $\delta^{13}C$ at the UK site, the Italian site, and the plasticity parameter between sites were performed by Anne Rae with R software (version 2.0.1, A Language and Environment Copyright, 2004) and were used to map the QTL by Anne Rae.

QTL mapping was carried out by Anne Rae using the web-based software QTLexpress (Seaton *et al.*, 2002), with the function Large Single Full-Sib Family Analysis (Tree) (http://qtl.cap.ed.ac.uk/). Chromosome-wide permutation tests with 1000 iterations were performed and the identification of a QTL was achieved using the resulting F value where $p<0.05$. Initially models incorporating the paternal, maternal and interaction parameters were run. If a parameter did not differ significantly from zero, it was removed from the model and the analysis re-run. Confidence intervals were obtained by taking the distance in cM corresponding to an F drop-off of two from the maximum F value as described previously in Rae *et al.* (2006).

The QTL were drawn using the software MapChart (Voorrips, 2002). Using adjacent markers on the genetic and physical maps, genes within QTL were identified and studied in order to identify candidate genes.

3.3. Results

3.3.1. Family 331 carbon isotope composition and stomatal conductance

3.3.1.1. Leaf carbon isotope composition

For the two experimental datasets (UK and Italy), planted with an identical experimental design in contrasting climatic zones of Europe (Fig. 3.1a, 3.1b) and SRC data re-analysed from Rae *et al.* (2009, Fig. 3.1c), leaf $\delta^{13}C$ values varied between the genotypes (Italy: $F_{186,217}=1.7$, $p<0.001$; UK: $F_{204,143}=2.0$, $p<0.001$; SRC: $F_{267,214}=2.3$, $p<0.001$). There was a consistent pattern across all three datasets in that *P. deltoides* showed a lower $\delta^{13}C$ value than *P. trichocarpa*. The absolute magnitude of this differed in each environment, suggesting an important environment as well as genotype effect determining this trait (Fig. 3.1a, 3.1b, 3.1c). The spread of the F_2 data for $\delta^{13}C$ also changed with the environment. In the UK-Italy study, the spread of isotopic discrimination was increased in Italy, where trees were subjected to periods of soil drying, varying between -23 and -29 ‰, while in the UK data for the F_2 only varied between -25 and -29 ‰, with many more genotypes falling within the mean frequency categories. These data suggest that the more stressful environment in Italy resulted in a wider expression of phenotypic plasticity but in contrast to this, the SRC-grown *Populus* revealed a similar spread of data to that in Italy and may reflect the intensive management practice with trees grown extremely tightly spaced. *P. trichocarpa* had higher $\delta^{13}C$ values compared to *P. deltoides* and the F1 parents at each site and considerable transgressive segregation was apparent for this trait with exteme F_2 phenotypes in the population (Rieseberg *et al.*, 1999).

3.3.1.2. Stomatal conductance

After logarithmic transformation, the data for g_s in Family 331 were normally distributed with significant differences between the genotypes ($F_{215,372}=1.9$, $p<0.001$). *P. trichocarpa* (240 mmol m^{-2} s^{-1}) had higher values on average than *P. deltoides* (135 mmol m^{-2} s^{-1}), and the F1 parents showed higher g_s than both species. The progeny had a large range of g_s values from 10 to 470 mmol m^{-2} s^{-1}, again with considerable transgressive segregation observed (Fig. 3.1d).

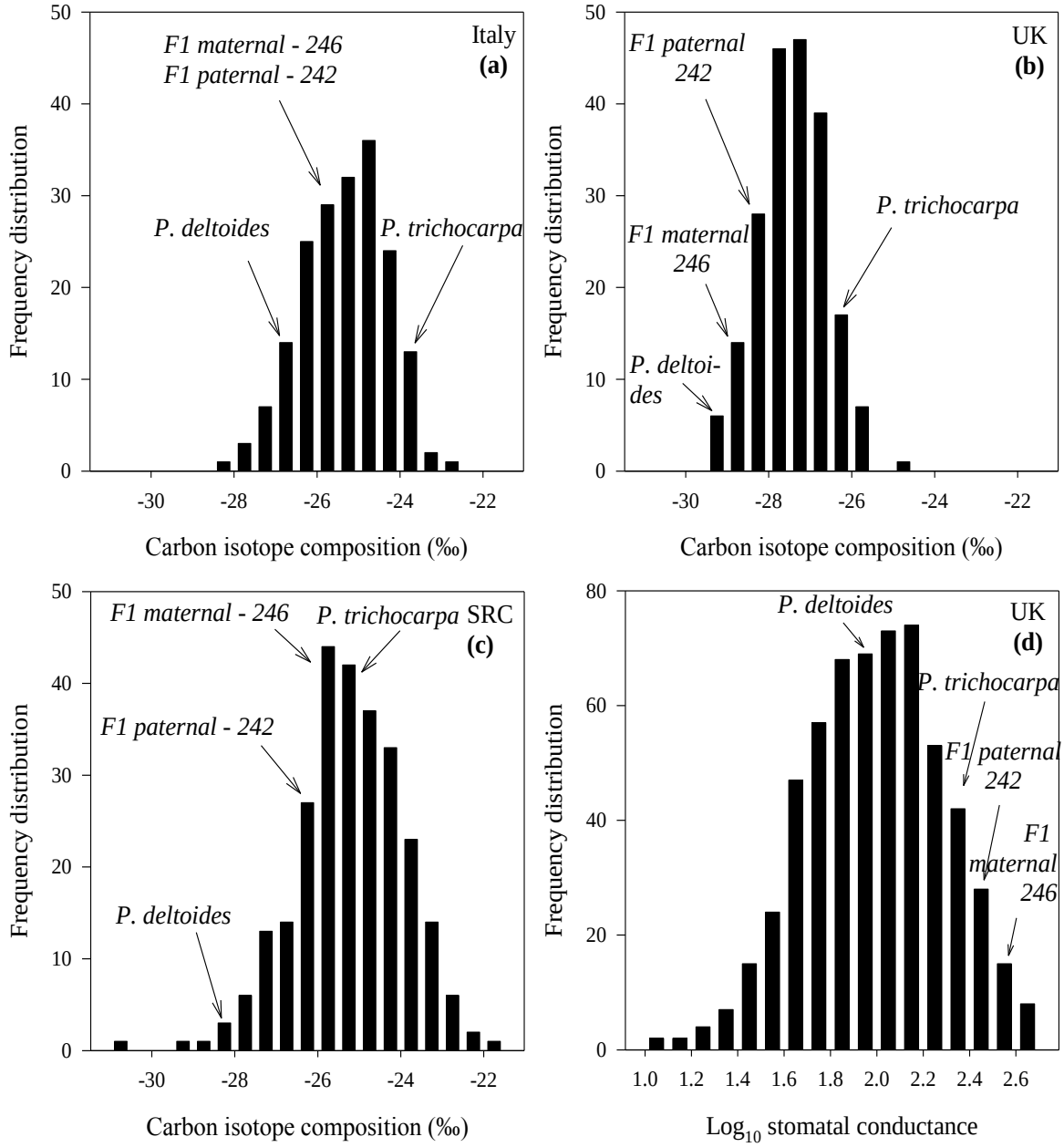


Figure 3.1: Frequency distribution of mean leaf carbon isotope composition, $\delta^{13}C$ (‰), in F_2 Family 331 of *Populus* for different environmental conditions and sites: single stem trees grown a fully replicated radomised and identical experiment in Italy **(a)** or in the UK **(b)** or grown in the UK as Short Rotation Coppice SRC **(c)** and mean $\log_{10}$ stomatal conductance from detached leaves of Family 331 trees from the single stem trees in the UK **(d)**.

3.3.2. QTL discovery and candidate genes

QTL were identified for $\delta^{13}C$ on nine LG (Fig. 3.2). At the Italian site six QTL were mapped for $\delta^{13}C$ explaining 41.1% of the total variation, on linkage group III, V, X, XII, XIII and XVII, while at the UK sites six QTL for $\delta^{13}C$ explained 30.2% of the total variation on LG IV, V, VI, X, XIII and XVII.

QTL representing the plasticity of $\delta^{13}C$ between environments collocated to all QTL mapped at the UK and Italian site with the exception of the QTL identified at the UK on LG VI. This suggests that with the exception of LG VI, genetic control of this trait was affected by the contrasting environments.

Two QTL for g_s measured at the UK site were mapped on LG VIII and XVI explaining 9.45% of the total variation (Table 3.1). This is a relatively small amount of variation accounted for by these QTL and reflects the difficulty of making measurements on large populations for physiologically based traits such as stomatal conductance, although it is also the case that many QTL identified are likely to be the largest effect QTL (Jacobsson *et al.*, 2005; Yang *et al.*, 2009; Ravi *et al.*, 2010). A QTL for the plasticity of $\delta^{13}C$ for single stemmed trees were compared between the UK and Italian site also maps to the stomatal conductance QTL on LG XVI.

LG VI, X and XVI were particularly interesting and deserved further study. LG VI contained one QTL for $\delta^{13}C$ in the UK, for both single stem and SRC trees (Fig. 3.3), as well as other QTL found in the literature, for example osmotic potential at full tugor (Tschaplinski *et al.*, 2006) close to the QTL hotspot on LG VI and several QTL for leaf development traits already published. Also, plasticity of $\delta^{13}C$ was not mapped on this LG which suggests genetic control of this trait was not affected by contrasting environments and that a 'constitutive QTL' is represented here which is less sensitive to environment. QTL for $\delta^{13}C$ were mapped consistently on LG X, in all three contrasting environments (Fig. 3.3). On LG XVI, one QTL for g_s, one QTL for $\delta^{13}C$ measured at the UK site and a plasticity QTL of $\delta^{13}C$ collocated with a leaf width to length ratio. 950, 758 and 979 genes were found within the 95% confidence intervals to which the QTL on LG VI, X and XVI were mapped respectively. A complete list of these genes is given in the Appendix CD1.

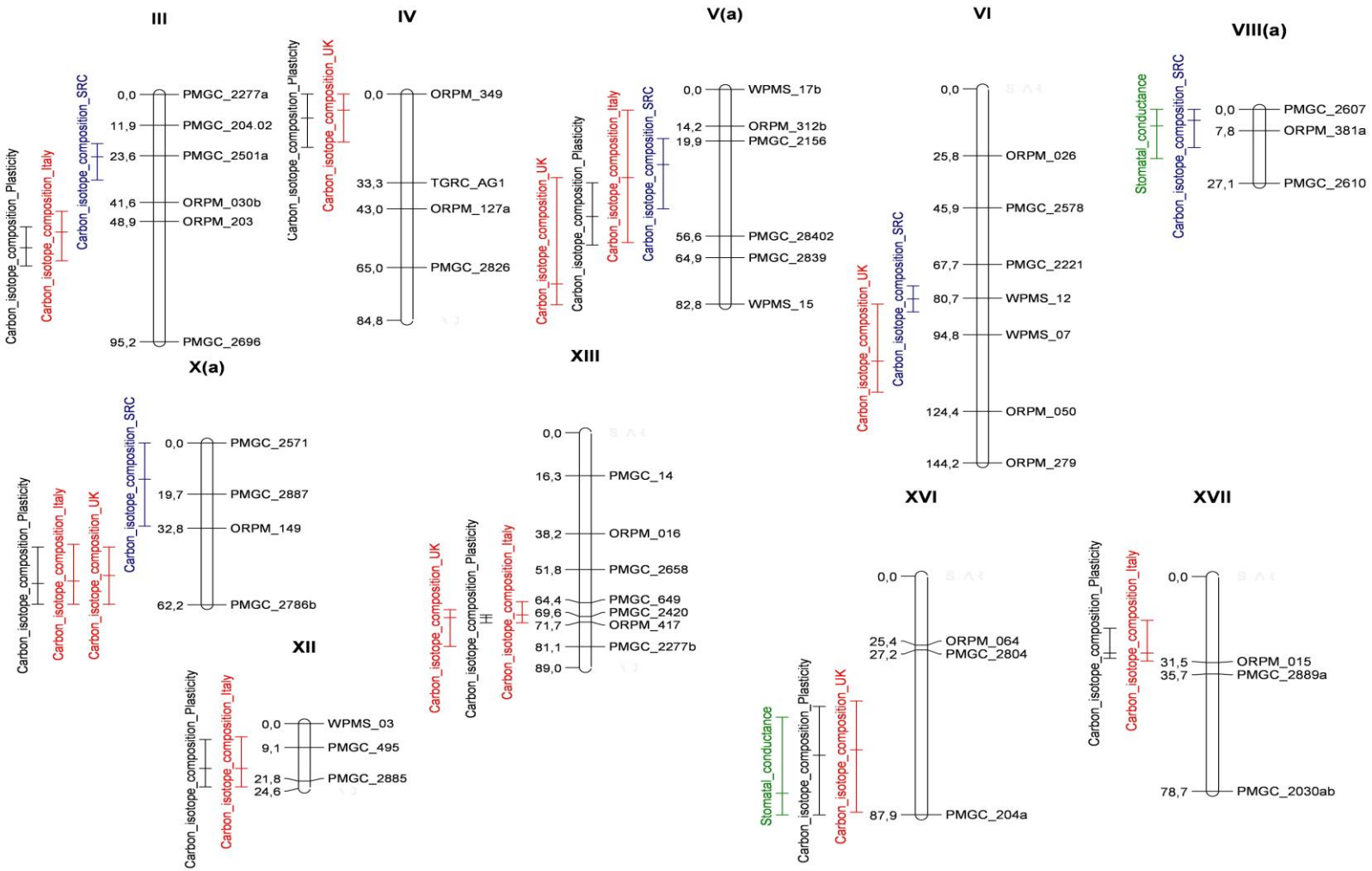


Figure 3.2: Quantitative Traits Loci (QTL) for $\delta^{13}C$ and g_s across the *Populus* genome. QTL mapped with the web-based software QTLexpress, with confidence intervals in cM and drawn in the software MapChart (Voorrips, 2002)

Table 3.1: The Quantitative Trait Loci (QTL) discovered for $\delta^{13}C$ and g_s found in the UK and Italy as single stem trees. QTL 'SRC' are modified from Rae *et al.* (2009) and were measured from trees grown in the UK as short rotation coppice.

Trait	Site	LG	Position	Confidence interval	p-value	Paternal effect	Maternal effect	Interac-tion effect	% VA
$\delta^{13}C$	Italy	III	53	45 - 64	<0.001	0.401	-	-	12.23
$\delta^{13}C$	Italy	Va	34	8 - 59	0.022	-0.200	-0.325	-0.285	6.33
$\delta^{13}C$	Italy	X	53	39 - 62	0.009	0.2700	-	-	4.47
$\delta^{13}C$	Italy	XII	17	10 - 24	0.001	-	0.294	-	6.31
$\delta^{13}C$	Italy	XIII	69	64 - 72	0.031	-0.226	-	-	4.03
$\delta^{13}C$	Italy	XVII	28	16 - 31	0.001	-0.220	0.235	-	7.73
$\delta^{13}C$	UK	IV	6	0 - 18	0.032	-	0.195	-	3.73
$\delta^{13}C$	UK	Va	75	34 - 83	0.025	-	-0.186	-	3.52
$\delta^{13}C$	UK	VI	105	83 - 117	0.001	0.248	-	-	6.77
$\delta^{13}C$	UK	X	51	40 - 62	0.001	0.241	0.233	-	9.03
$\delta^{13}C$	UK	XIII	70	67 - 81	0.037	-0.166	-	-	3.62
$\delta^{13}C$	UK	XVI	64	46 - 87	0.023	-	0.245	-	3.54
$\delta^{13}C$	Plasticity	III	59	51 - 66	<0.001	0.313	-	-	11.95
$\delta^{13}C$	Plasticity	IV	9	0 - 20	0.019	-	0.216	-	4.97
$\delta^{13}C$	Plasticity	Va	49	36 - 60	0.008	-	-0.222	-	5.88
$\delta^{13}C$	Plasticity	X	54	40 - 62	<0.001	0.266	0.109	-	9.43
$\delta^{13}C$	Plasticity	XII	17	6 - 24	0.025	-	0.157	-	3.34
$\delta^{13}C$	Plasticity	XIII	69	67 - 72	0.003	-0.199	-	-	6.36
$\delta^{13}C$	Plasticity	XVI	66	48 - 88	0.016	-	0.244	-	4.42
$\delta^{13}C$	Plasticity	XVII	28	19 - 30	0.005	-0.141	0.184	-	7.42
$\delta^{13}C$	SRC	III	24	19 - 33		-	0.23		2.4
$\delta^{13}C$	SRC	Va	29	19 - 46	0.06	-0.3586	-0.1417		4.5
$\delta^{13}C$	SRC	VI	81	76 - 86	0.05	-0.3122	-		4.8
$\delta^{13}C$	SRC	VIIIa	4	0 - 14	0.01	-0.3307	-0.14		4.9
$\delta^{13}C$	SRC	Xb	14	0 - 32	0.02	0.2376	0.3297		5
gs	UK	VIII(a)	6	0 - 18	0.022	8.983	-	-	2.55
gs	UK	XVI	80	52 - 88	0.005	7.945	-10.597	-15.981	6.90

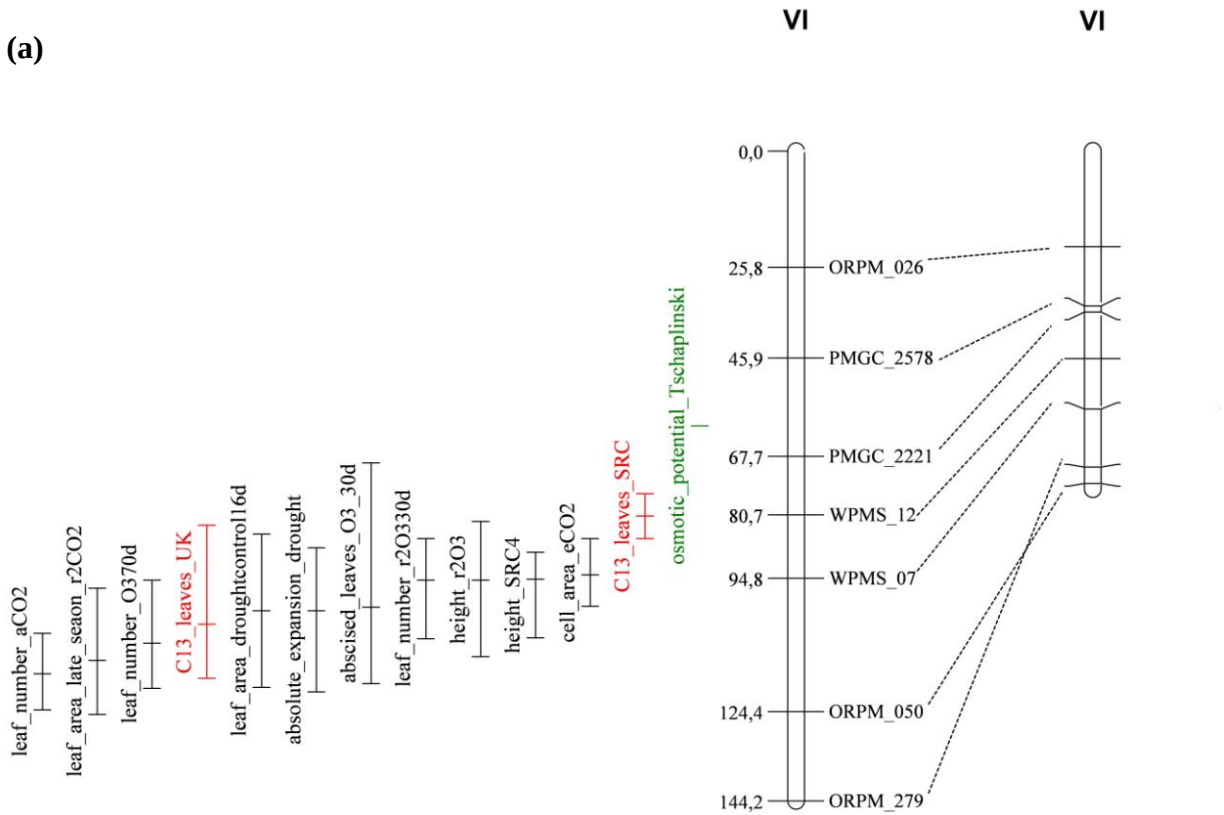


Figure 3.3: QTL 'hotspots' on linkage group VI **(a)**, X **(b)** and XVI **(c)**. The traits are $\delta^{13}C$ and g_s (UK and Italy). QTL mapped with the web-based software QTLexpress, with confidence interval in cM and drawn in the software MapChart (Voorrips, 2002). Other QTL were collected from the literature: osmotic potential at full tugor (Tschaplinski *et al.*, 2006), response to ozone stress (Street *et al.*, 2010), response to elevated CO_2 (Rae *et al.*, 2006), response to drought stress (Street *et al.*, 2006) and trees grown as SRC (Rae *et al.*, 2009). Detailed explanations of published traits are given in the Appendix A.

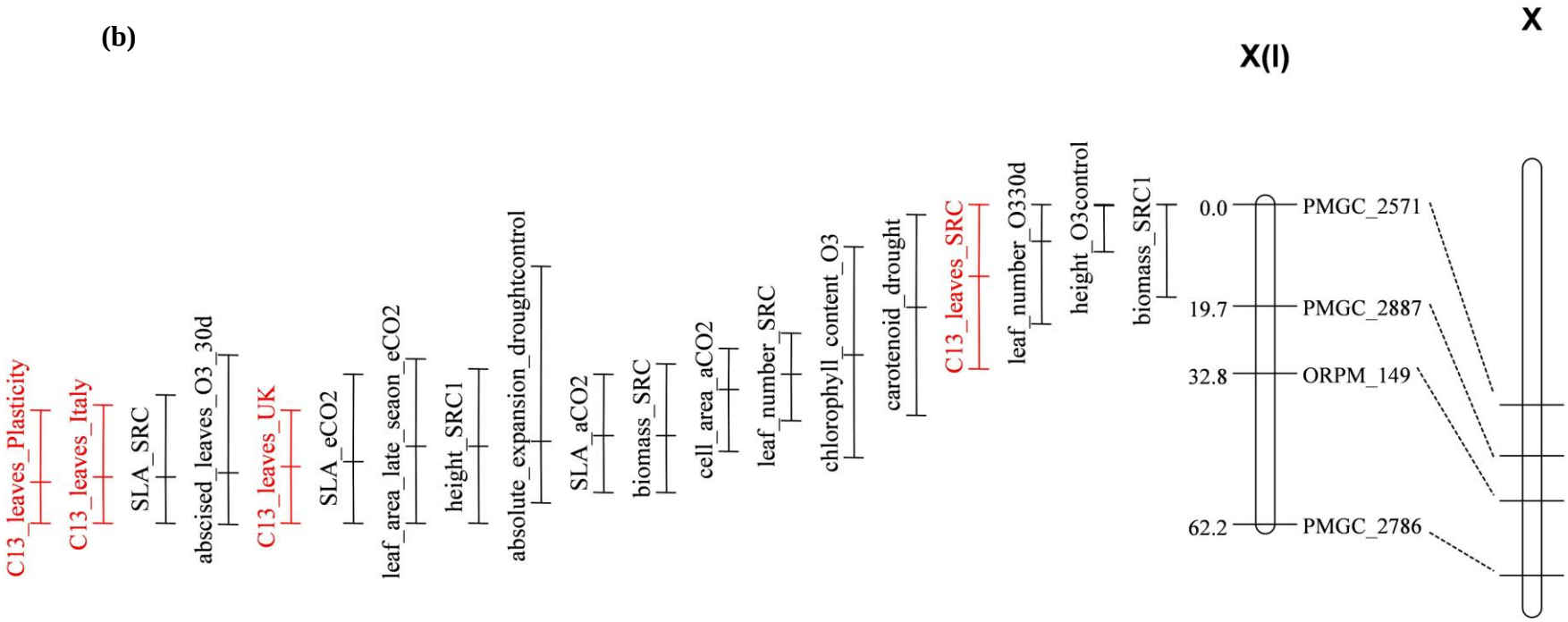


Figure 3.3 continued

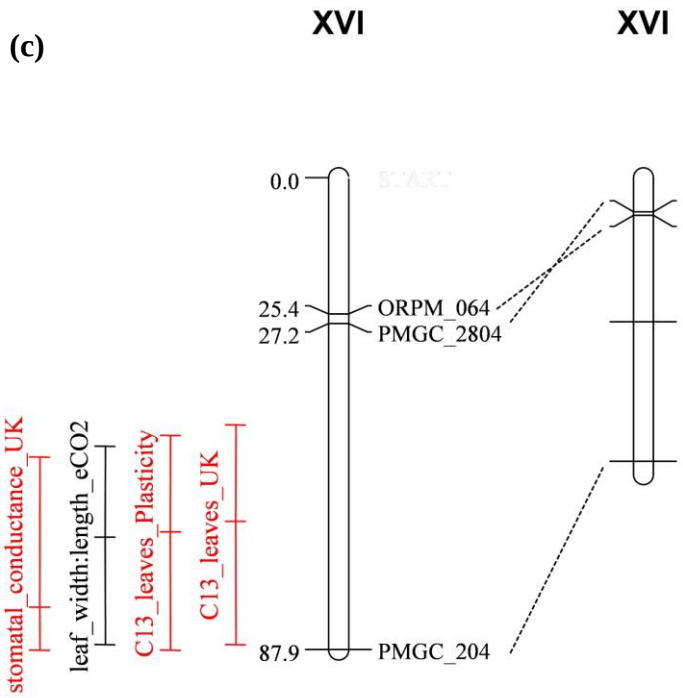


Figure 3.3 continued

In order to reduce the number of genes from this list within the hotspots and to identify novel targets in this mapping pedigree, published microarray data (Supplementary documents, Street *et al.*, 2006) of gene expression in response to drought, from the same genotypes of *P. deltoides* and *P. trichocarpa* reported here, were compared to the list of genes within the QTL hotspots. The genes from the microarray data were expressed in response to drought, but either showed a consistent response across both species (common) or were differentially expressed between them (differential). Forty seven genes from the microarray data were found within the QTL and twenty three within the QTL hotspots: 7 on LG VI, 6 on LG X and 9 on LG XVI (Table 3.2). It included a universal stress protein (UspA), ubiquitin and chaperone proteins (e.g. C3HC4-type RING finger), a translation initiation factor (elF-5A), serine/threonine protein kinases, an ankyrin repeat family protein and a late embryogenesis abundant (LEA) group 1.

Table 3.2: List of genes from the microarray analysis (Street *et al.*, 2006) highly expressed in response to drought which are also contained in the QTL hotspots on LG VI and X. The genes were either commonly expressed by *P. deltoides* and *P. trichocarpa* (up or down) or were differentially expressed by the two species in response to drought.

LG	**Protein Name**	**Description**	**Expression in response to drought for *P. deltoides* and *P. trichocarpa***
LG_III	estExt_Genewise1_v1.C_LG_III2205	Armadillo/beta-Catenin/plakoglobin	commonly expressed down
LG_III	eugene3.00030749	Ribonuclease, putative	differentially expressed
LG_III	gw1.III.2058.1	Expressed protein	commonly expressed down
LG_III	estExt_Genewise1_v1.C_LG_III0117	Microtubule-associated anchor protein involved in autophagy and membrane trafficking	commonly expressed up
LG_IV	estExt_Genewise1_v1.C_LG_IV1582	Lactoylglutathione lyase, putative / glyoxalase I, putative	commonly expressed up
LG_IV	eugene3.00040033	F0F1-type ATP synthase, gamma subunit, chloroplast precursor, Energy production and conversion	commonly expressed down
LG_IV	eugene3.00040082	Polyubiquitin (UBQ14), Posttranslational modification, protein turnover, **chaperones**	differentially expressed
LG_IV	estExt_fgenesh4_pm.C_LG_IV0060	Oxygen evolving enhancer 3	commonly expressed down
LG_V	estExt_fgenesh4_pm.C_LG_V0246	Squalene monooxygenase 2, Lipid transport and metabolism	commonly expressed up
LG_V	grail3.0027014001	Unknown	commonly expressed up
LG_V	estExt_Genewise1_v1.C_LG_V4699	RNA recognition motif (RRM)	commonly expressed down
LG_V	estExt_fgenesh4_pm.C_LG_V0368	Unknown	commonly expressed up
LG_V	estExt_fgenesh4_pm.C_L	60S ribosomal protein L3 and related proteins, Translation, ribosomal structure	commonly expressed down

	G_V0429	and biogenesis	
LG_V	estExt_Genewise1_v1.C_LG_V0611	Protein Mei2, essential for commitment to meiosis, and related proteins, Cell cycle control, cell division, chromosome partitioning	commonly expressed up
LG_V	estExt_fgenesh4_pg.C_LG_V1271	Unknown	commonly expressed up
LG_V	gw1.V.1264.1	Cytosolic sorting protein GGA2/TOM1, Intracellular trafficking, secretion, and vesicular transport	commonly expressed up
LG_V	eugene3.00051349	Armadillo/betacatenin like repeats	commonly expressed up
LG_V	estExt_fgenesh4_pg.C_LG_V1578	Thioredoxin, nucleoredoxin and related proteins	commonly expressed up
LG_V	fgenesh4_pg.C_LG_V001651	1,4 alpha glucan branching enzyme/starch branching enzyme II, Carbohydrate transport and metabolism	differentially expressed
LG_VI	gw1.VI.808.1	Ubiquitin C terminal hydrolase, Posttranslational modification, protein turnover, **chaperones**	commonly expressed down
LG_VI	estExt_fgenesh4_pg.C_LG_VI1067	ABC transporter family protein, Secondary metabolites biosynthesis, transport and catabolism	differentially expressed
LG_VI	estExt_Genewise1_v1.C_LG_VI0968	Translation initiation factor 5A (eIF5A), Translation, ribosomal structure and biogenesis	commonly expressed up
LG_VI	estExt_Genewise1_v1.C_LG_VI2117	Zinc finger (C3HC4 type RING finger) protein family, Posttranslational modification, protein turnover, **chaperones**, Predicted E3 ubiquitin ligase	differentially expressed
LG_VI	fgenesh4_pm.C_LG_VI000651	50S ribosomal protein L4, chloroplast (CL4), Translation, ribosomal structure and biogenesis	commonly expressed down
LG_VI	estExt_fgenesh4_pm.C_LG_VI0678	Glycine dehydrogenase (decarboxylating), Amino acid transport and metabolism	commonly expressed down
LG_VI	gw1.VI.198.1	MEKK and related **serine/threonine protein kinases**, Signal transduction mechanisms	commonly expressed up
LG_VI	estExt_Genewise1_v1.C_LG_VI2708	**Serine/threonine protein kinase**, Signal transduction mechanisms	differentially expressed

LG_VIII	estExt_Genewise1_v1.C_LG_VIII0367	**Serine/threonine protein phosphatase**, protein phosphatase 2C (PP2C)	commonly expressed up
LG_X	estExt_fgenesh4_pg.C_LG_X1117	Unknown	commonly expressed up
LG_X	grail3.0006045701	Unknown	differentially expressed
LG_X	gw1.X.2081.1	26S proteasome regulatory complex, ATPase RPT4, Posttranslational modification, protein turnover, **chaperones**	commonly expressed down
LG_X	estExt_Genewise1_v1.C_LG_X2383	Ankyrin repeat family protein, Encodes a protein with an ankyrin motif and transmembrane domains that is involved in **salt tolerance**	commonly expressed up
LG_X	estExt_Genewise1_v1.C_LG_X2607	Chlorophyll A B binding protein / LHCI type I (CAB)	commonly expressed down
LG_X	estExt_fgenesh4_pg.C_LG_X2053	Serine carboxypeptidases (lysosomal cathepsin A), Amino acid transport and metabolism	commonly expressed up
LG_X	estExt_Genewise1_v1.C_LG_X3317	Molecular **chaperone** (DnaJ superfamily), Posttranslational modification, protein turnover, chaperones	commonly expressed up
LG_X	estExt_fgenesh4_pg.C_LG_X2233	bZIP protein, Oxidoreductase activity, Aldehyde dehydrogenase	commonly expressed down
LG_XII	fgenesh4_pg.C_LG_XII000590	RNA splicing factor Slu7p, RNA processing and modification	commonly expressed up
LG_XII	grail3.0015004101	Microtubule binding protein involved in cell cycle control, EB1 protein, Cell cycle control, cell division, chromosome partitioning	commonly expressed up
LG_XIII	estExt_fgenesh4_pm.C_LG_XIII0455	Alpha expansin 2, cell wall organization and biogenesis	differentially expressed
LG_XVI	eugene3.00160262	mRNA binding protein Encore, Single-stranded nucleic acid binding R3H	commonly expressed up
LG_XVI	grail3.0004009401	RRM domain, **Late embryogenesis abundant (LEA) group 1**	commonly expressed up
LG_XVI	estExt_fgenesh4_pg.C_LG_XVI0528	ABC transporter family protein, Secondary metabolites biosynthesis, transport and catabolism	differentially expressed

LG_XVI	eugene3.00160593	HAD superfamily hydrolase, subfamily IIB	differentially expressed
LG_XVI	eugene3.00160660	Alkyl hydroperoxide reductase, **thiol specific antioxidant** and related enzymes, Posttranslational modification, protein turnover, **chaperones**	commonly expressed down
LG_XVI	eugene3.00161000	**UspA, response to stress**	commonly expressed up
LG_XVI	eugene3.00161195	Expressed protein	commonly expressed up
LG_XVI	estExt_fgenesh4_pg.C_LG_XVI1239	Unknown	differentially expressed
LG_XVI	estExt_Genewise1_v1.C_LG_XVI0292	Unknown	commonly expressed up
LG_XVII	gw1.XVII.194.1	Carbonic anhydrase family protein / carbonate dehydratase family protein, Predicted carbonic anhydrase **involved in protection against oxidative damage**, Inorganic ion transport and metabolism	commonly expressed up

3.3.3. Differences in stomatal behaviour between P. deltoides and P. trichocarpa

3.3.3.1. Slow droughting and diurnal changes

Diurnal patterns of g_s in mature leaves of *P. trichocarpa* and *P. deltoides* trees in a greenhouse both showed marked variation of g_s during the day (Fig. 3.4a, 3.4b). There was a maximum stomatal opening in the morning between 06:00 and 10:00 but there were differences between the two species in response to the treatment (after 15 days with no water) and the stomatal behaviour in the afternoon and the evening.

Under drought stress, *P. deltoides* showed a decrease in g_s when compared to the well-watered plants. For example at the peak time (08:00), g_s reached 700 mmol m^{-2} s^{-1} in well-watered plants and 250 mmol m^{-2} s^{-1} in droughted trees (Fig. 3.4a). The treatment effect was significant ($F_{1,41}$=4.65, p<0.001), as well as time ($F_{13,41}$=14.00, p<0.001) and their interaction treatment x time ($F_{13,41}$=0.17, p<0.05). After the morning, g_s of *P. deltoides* slowly decreased for well-watered trees reaching nearly zero after 21:00 while the droughted trees rapidly closed their stomata from 10:00 until 23:00 (Fig. 3.4a).

Stomatal conductance for *P. trichocarpa* also varied during the day ($F_{13,52}$=13.83, p<0.001; Fig. 3.4b) but did not show any significant differences between well-watered and drought treatments ($F_{1,56}$=0.14, p=0.709) and no significant interaction of treatment x time ($F_{13,56}$=0.46, p=0.938). For both treatments, a peak of g_s was observed between 06:00 and 09:00 with a maximum at 07:00 of 605 mmol m^{-2} s^{-1} (well-watered) and 623 mmol m^{-2} s^{-1} (drought) in average (Fig. 3.4b). Between 10:00 and 23:00, there were small differences in g_s for *P. trichocarpa* (300-400 mmol m^{-2} s^{-1}).

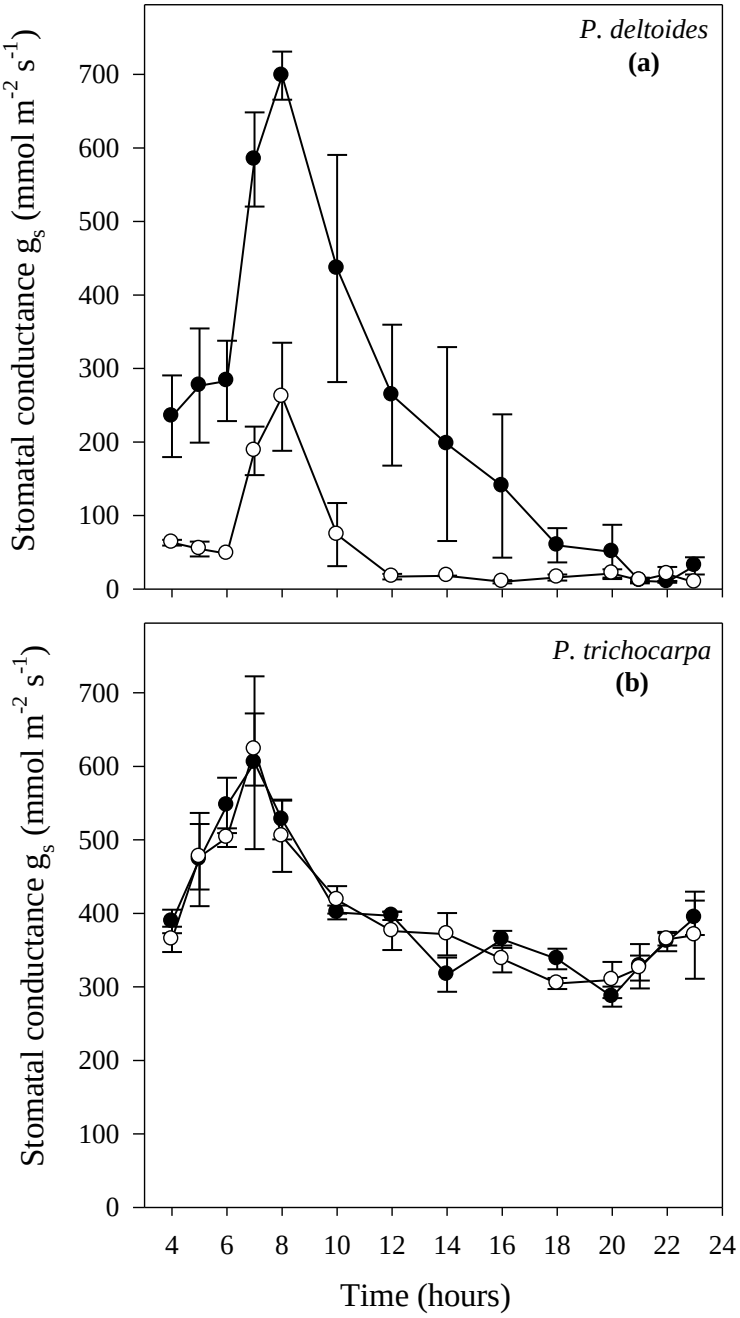


Figure 3.4: Diurnal time course of stomatal conductance, g_s (mmol m^{-2} s^{-1}), for leaves of *P. deltoides* **(a)** and *P. trichocarpa* **(b)** when well-watered (●) or after 15 days of drought (○) over a period of 19 hours from 4:00 to 23:00. Each value with bars represents the average ± standard error

3.3.3.2. Thermal imaging

Prior to treatments, leaves of both species under all conditions in the experiment had similar temperatures (Fig. 3.5a). Subsequently, the excised and ABA-treated leaves of *P. deltoides* first decreased rapidly in temperature, in a process referred as the 'Iwanoff effect' (Iwanoff, 1928) and is due to a sudden loss in epidermal turgor (Kaiser & Grams, 2006) then showed a rise in temperature after 30 minutes indicating stomatal closure (Fig. 3.5b and see video available in the Appendix CD2) while those of *P. trichocarpa* did not change, even after 2 hours (data not presented). Under control, well-watered conditions, mature leaves of both species showed similar values of temperature over time. However, the responses to ABA and excision treatments differed considerably between the two species.

Leaf temperature for *P. deltoides* decreased briefly for excised leaves and then increased rapidly, stabilizing after approximately 10 minutes for excised leaves. Leaves treated with ABA increased in leaf temperature and after 15 minutes leaf temperature was stable. Leaves of *P. trichocarpa* showed very limited responses to excision or ABA, compared to those observed in *P. deltoides* (Fig. 3.5) and leaf temperatures remained relatively constant.

Using the leaf and reference temperatures, relative conductance, g', was also calculated (Appendix B). For *P. deltoides*, g' responded to drought and ABA solution by decreasing from 0.8 to 0.2 while g' of *P. trichocarpa* remained constant over time after stress (Appendix B).

Relative leaf conductance g' was also studied and compared between a steady period prior to treatment and the end of the experiment for each genotype and at each treatment. Only the excised treatment in *P. deltoides* showed a significant difference between the initial and the final time (Fig. 3.6). Although, ABA-treated leaves of *P. deltoides* showed a decrease over time, more replicates would be necessary to be significant statistically.

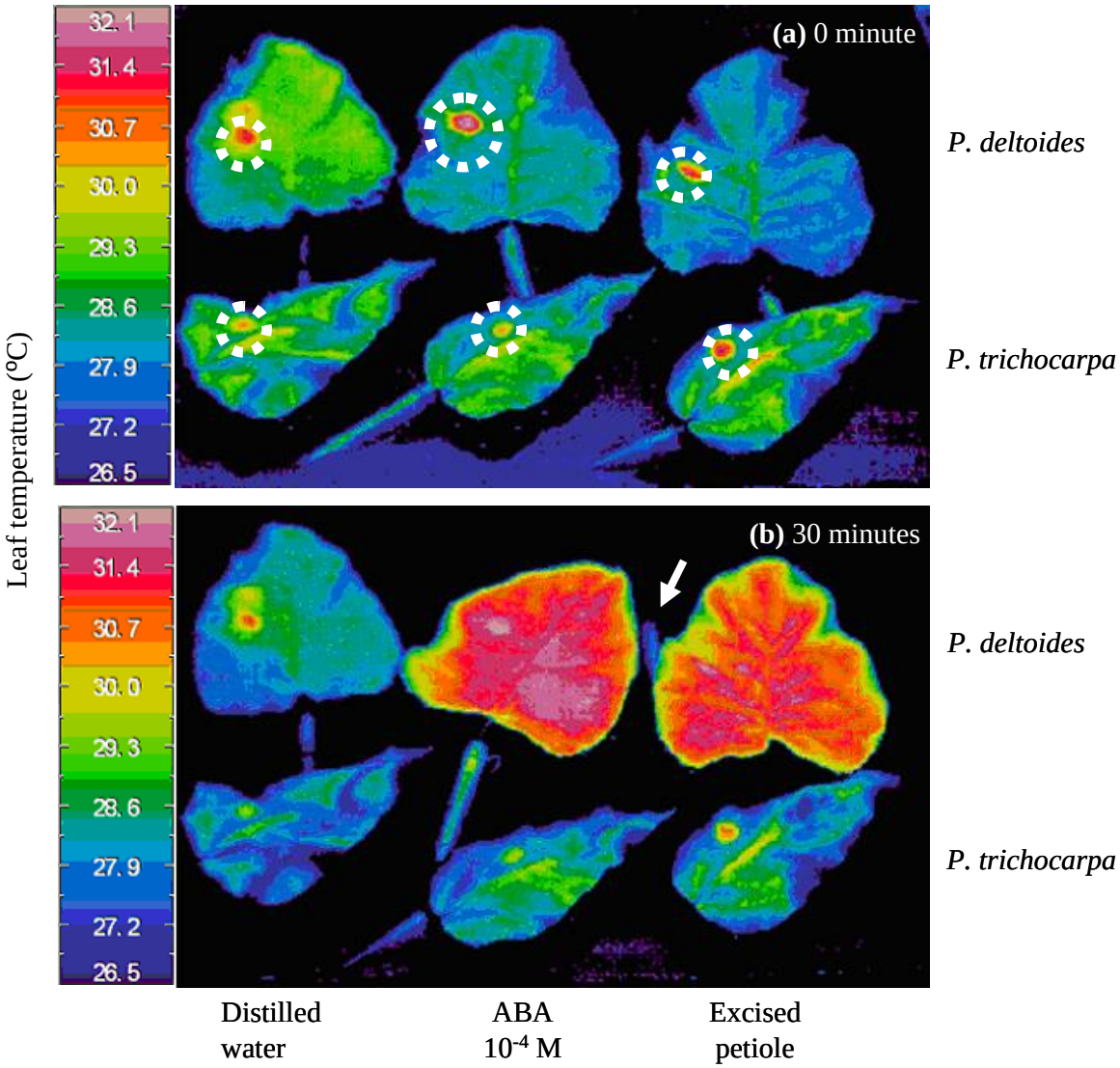


Figure 3.5: Infrared images of leaf temperature of mature leaves of *P. deltoides* and *P. trichocarpa* under three different treatments: leaves in distilled water (left), in ABA 10^{-4} M solution (middle) and with petiole excised simulating acute dehydration (right); **(a)** at the start of the treatments and **(b)** 30 minutes later. The left-hand scale shows leaf temperature in ºC. A grease spot was applied to each leaf as a dry reference (white dashed circle) and pieces of wet filter paper (white arrow) were used as a wet reference surface. A video of this is given in the Appendix CD2.

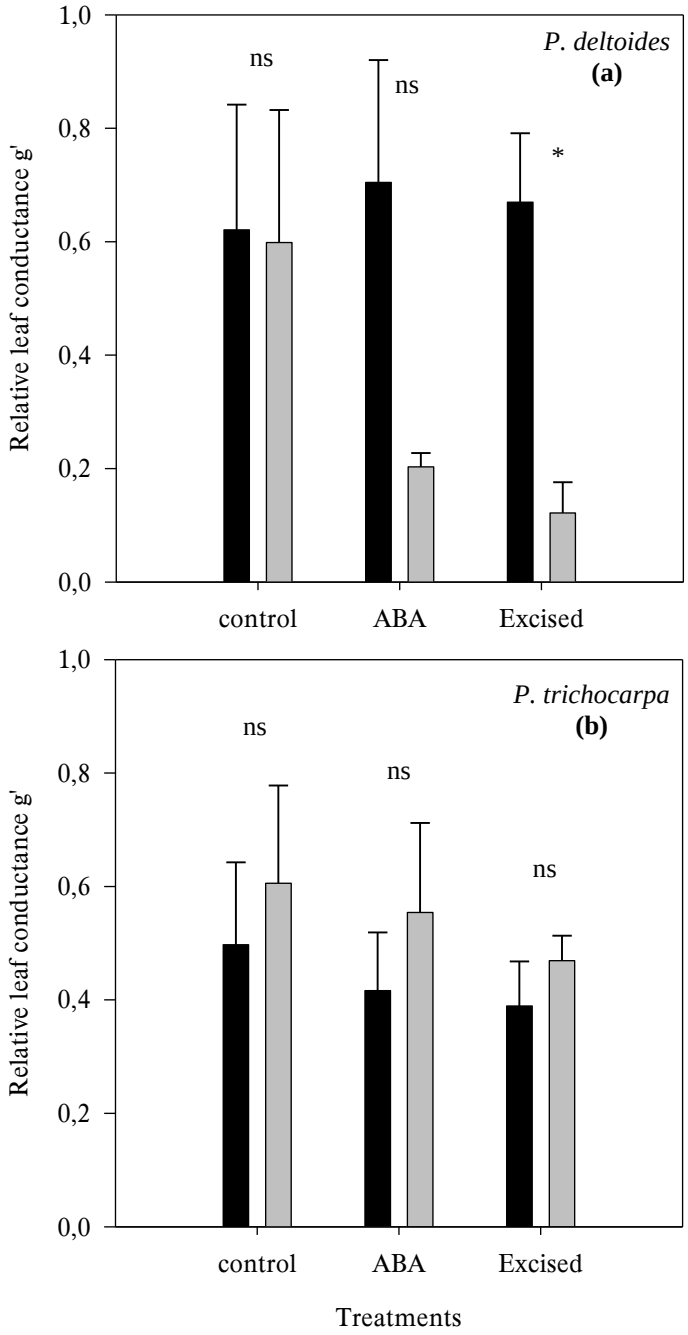


Figure 3.6: Relative conductance (g') calculated from leaf and reference temperatures in mature leaves of **(a)** *P. deltoides* and **(b)** *P. trichocarpa* in control condition, ABA of 10^{-4}M solution and excised condition at the initial (black column) and final time (grey column) of the experiment. Each value with bars represents the average ± standard error. ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$, n.s. non-significant

3.4. Discussion

The aim of this study was to gain a better understanding of the genetic basis of water use efficiency within *Populus* since it is already recognised that WUE is a valuable trait for breeding plants that are able to tolerate conditions with a reduced water supply (Collins *et al.*, 2008). It is also likely that water will be a considerable limiting factor for forest ecosystems across a wide scale in the future and that trees adapted to restricted water supply are likely to be of increasing importance. This includes bioenergy feedstock deployment (Somerville *et al.*, 2010), as well as conservation of semi-natural ecosystems (Gitlin *et al.*, 2006).

We have quantified the phenotypic plasticity in water use efficiency using the measurement of carbon isotope composition, for which there is extensive theoretical and empirical data to suggest that this composition is positively correlated with water use efficiency, for a wide range of crops, such as wheat (Farquhar & Richard, 1984; Condon *et al.*, 2002), coffee (DaMatta *et al.*, 2003), rice (Impa *et al.*, 2005; Xu *et al.*, 2009), sugar beet (Rajabi *et al.*, 2009), oak (Ferrio *et al.*, 2003; Brendel *et al.*, 2008), poplar (Ripullone *et al.*, 2004; Marron *et al.*, 2005; Monclus *et al.*, 2005; Dillen *et al.*, 2008). Here we were able to quantify phenotypic plasticity in the F_2 population for leaf $\delta^{13}C$, revealing considerable variation, dependent upon both genotype and the varying environments and management conditions to which the trees were subjected. A weak positive correlation between the dataset for the UK and Italy grown as single stem trees was observed ($r_s = 0.297$, $p<0.001$). Although few genotypes expressed high leaf $\delta^{13}C$ in Italy and low leaf $\delta^{13}C$ in the UK or inversely, genotypes with extreme values of leaf $\delta^{13}C$ (high or low) were generally the same for both sites. Given this variation, as with other studies, QTL were evident for $\delta^{13}C$, providing some insight into the genetic basis of water use efficiency. Our QTL accounted for a reasonably large amount of genetic variation. Carbon isotope measurements have also been mapped in other plants such as *Arabidopsis* (Masle *et al.*, 2005), rice (Xu *et al.*, 2009), chestnut (Casasoli *et al.*, 2004), maritime pine (Brendel *et al.*, 2002), oak (Brendel *et al.*, 2008).

Here QTL for stomatal conductance and carbon isotope composition measurements in the F_2 Family 331 were determined for these water use efficiency traits for the first time to our knowledge in *Populus*. Few QTL exist for stomatal conductance in the published literature in other plants (Ulloa *et al.*, 2000; Hervé *et al.*, 2001; Fracheboud *et al.*, 2002), reflecting the difficulty of measuring this trait on many hundreds on individuals in replication QTL mapping population experiments. Thus our attention was focused on carbon isotope composition as a surrogate for WUE. For three regions of the genome in particular, 'QTL hotspots' were defined where at least one QTL explained > 5% of the variation and where multiple QTL were present. These QTL hotspots were mapped to LG VI, X and XVI and contained two and four $\delta^{13}C$ QTL on LG VI and X respectively and two QTL for $\delta^{13}C$ and one for g_s on LG XVI. More QTL were found within these three areas of the genome from the literature for different traits that may be related to WUE: cell area (Rae *et al.*, 2006), leaf area (Rae *et al.*, 2006; Street *et al.*, 2006; Rae *et al.*, 2009), stomatal density (Rae *et al.*, 2006), biomass (Rae *et al.*, 2009; Street *et al.*, 2010) and osmotic potential at full tugor (Tschaplinski *et al.*, 2006).

Markers linking the genetic and physical map of the F_2 pedigree were used to determine gene models localised within these QTL 'hotspots' in the *Populus* genome (Tuskan *et al.*, 2006). 950, 758 and 979 genes were found within the 95% confidence intervals to which the QTL on LG VI, X and XVI were mapped respectively (Appendix CD1). Several candidate genes with a putative role in determining tolerance to drought stress were apparent in this list. Indeed two gene models on LG X and one on LG XVI had a dehydration description such as dehydration-responsive family protein. The hotspots also contained one universal stress protein (USP) on LG VI and two USPs on LG XVI. Five and six gene models related to response to oxidative stress were present on LG VI and XVI respectively and one related to response to low temperature and salt stresses were present on LG VI and X. Other examples on LG VI were an *AP2* transcription factor and *MYB* family transcription factor which are known to be involved in drought stress response pathways (Shinozaki & Yamaguchi-Shinozaki, 2007) and *ERECTA*, a gene found to be related to the regulation of plant transpiration efficiency as well as leaf organogenesis, cell expansion or cell division (Masle *et al.*, 2005). Within the confidence intervals for the QTL on LG X, gene models of interest included several heat-shock and chaperones which have a functional role in drought stress to repair

damaged proteins (Bartels & Sunkar, 2005), as well as early-response to dehydration protein-related genes which play a role in the regulation of the drought stress response within the ABA-independent pathway (Shinozaki & Yamaguchi-Shinozaki, 2007). A late embryogenesis abundant (LEA) group 1 was present within the hotspot on LG XVI. LEA proteins respond to abiotic stress, such as dehydration, osmotic stress and ABA (Hong-Bo *et al.*, 2005; Bartels & Sunkar, 2005) and their main role is to protect the cells from dehydration damages.

Combining microarray data (Street *et al.*, 2006) and genes within QTL hotspots, forty seven gene models were found within the QTL and 23 on LG VI, X and XVI which were also highly expressed in response to dehydration from the microarray analysis (Table 2): ten were up-regulated in *P. deltoides* and *P. trichocarpa* (2 on LG VI, 3 on LG X and 5 on LG XVI), seven were down-regulated in both species (3 on LG VI, 3 on LG X and 1 on LG XVI) and six were differentially regulated by the two species in response to drought (3 on LG VI and 3 on LG XVI). Many of those gene models were stress or water use related. For example, C3HC4-type RING finger genes are induced by drought and mediated by ABA-dependent pathways (Ma *et al.*, 2009a) and elF-5A is a translation initiation factor which has been observed in *Arabidopsis* mutants to improve growth under osmotic stress (Ma *et al.*, 2010). Other genes were also involved in signalling (serine/threonine protein kinases) and protein turnover such as chaperones and ubiquitins which are involved in drought stress response pathways (Shinozaki & Yamaguchi-Shinozaki, 2007). On LG X, a model gene commonly expressed in response to drought for *P. deltoides* and *P. trichocarpa* was an ankyrin repeat family protein involved in salt tolerance (Table 2). A universal stress protein (UspA) was also on LG XVI and up-regulated in both species. Taken together this gene list provides a valuable resource for further analysis, either through developing markers for these genes and testing them in different genetic backgrounds, such as for example willow bioenergy breeding programmes (Karp *et al.*, 2011) or reverse genetic approaches in *Arabidopsis* and *Populus* to test proof of concept which manipulation of these genes may lead to an altered phenotype (Du *et al.*, 2009; Behnke *et al.*, 2010; Mohamed *et al.*, 2010).

Stomatal response to slow soil droughting differed dramatically between *P. trichocarpa* and *P. deltoides*. *P. deltoides* closed their stomata in response to drought, while stomata

on *P. trichocarpa* mature leaves did not respond to dehydration. Continuous thermal imaging of whole leaves demonstrated the pronounced and rapid stomatal responses to excision simulating rapid dehydration and to exogenous ABA treatment in *P. deltoides* compared to *P. trichocarpa*. ABA is a stress hormone accumulated during drought and is involved in signal transduction in response to water deficit (Bray, 1997) and stomatal closure. Decreased stomatal conductance following leaf excision and exposure to ABA was observed for *P. deltoides* but our results confirm other observations that *P. trichocarpa* and its hybrids show limited ability for rapid stomatal closure in response to water deficit (Schulte & Hinckley, 1987a; Schulte & Hinckley, 1987b; Schulte *et al.*, 1987; Hinckley *et al.*, 1989; Kim *et al.*, 2008). The hybrid poplar 'Peace' (*P. koreana* x *P. trichocarpa*) showed little variation in stomatal closure under severe water stress, while in contrast, the genotype I-214 (*Populus euramericana*) showed a dramatic decline in g_s when drought was applied (Furukawa *et al.*, 1990). It appears that this limited stomatal response can partly be explained by stomatal insensitivity to ABA. Although increase in ABA concentration has been observed for both species under stress conditions (Schulte & Hinckley, 1987b), mature leaves of *P. trichocarpa* appear unable to respond to this ABA produced and here we have shown that exogenous ABA application was ineffective in inducing stomatal closure in *P. trichocarpa* compared to *P. deltoides*. However, stomatal behaviour was modified when trees of *P. trichocarpa* were preconditioned with water stress, followed by re-watering (Schulte *et al.*, 1987; Schulte & Hinckley, 1987a). If *P. trichocarpa* is exposed to drought, ABA production during the period of stress will influence developing leaves and enable stomata to function correctly (Schulte & Hinckley, 1987b).

Measurements of leaf $\delta^{13}C$ in the field in Italy and UK showed higher values for *P. trichocarpa* than for *P. deltoides*, implying a higher WUE for *P. trichocarpa*, and yet this would be counter-intuitive from our and others data on stomatal behaviour in these two species, that suggests a poor control of water loss in *P. trichocarpa* and poor WUE. However, $\delta^{13}C$ is controlled by both water loss and/or photosynthetic rate. Stomatal conductance was higher for *P. trichocarpa* in the UK site which could explain a better WUE for this clone. Even though *P. deltoides* has a better stomatal control and responds to water stress by closing its stomata, leaf $\delta^{13}C$ was also higher in Italy for *P. trichocarpa*. It has been suggested by Warren and Adams (2006), that caution should be

used in the interpretation of data on carbon isotopic discrimination, in particular, when cross-species comparisons are made, where differences between leaf anatomy and structure are likely. This is because the relationship between WUE and carbon isotope discrimination is influenced by the leaf internal conductance, a term rarely quantified but problematic in an intra-specific cross such as the one described here, where differences in leaf anatomy, cell size and intercellular spaces as well as leaf thickness are well known (Ferris *et al.*, 2002; Rae *et al.*, 2004; Street *et al.*, 2006). Internal conductance ($g_i=A/(C_i–C_c)$) is the diffusion of carbon from the substomatal cavities (C_i) to the sites of carbon fixation (C_c) (Warren, 2008) and may vary between species which would influence the relationship between $\Delta^{13}C$ and WUE (Warren & Adams, 2006). Despite this, it is also possible that once *P.* trichocarpa is pre-conditioned to drought, the WUE of emerging leaves may indeed be higher than that of *P. deltoides* as Schulte *et al.* (1987) demonstrated, developing stomata of *P. trichocarpa* after precondition by water stress responded very well to drought.

In conclusion, we have revealed wide variation in stomatal behaviour, WUE and response to drought within a *Populus* pedigree, that has enabled three 'hotspots' within the genome linked to water use efficiency to be identified. Data analysis has already identified a small number of candidate genes in these hotspots that provide targets for future molecular breeding and improved drought adaptation in *Populus*.

Chapter 4: Natural variation in water use efficiency, leaf development, cell and stomatal traits in *Populus nigra* L.

Abstract

A common garden in Belgium was established in 2004 for a study of wild genotypes of *Populus nigra* collected in Europe from contrasting environments. After two years of growth, natural variation in leaf area was revealed depending on their latitude of origin. In this chapter, extreme genotypes in leaf area discovered from this previous research in the common garden were used to investigate leaf development and variation in cell and stomatal traits between the two groups of genotypes. Eight genotypes with 'large leaves' and eight with 'small leaves' were grown in a greenhouse experiment at the University of Southampton. Genotypes from drier locations (Spain and Southern France) had small leaves, bigger cells and lower stomatal density than genotypes from wet environments such as North Italy and The Netherlands. These variations in Spanish genotypes could be the expression of an adaptation to drought stress. Carbon isotope discrimination ($\Delta^{13}C$) is an indirect negative measurement of water use efficiency (WUE). Wood and leaf were sampled to measure $\Delta^{13}C$ in the association population composed by 500 genotypes grown in the common garden. Genotypes varied in $\Delta^{13}C$ depending on their latitude of origin. *P. nigra* is a species present in Europe from the United Kingdom to central Asia. However this tree is threatened by human activities, habitat loss and global change. It is essential to understand the natural variation in this species for management purposes and breeding programs.

4.1. Introduction

Stomatal development is essential for plant survival and adaptation to changing environments by opening or closing their pore (Barton, 2007; Casson & Hetherington, 2010). Stomata control the CO_2 uptake and water vapour exchange through transpiration between the plant and the atmosphere (Boudolf *et al.*, 2004).
They are cellular structures developing on aerial parts of the plants such as leaf and stem. They consist of two symmetrically opposed cells called guard cells. The formation of stomata is complex and controlled by many genes including TMM (*Too Many Mouths*), FLP (*Four Lips*), YODA, SDD1 (*Stomatal Density And Distribution 1*) and *ERECTA* (Boudolf *et al.*, 2004; Bergmann & Sack, 2007). Three main steps are necessary for the formation of a stoma. Firstly, a neutral prodermal cell becomes a meristemoid mother cell (MMC) which divides into a small (meristemoid) and a large daughter cell called stomatal lineage ground cell (Barton, 2007). Secondly, the meristemoid can transit into a guard mother cell (GMC) which can thirdly divide symmetrically to mature guard cells (Boudolf *et al.*, 2004; Barton, 2007; Bergmann & Sack, 2007).

Stomatal closure is a short-term adaptation to changing environments such as dehydration but the control of stomata number is a long-term adaptation (Casson & Hetherington, 2010). Environmental stresses can be sensed by mature leaves which will adjust by increasing or decreasing the stomatal density (SD) or stomatal index (SI) in their developing leaves (Brownlee, 2001). Under well-watered conditions, SD has been observed to be positively correlated with water use efficiency (Hardy *et al.*, 1995). Indeed, large SD could increase stomatal conductance which would increase CO_2 assimilation and photosynthetic rate would eventually rise (Schlüter *et al.*, 2003; Afas *et al.*, 2006).

Instantaneous water use efficiency (WUE) is the ratio between the net CO_2 assimilation and the transpiration rate. It is positively correlated with carbon isotope composition ($\delta^{13}C$), hence negatively correlated with carbon isotope discrimination ($\Delta^{13}C$) which has been widely used as an indirect measurement for WUE in many plants (Farquhar & Richard, 1984; Farquhar *et al.*, 1989; Condon *et al.*, 2002; DaMatta *et al.*, 2003; Ferrio *et al.*, 2003; Impa *et al.*, 2005; Brendel *et al.*, 2008; Xu *et al.*, 2009) including poplar

(Ripullone *et al.*, 2004; Marron *et al.*, 2005; Monclus *et al.*, 2005; Dillen *et al.*, 2008). It has been used in breeding programs to develop plants adapted to drought (Impa *et al.*, 2005; Stiller *et al.*, 2005).

Populus nigra is a typical poplar tree found along the rivers in Europe but also expands from the UK until central Asia (Muller *et al.*, 2002). It has many economical uses, including domestic plantations, for soil protection and also as a parent for breeding programs (Vanden Broeck, 2003). Loss of its habitat due to human perturbations is responsible for this species to be near extinction in Europe, especially in Belgium and the UK (Muller *et al.*, 2002; Vanden Broeck, 2003; Gaudet *et al.*, 2008). It is also a tree abundant in riparian woodlands (Vanden Broeck, 2003) and by consequence sensitive to drought stress (Aylott *et al.*, 2008).

In this chapter, we study the natural variation in leaf area, carbon isotope discrimination, cell and stomatal measurements in a natural population of *Populus nigra*. This population consists of wild black poplars collected from five different European countries thus from contrasted latitude of origin in precipitation and light intensity but grown in the same environment. Comparison between field and greenhouse conditions was made for leaf area and cell/stomata traits. This important genus for forest ecology and economy is under threat with habitat loss and future climate change such as summer droughts. It is therefore important to study the natural variation of *P. nigra* for management and conservation.

4.2. Material and Methods

4.2.1. Plant material and condition of growth

This chapter is divided into two experiments: a field trial in Belgium and a greenhouse experiment in South England.

4.2.1.1. Belgium experiment

Cuttings from wild trees of *Populus nigra* were collected to generate an association population of 479 genotypes from five different European countries: Spain, France, Italy, Germany and The Netherlands, representing a wide range of precipitation and temperature (Fig. 2.2, Chapter 2). Genotypes were grouped into 11 river populations related to the river system where the collection was made (Table 4.1). The field plantation was part of the Popyomics project (http://www.soton.ac.uk/~popyomic/index.htm). Cuttings were planted in a common garden in Belgium, Geraardsbergen (50º 46‟51.23”N) in the spring 2004, cut at the base in the spring 2005 and in June 2005 side stems were cut so the trees grew as single stem. The field was divided into 6 blocks each containing a replicate of each genotype with a double row of *Populus* ‘Muur’ planted around the 6 blocks in order to reduce the edge effect.

Table 4.1: Summary of the river population of *P. nigra* with their country of origin

River population	Country	Colour code
Ebro1	Spain	Red
Ebro2	Spain	Red
Loire Est	France	Yellow
Loire W	France	Yellow
Drôme1	France	Yellow
Drôme6	France	Yellow
Durance	France	Yellow
Ticino W	Italy	Purple
Ticino Est	Italy	Purple
Rhine	Germany	Blue
Netherlands	Netherlands	Green

Colours were allocated for each country and used for all the graphs produced in this chapter (Table 4.1).

4.2.1.2. Greenhouse experiment

From the measurements done in the field in Belgium, 16 extreme genotypes were selected from the size of their mature leaves, eight with the smallest leaves and eight with the largest (Table 4.2).

A greenhouse experiment using these extreme genotypes was undertaken in partnership with Harriet Trewin. Settings in the greenhouse were kept at 16h:8h, light:dark, 22.5ºC:21ºC. Unrooted cuttings were planted in pots of 19L with replicates for each genotype. Two rows of *P. nigra* guard trees surrounded the experimental trees on two sides (left and right) and five rows on the other sides (top and bottom) reducing the edge effect. Labelling errors occurred during the planting of the population in the common garden in Belgium. Leaf samples were not collected for DNA extraction and SSR for the trees used in the greenhouse experiment. The lack of marker information on some Spanish genotypes explains the decision to combine five genotypes from Spain into a separate group named 'Other Spanish'.

Table 4.2: Summary of the extreme genotypes of *P. nigra* and their origin

Genotype	River population	Country of origin
Other Spanish	Ebro1 and Ebro2	Spain
CART2	Ebro2	Spain
71092-36	Durance	France
71095-1	Durance	France
N30	Ticino W	Italy
N38	Ticino W	Italy
N53	Ticino W	Italy
N56	Ticino W	Italy
N66	Ticino W	Italy
SN19	Ticino Est	Italy
NI1682	Netherlands	Netherlands
NVHOF5-16	Rhine	Germany

The same colour code was used for the graphs with red for Spain, yellow for France, purple for Italy and green for Netherlands.

4.2.2. Carbon isotope discrimination

Wood was collected in March 2007 in the Belgium site. Sections of 30cm were cut from 40cm above ground. Each log was put in a plastic bag and stored in a cold room. Samples were debarked and cut in small pieces in the University of Southampton for carbon isotope discrimination measurements. They were dried in the oven for 18h at 70°C and ground using a ball grinder (Cyclotec, Helsinki, Finland) at the Forestry Commission in Alice Holt. Ground samples were weighed and 1mg was placed into a 6 x 4 mm tin capsule (Ultra-clean pressed tin capsules, Elemental Microanalysis, Devon, UK). A SerCon 20-20 Stable Isotope Analyzer with ANCA-GSL Solid/Liquid Preparation Module (SerCon, Crewe, UK) was used at the Scottish Crop Research Institute (Dundee, UK) to analyse the samples to calculate carbon isotope composition. The latter was determined using the formula: $\delta^{13}C$ (‰) = δ_{plant} = [(R_{sample} - $R_{reference}$) / $R_{reference}$] × 1000, where R_{sample} and $R_{reference}$ are the $^{13}C/^{12}C$ ratios of the sample and the reference respectively, in VPDB (Vienna Pee Dee Belemnite) units (Scrimgeour & Robinson, 2004). Carbon isotope discrimination was calculated as $\Delta^{13}C$ (‰) = [(δ_{air} - δ_{plant})/(1+(δ_{plant} /1000)] with δ_{air} assumed to be close to -8‰ (Farquhar & Richard, 1984; Monclus *et al.*, 2006).

Leaf samples were also collected from the fields in Belgium and placed in a paper bag for each sample by Harriet Trewin in August 2006. They were dried in the oven for 48h at 80°C and ground using a ball grinder (Glen Creston ball, Retsch MM300). Three replicates of three genotypes per country were selected and sent for $\Delta^{13}C$ the same way as for wood.

4.2.3. Leaf and cell measurements

A day before measurements started, the first emerging leaf was tagged with cotton and was labeled L1. The leaves following L1 were named L2, L3 until L10 as being the youngest (Fig. 4.1). Every other day, leaf area was measured following the leaf growth of L1 to L10 between February 13th and March 29th 2007, by drawing around them on a white paper and using ImageJ (Abramoff *et al.*, 2004) to calculate the leaf area.

On March 30th, L - 10 (10th leaf counted down from L1) was collected to measure mature leaf area and used for cell imprints on the abaxial and adaxial side of the leaf. Nail vanish was applied in the same area of the leaf for each replicate, left to dry,

carefully removed and place on a microscope slide (Gardner *et al.*, 1995; Trewin, 2008). Imprints were collected with a Zeiss microscope at x40 magnification attached with a camera capturing the images. Cell number (CN), cell area and stomata number were measured using ImageJ (Abramoff *et al.*, 2004). From these values, stomatal density (SD) and stomatal index (SI) were calculated. Cell area (CA) was the average of 10 cell area (μm^2) in the field of view.

SD = Σ stomata number / Field of view

SI = 100 x [Σ stomata number / (Σ cell number + Σ stomata number)]

Cell number per leaf on the abaxial and adaxial side was measured using the cell number in the field of view converted in mm^2 and mature leaf area (mm^2)

CN per leaf = CN / leaf of view * leaf area

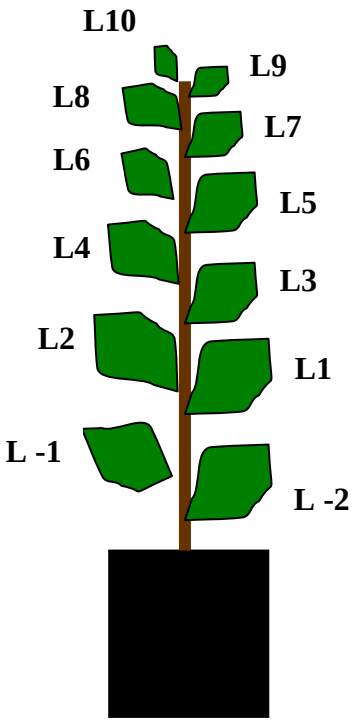


Figure 4.1: Diagram representing the labeling of the leaves during the greenhouse experiment

4.2.4. Statistical analysis

Statistical analysis was done using the SPSS software package (SPSS, Chicago, IL, USA). Normality (Kolmogorov-Smirnov test) was tested for all data and transformation (log_{10}) was carried when required to improve normality.

For the wood $\Delta^{13}C$ analysis, a GLM tested the effects of block and river population and also for genotype. A comparison of means was carried out between river populations using a Student-Newman-Keuls post-hoc. For the leaf $\Delta^{13}C$ analysis, a GLM test was used for country effect and Student-Newman-Keuls post-hoc compared the means between countries.

For the leaf area in the greenhouse, a GLM with repeated measures with time as a factor was testing the effects of genotype, leaf age and time and also leaf size (small or large), leaf age and time with their interactions. Statistical differences in cell and stomatal measurements were tested with a GLM for genotype or leaf size effects.

4.3. Results

4.3.1. Wood and leaf carbon isotope discrimination

An association population of 497 genotypes of *P. nigra* were used to study natural variation in wood carbon isotope discrimination ($\Delta^{13}C$) grown under well-watered conditions in a Belgium field (Fig. 4.2).

Differences between genotypes were observed ($F_{447,852}$=6.22, p<0.001). The genotype with the lowest average in $\Delta^{13}C$ was from The Netherlands (NI1421) at 19.66‰ while the highest average was 23.10‰ from the east side of the Loire River in France (925103). To study and understand these differences, genotypes were grouped into 11 populations defined by their native location along rivers.

Variation was also observed between river populations ($F_{10,1230}$=48.92, p<0.001). $\Delta^{13}C$ decreased along the latitude gradient meaning that under well-watered conditions, water use efficiency was higher for genotypes from more eastern latitude (e.g. The Netherlands and Germany). The post-hoc test revealed that Spanish and French genotypes from drier environments such as Durance were significantly similar in $\Delta^{13}C$ between 21.7‰ and 21.9‰ while French eastern genotypes such as Drôme1 and Drôme6 with an average of 21.3‰ were closer to Northern Italian ones (21.15‰). Black poplars from Germany and Netherlands had the lowest values of $\Delta^{13}C$ with an average of 20.8‰.

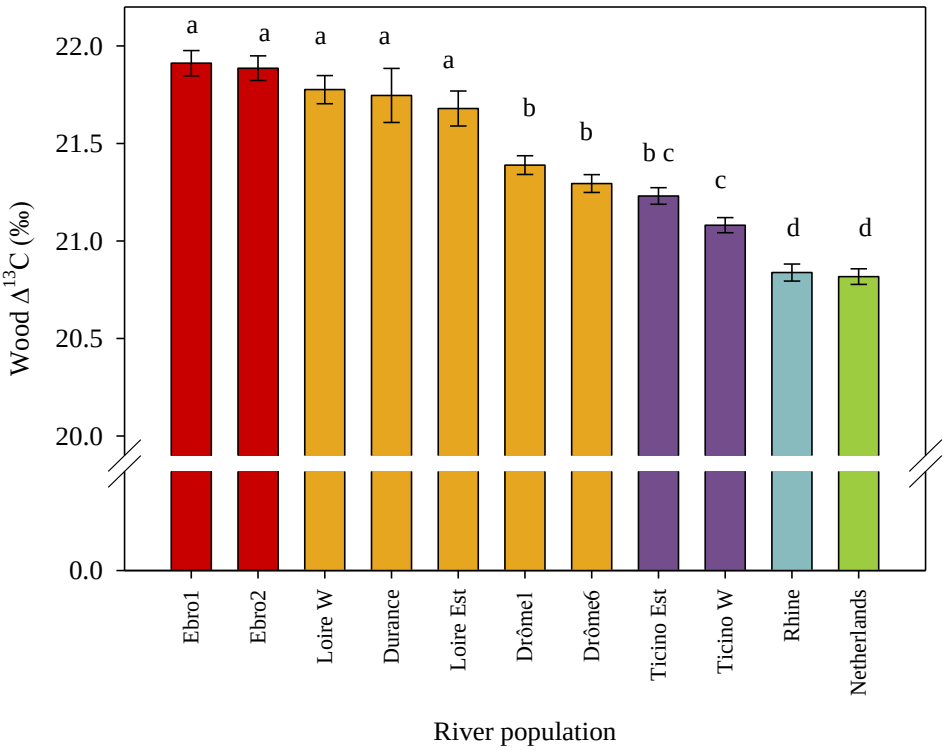


Figure 4.2: Wood carbon isotope discrimination for each river population grown in a common garden in Belgium. Same letter indicates no significant difference at 5% level, Student-Newman-Keuls post-hoc testing. Each value with bars represents the average ± standard error.

Leaves were also collected in the common garden and $\Delta^{13}C$ was measured with three genotypes per country. Values in $\Delta^{13}C$ were similar between wood and leaf values. Spanish and Southern French genotypes were significantly higher than North Italian, Germany and the Netherlands genotypes ($F_{4,40}=14.4$, $p<0.001$): Spain>South France>Italy>Netherlands>Germany (Fig. 4.3). The genotype with the highest $\Delta^{13}C$ was from Spain with values around 22.5‰ and the lowest was from Germany at an average of 19.2‰.

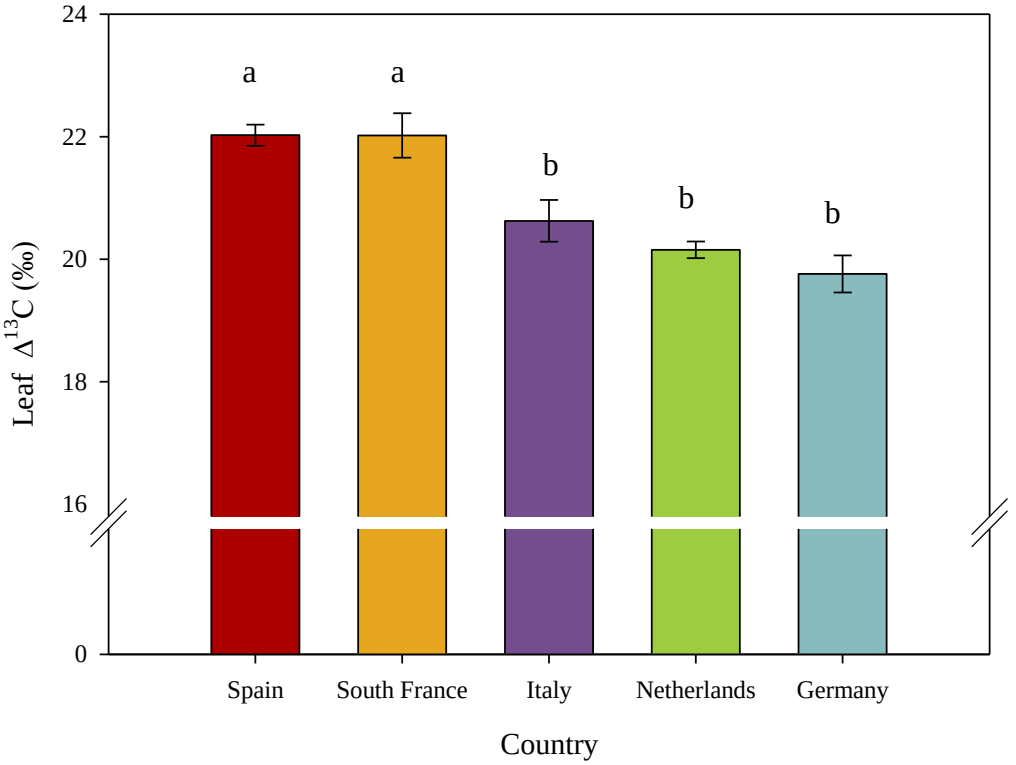


Figure 4.3: Leaf carbon isotope discrimination per country grown in a common garden in Belgium. Same letter indicates no significant difference at 5% level, Student-Newman-Keuls post-hoc testing. Each value with bars represents the average ± standard error.

4.3.2. Leaf growth

An experiment was then conducted in a control environment in the University of Southampton to study the leaf growth of extreme leaf size genotypes.

Figure 4.4 represents the averages of five replicates of leaf development from initiation until maturity for the first emerging leaf L1 for each 16 "extreme" genotypes. Unsurprisingly, 'large leaves' genotypes developed the largest leaf area with a maximal area between 3000 and 5000 mm^2. They were all originated from wet regions of Europe, The Netherlands, Germany and North Italy. The 'small leaves' genotypes had the smallest leaf areas between 400 and 1000 mm^2. They were from Southern France and Spain, hotter and drier European regions. Genotypes were significantly different ($F_{11,274}$=60.85, $p<0.01$) and an effect between large and small leaves was also observed ($F_{1,284}$=573.86, $p<0.01$). A post-hoc test also revealed the genotypes with 'large leaves' were significantly different in leaf area for L1 from the genotypes 'small leaves'. Interestingly, these genotypes showed a variation in leaf area depending on their latitude of origin, the smallest leaves being from low precipitation regions and the largest from wet environments.

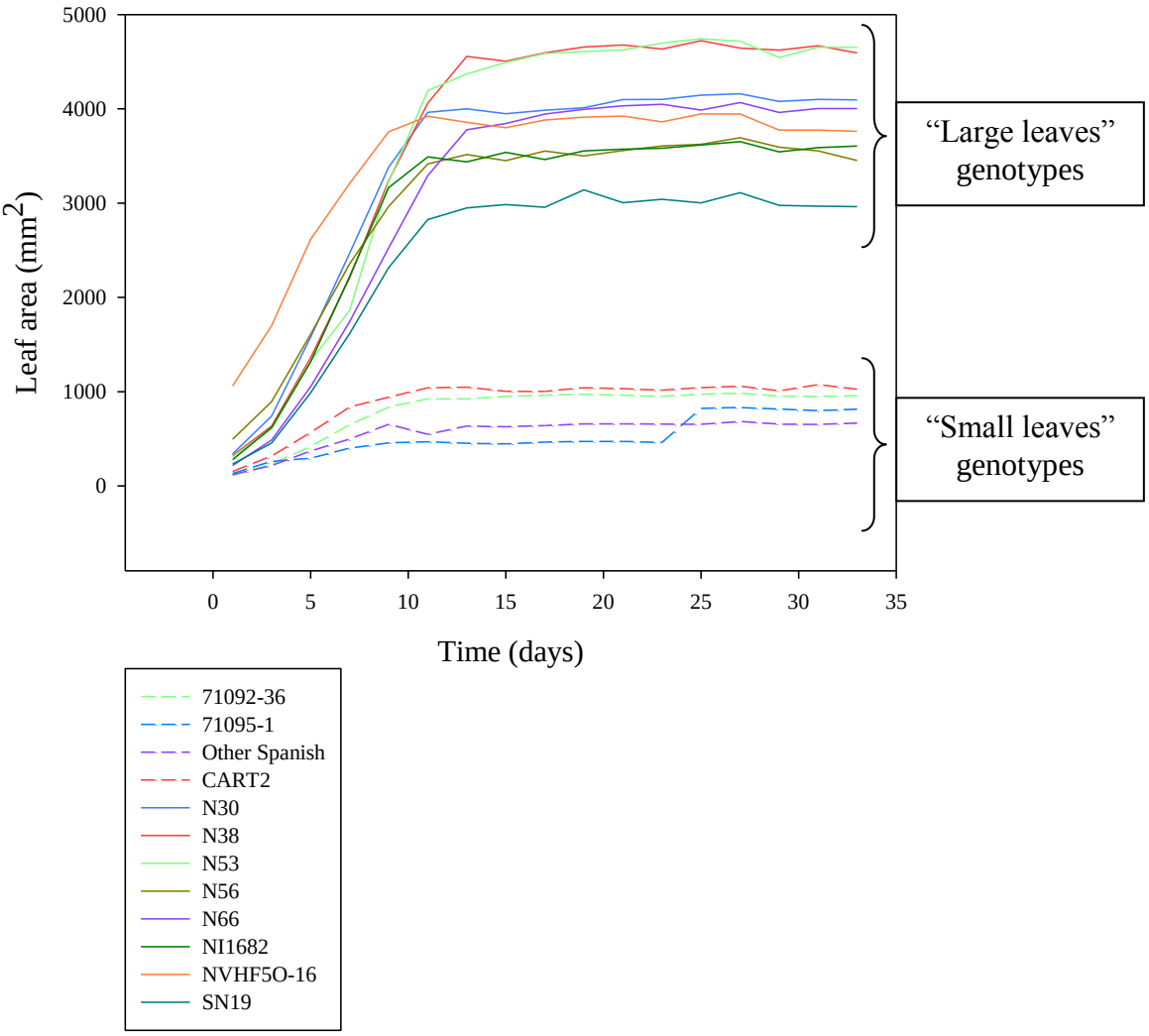


Figure 4.4: Change in mean leaf area (mm²) over time (days) for the first emerging leaf L1 for the 16 'extreme' genotypes

Observations of leaf development were also done for 10 leaves from leaf initiation until leaf maturity for 33 to 39 days (Fig 4.5 & 4.6).
Leaf area was significantly different between leaf ages ($F_{9,652}$=146.07, p<0.001) and between genotypes ($F_{11,652}$=286.65, p<0.001). A post-hoc revealed that the Spanish and French genotypes were significantly different in leaf area from the Italian, German and Dutch genotypes. When grouping the genotypes into two groups depending on the size of their leaves ('small leaves' versus 'large leaves'), leaf size had a significant effect on leaf area ($F_{9,752}$=78.53, p<0.001). Average leaf area at maturity varied between 600 and 1500 mm^2 for 'small leaves' genotypes (Fig. 4.5) and between 3000 and 6000 mm^2 for the 'large leaves' genotypes (Fig. 4.6).

For all genotypes, leaf growth was linear from the initiation until it reached maturity. Maximum growth was measured in days from initial until maximum leaf area on L1 for each genotype as it was the only leaf for some genotypes monitored until complete growth. It was also achieved quicker for 'small leaves' genotypes with an average of 12.75 days and 17.4 days for 'large leaves' genotypes.

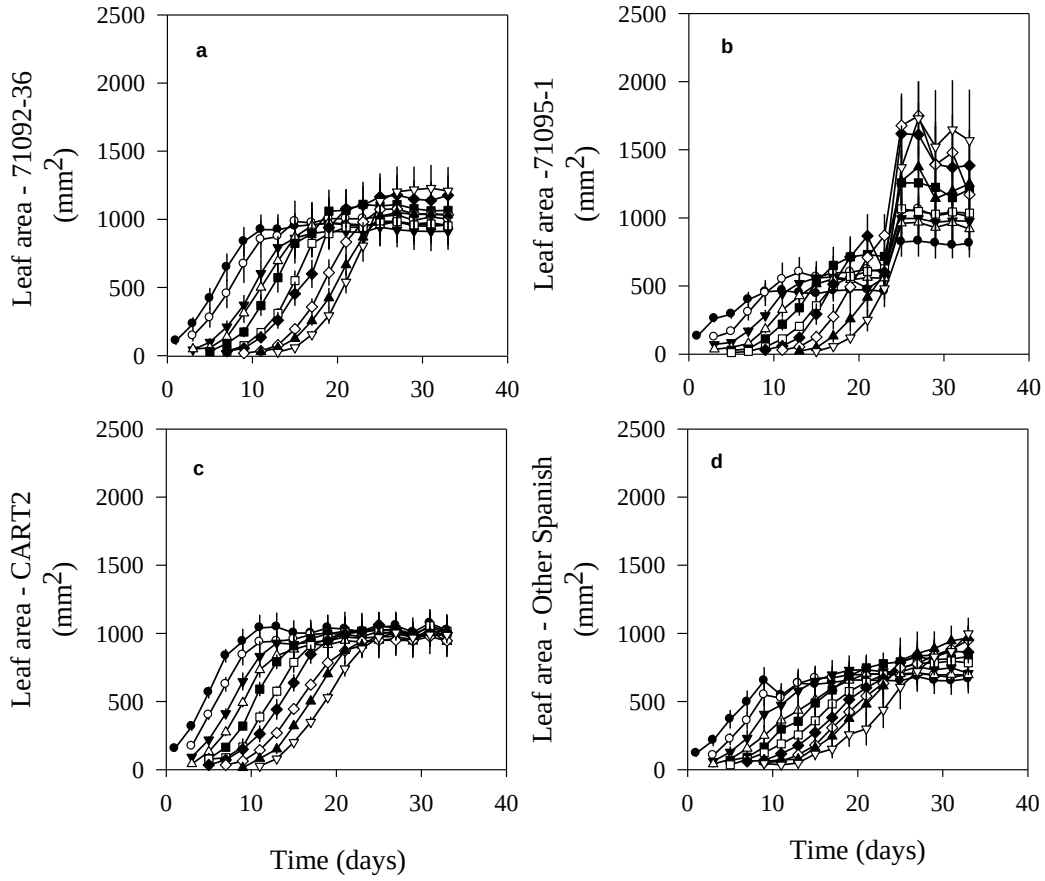


Figure 4.5: Mean leaf area (mm²) overtime (days) for each small-leaf genotypes: 71092-36 (a), 71095-1 (b), CART2 (c), Other Spanish genotypes (d) for the 10 leaves: ● L1, ○ L2, ▼ L3, ∆ L4, ■ L5, □ L6, ♦ L7, ◊ L8, ▲ L9, ▽ L10. Each value with bars represents the average ± standard error.

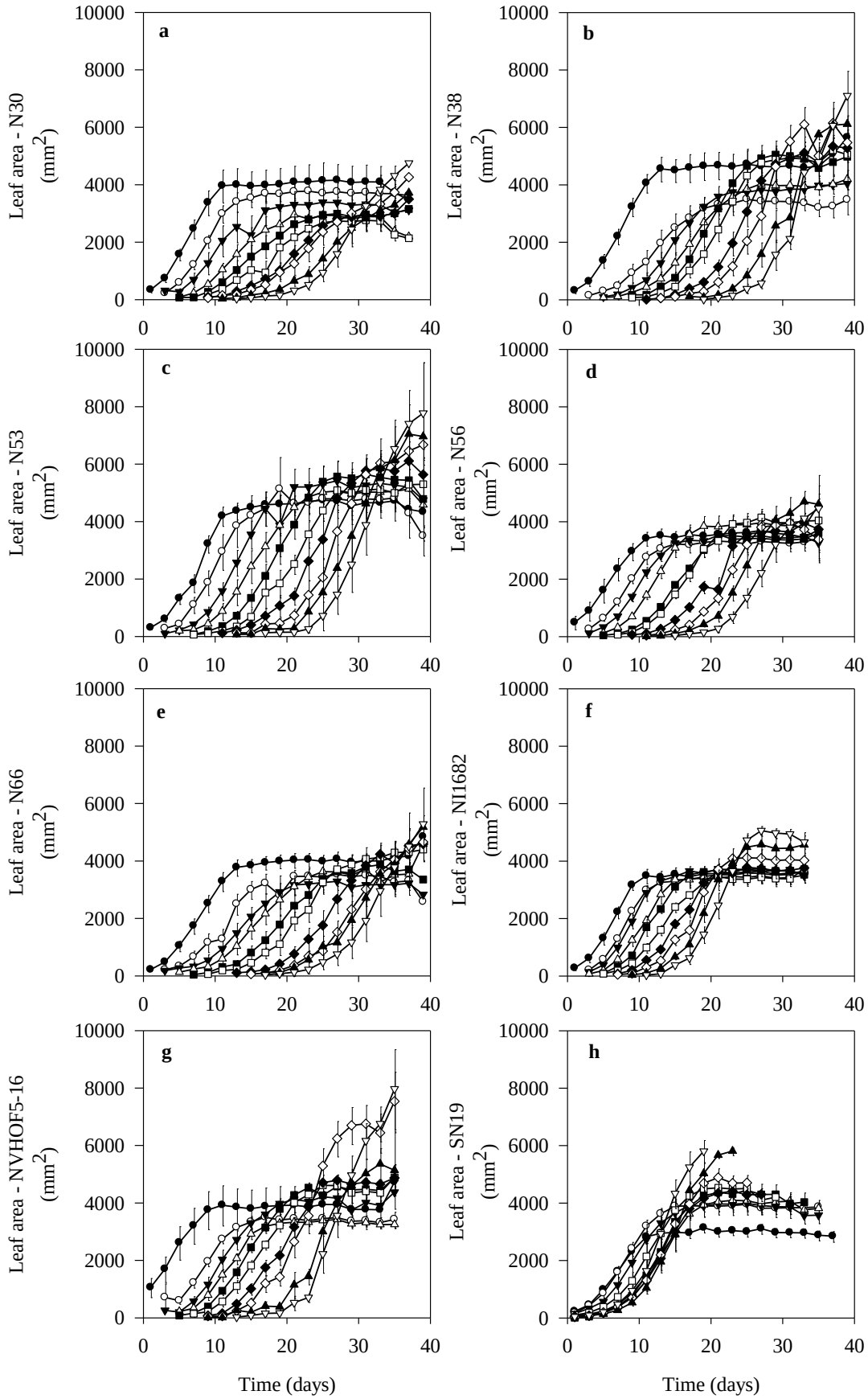


Figure 4.6: Mean leaf area (mm²) over time (days) for each large-leaf genotypes: N30 (a), N38 (b), N53 (c), N56 (d), N66 (e), NI1682 (f), NVHOF5-16 (g), SN19 (h), for the 10 leaves: ● L1, ○ L2, ▼ L3, Δ L4, ■ L5, □ L6, ♦ L7, ◊ L8, ▲ L9, ▽ L10. Each value with bars represents the average ± standard error.

4.3.3. Cell and stomata measurements

Cell imprints were made on one mature leaf for each replicate of *P. nigra* leaf extreme genotypes and used to calculate stomatal density (SD), average cell area (CA) and stomatal index (SI) on the abaxial (bottom) and adaxial (top) side of the leaf (Fig. 4.7, Fig. 4.8 & Table 4.3).

Cell area showed variation between genotypes (abaxial: $F_{12,58}$=6.29, p<0.001; adaxial: $F_{12,60}$=4.22, p<0.001) and also between leaf size groups (abaxial: $F_{1,69}$=46.51, p<0.001; adaxial: $F_{1,71}$=21.81, p<0.001) both sides of the leaf (Table 4.3). Cell area was also larger for the adaxial side (721.6 μm^2) compared to the abaxial (544.1 μm^2). Genotypes with small leaves had larger cells with an average of 659.7 μm^2 (abaxial) and 802.3 μm^2 (adaxial) while large leaves genotypes develop smaller cells, 428.5 μm^2 on the abaxial (Fig. 4.7A) and 641.0 μm^2 on the adaxial side of the leaf (Fig. 4.7B). The genotype C15 from Spain had the largest cells with an average of 742.2 μm^2 (abaxial) and 1024.2 μm^2 (adaxial). N56 (Italy) had the most extreme pattern as it developed the smallest cell areas at 374.8 μm^2 (abaxial) and 546.8 μm^2 (adaxial).

Cell number per leaf was higher on the abaxial size than adaxial with an average of 2.2 millions cells and 1.6 millions cells respectively (Fig. 4.7C and 4.7D). Differences were observed between genotypes on the abaxial ($F_{12,56}$=25.12, p<0.001) and the adaxial ($F_{12,58}$=21.3, p<0.001). N53 from North Italy have the largest number of cells per leaf (4.9 millions cells on the abaxial and 3.6 millions on the adaxial) while B7 from Spain had the lowest number on the abaxial (0.6 millions cells) and C15 (Spain) on the adaxial (0.4 millions cells). 'Large leaves' genotypes also developed significantly more cells on both sides of their leaves than genotypes with small leaves (abaxial: $F_{1,67}$=207.31, p<0.001; adaxial: $F_{1,69}$=201.57, p<0.001).

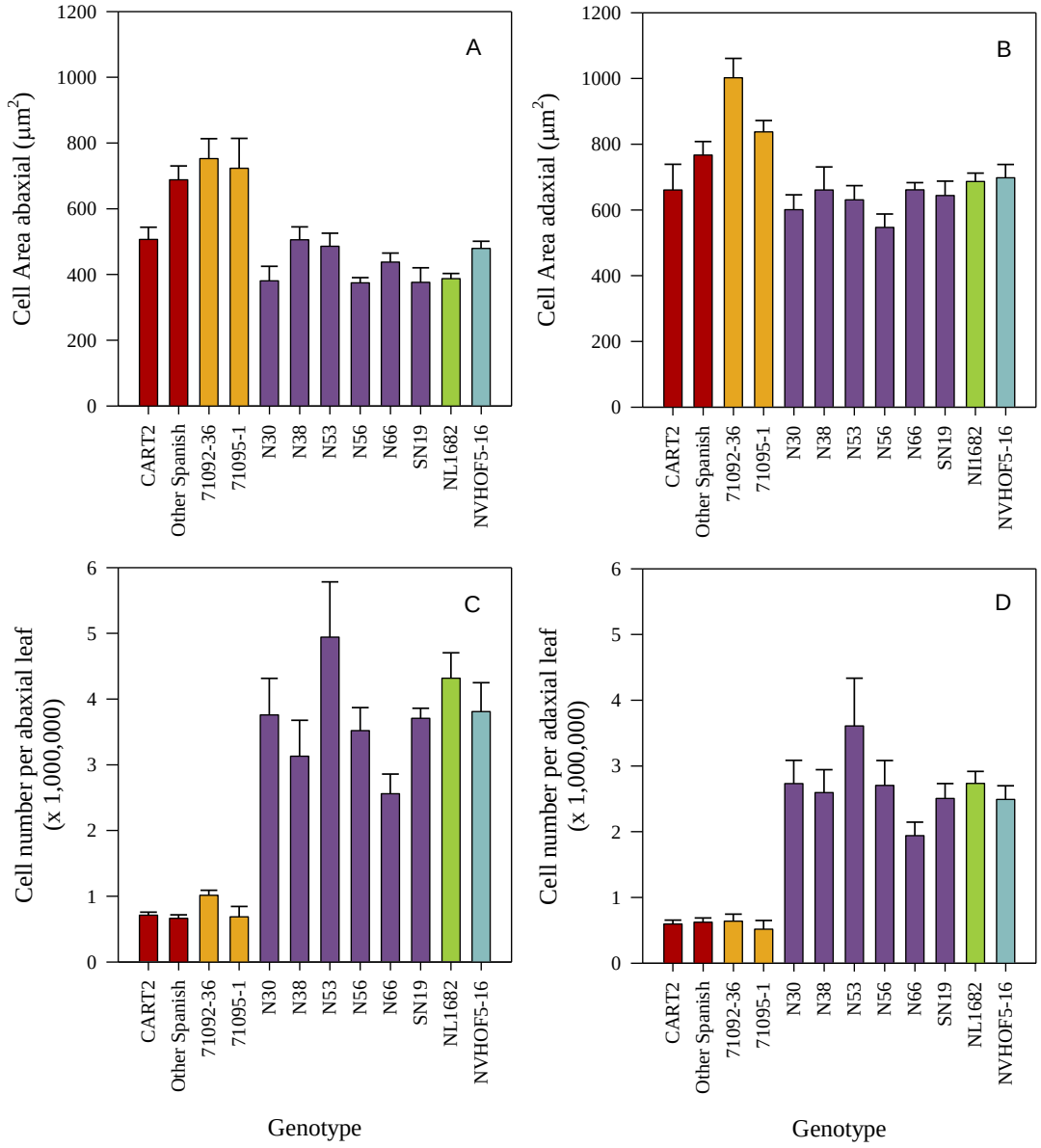


Figure 4.7: Cell measurements for each genotype growth in the greenhouse: cell area abaxial (A) and adaxial (B) in µm^2, and cell number per abaxial leaf (C) and adaxial leaf (D); Each value with bars represents the average ± standard error.

Table 4.3: Summary of the statistical results for cell and stomatal measurements done in the greenhouse experiment on the extreme genotypes. p-values in bold are significant ($p<0.05$).

	Genotype			Leaf size		
Traits	F	df	p	F	df	p
Stomatal Density abaxial	8.87	12,58	**<0.001**	35.23	1,69	**<0.001**
Stomatal Density adaxial	3.49	12,64	**<0.01**	0.75	1,75	0.389
Cell Area abaxial	6.29	12,58	**<0.001**	46.56	1,69	**<0.001**
Cell Area adaxial	4.22	12,60	**<0.001**	21.81	1,71	**<0.001**
Stomatal Index abaxial	0.89	12,58	0.562	0.00	1,69	0.99
Stomatal Index adaxial	1.997	12,60	**0.040**	2.44	1,71	0.12
Cell Number per leaf abaxial	25.12	12,56	**<0.001**	207.31	1,67	**<0.001**
Cell Number per leaf adaxial	21.3	12,58	**<0.001**	201.57	1,69	**<0.001**

SD was higher for abaxial than for adaxial with an average between all genotypes of 134.6 and 54.6 respectively (Fig 4.8 A&B). Variation in SD was also observed between genotypes for abaxial ($F_{12,58}=8.87$, $p<0.001$) and adaxial side ($F_{12,64}=3.49$, $p<0.01$) but when genotypes were separated between small and large leaves, differences between the leaf size in SD was only significant for abaxial ($F_{1,69}=35.23$, $p<0.001$) and not adaxial ($F_{1,75}=0.75$, $p=0.389$). Thus genotypes with large leaves had a significantly higher stomatal density on the abaxial side of the leaf (156.9) compared to the 'small leaves' genotypes (112.3). A Southern French genotype (71095-1) had the lowest SD with an average of 87.25 while N56 from North Italy was the highest with 198.5 in average.

SI was calculated taking into account the number of cells and stomata. Adaxial SI was lower than the abaxial SI with 4.2 and 6.6 respectively. On the abaxial side (Fig. 4.8C), SI showed no variation between genotypes ($F_{12,58}=0.89$, $p>0.05$) but also no variation between leaf size ($F_{1,69}=0.00$, $p>0.05$). For the adaxial (Fig. 4.8D), only genotype effect was significant ($F_{12,60}=1.997$, $p<0.05$). On the other hand, leaf size had no significant effect ($F_{1,71}=2.44$, $p>0.05$) . Although 'small leaves' genotypes had a higher SI in average, some genotypes had a small stomatal index such as RIN2 and C15 from Spain with 2.8 and 3.1 respectively. Conversely few 'large leaves' genotypes had a high SI, N56 for example had a SI of 5.3 while NVHOF5-16 stomatal index was in average 2.0.

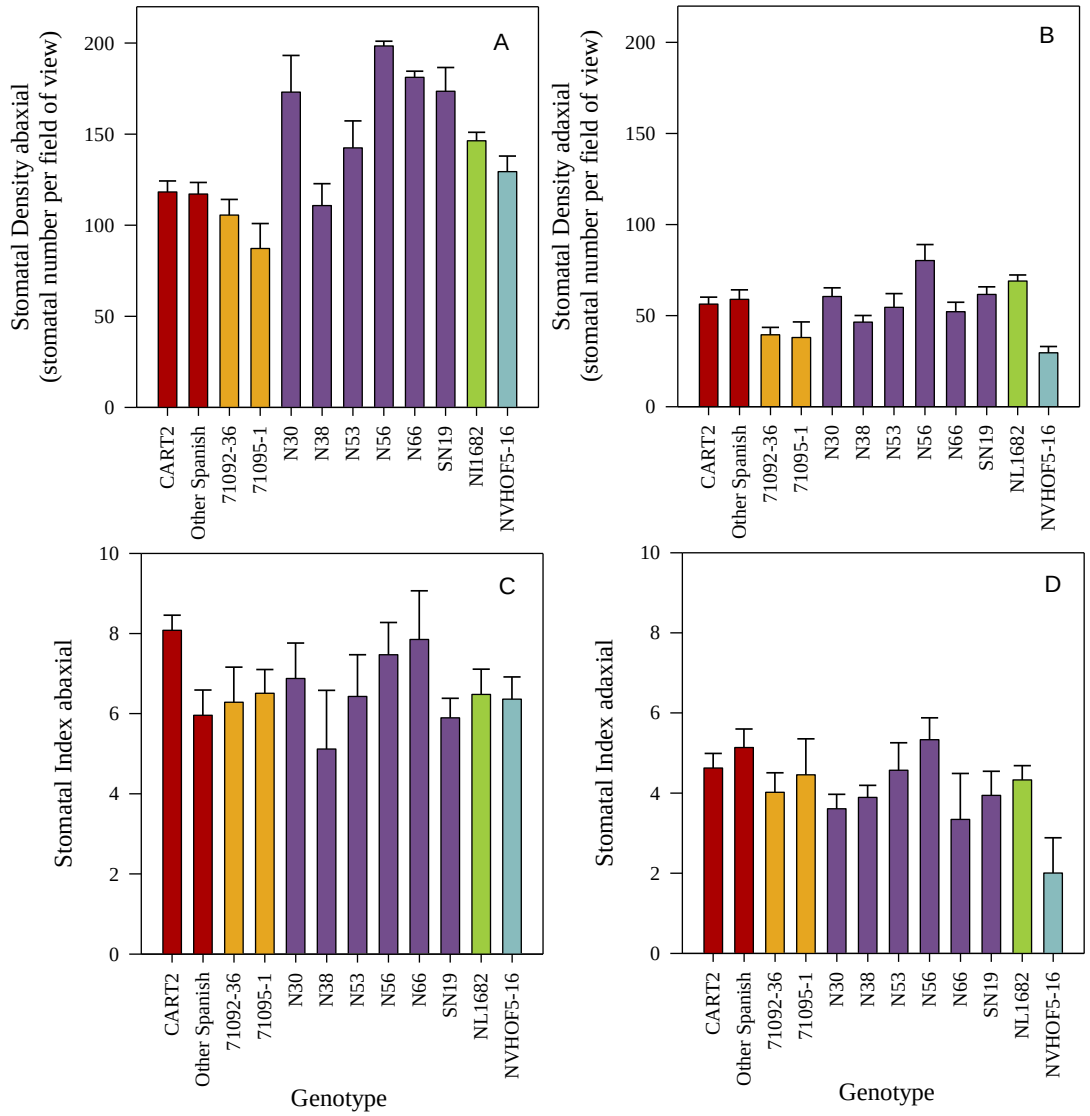


Figure 4.8: Stomatal measurements for each genotype grown in the greenhouse: stomatal density abaxial (A) and adaxial (B) and stomatal index abaxial (C) and adaxial (D); Each value with bars represents the average ± standard error.

A multivariate analysis was performed using all the stomatal and cell trait data as well as the leaf area of L1 on day 33. From the graph, three groups appeared (Fig. 4.9). Leaf area was grouped closer to cell number traits, while stomatal traits were clusted together and cell area traits in another cluster.

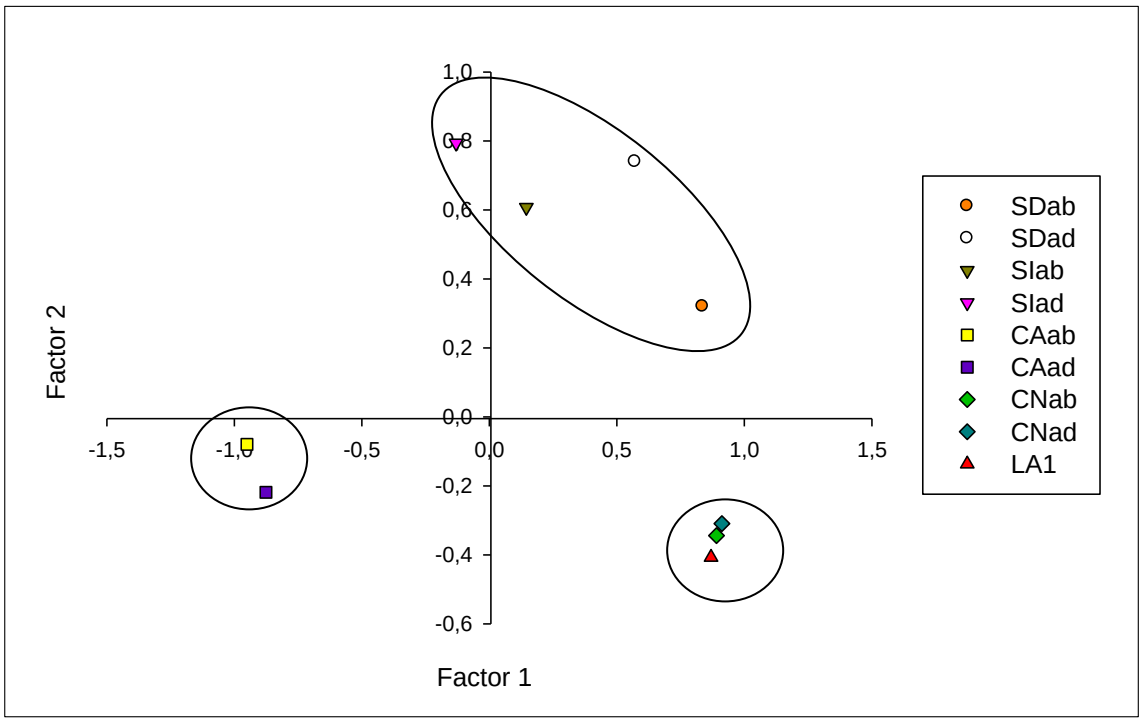


Figure 4.9: Multivariate analysis containing two principal components (Factor 1 and Factor 2)

4.4. Discussion

In this study, natural variation in carbon isotope discrimination, leaf, cell and stomatal development was observed in *P. nigra* sourced from various locations in Europe, when grown under well-watered conditions in a common garden in Belgium and also in a greenhouse experiment in South England.

Although *Populus* is sensitive to drought, genotypic variation was observed in the literature for this genus in traits related to drought tolerance and water stress responses such as osmotic adjustment (Marron *et al.*, 2002; Tschaplinski *et al.*, 2006), leaf expansion (Street *et al.*, 2006), leaf abscission (Street *et al.*, 2006) and WUE (Rae *et al.*, 2004; Voltas *et al.*, 2006; Monclus *et al.*, 2005; Monclus *et al.*, 2006; Dillen *et al.*, 2008).

For the population of *P.* nigra, genotypic variation was clear in water use efficiency using carbon isotope discrimination and varied with their latitude of origin. Wood and leaf carbon isotope discrimination had lower values in populations from the North East of Europe, such as The Netherlands and Germany, and the East, such as North Italy and the Drôme region in France, and consequently had the highest water use efficiency (WUE). These trees were collected from wet environments in Europe, their latitude of origin is comparable to the conditions in the fields in Belgium. On the other hand, Spanish and Southern French populations had the highest $\Delta^{13}C$ therefore the lowest WUE. However, this study was conducted in a wet environment in Belgium and had extremely different conditions compared to their origin for genotypes from Spain or Southern France. $\Delta^{13}C$ varies with weather such as precipitation and soil water regime. Indeed, wood and leaf $\Delta^{13}C$ respond positively to precipitation (Ferrio *et al.*, 2003; Otieno *et al.*, 2005) and WUE decreases with enhancement of precipitation (Li *et al.*, 2007), but genotypic variation exists and can vary in response to drought (Otieno *et al.*, 2005; Monclus *et al.*, 2006). Genotypes from Spain or Southern France, a location where precipitation is lower than Belgium, could then improve their WUE under drought while others from a similar environment to Belgium such as North Italy or The Netherlands would decrease WUE in water stress conditions. Genotypic diversity in carbon isotope discrimination is common in poplars (Rae *et al.*, 2004; Voltas *et al.*, 2006) but is not always correlated with productivity (Monclus *et al.*, 2005; Monclus *et*

al., 2006; Dillen *et al.*, 2008). Selecting genotypes that combine high productivity and high water use efficiency is then possible (Monclus *et al.*, 2005; Monclus *et al.*, 2006; Dillen *et al.*, 2008).

Variation was also observed in leaf area with small leaf size genotypes from Spain and Southern France where precipitation is low compared to North Italy, Germany or The Netherlands which genotypes developed larger leaves. Similar results were found in the field in Belgium after three years of growth (Trewin, 2008). Many studies show variation in physiological traits in plants collected from different locations but grown under the same conditions such as field, nursery or greenhouse (Christersson, 1996; Viveros-Viveros *et al.*, 2009). For example, Mergen (1963) found a correlation in *Pinus strobus* L. between the needle length and the latitude of the source of the seed. The same relationship was observed between latitude and stomatal number (Mergen, 1963). Growth in poplar differed depending on the original latitude (Pauley & Perry, 1954; Ceulemans & Deraedt, 1999).

Breeding programs use variation in leaf size as a drought related trait (Levi *et al.*, 2009; Ashraf, 2010). Indeed Radin *et al.* (1994) observed a negative correlation between leaf size and water potential (Ψ_W) suggesting small leaves would present higher Ψ_W. This phenomenon improves photosynthetic rate and consequently heat or drought resistance (Radin *et al.*, 1994; Levi *et al.*, 2009; Ashraf, 2010). Small leaves also have greater dissipation of heat and control of water loss by stomatal closure (Jarvis & McNaughton, 1986; Chaves *et al.*, 2003). ABA is a hormone accumulated during drought and is transported for signal transduction in response to water deficit (Bray, 1997). A negative correlation was also found between leaf size and ABA accumulation in response to dehydration in detached leaves of rice - *Oryza sativa* L. (Henson, 1983; Henson, 1985) which was later explained by a genetic linkage with a QTL association between ABA accumulation and leaf size (Quarrie *et al.*, 1997). Therefore, Spanish and Southern French genotypes could be developing small leaves as an adaptation to the drought stress they experience in their native environment. Similar observations were drawn in two other genotypes of *P. nigra* from contrasting environments in Italy. The Northern genotype had large leaves while the Southern genotype had smaller leaves and were

better adapted to defend itself against water stress (Regier *et al.*, 2009; Cocozza *et al.*, 2010).

Leaf size is determined for these genotypes by cell area and cell production. Leaf development occurs in two phases of time, first the cell production taking 2/3 of the time while cell expansion is slow then secondly the rapid cell expansion (1/3 of the developmental time) when cell production is essentially finished (Lecoeur *et al.*, 1995). Both cell number and cell area were different between the two types of leaf size. 'Small leaves' genotypes had larger cells on the abaxial and adaxial but had a lower cell production on each of the leaf side while 'large leaves' genotypes had more small cells. The same measurements were taken for trees grown in the field and show the same results with a relationship between latitude of origin and cell traits (Trewin, 2008). Poplars from lower latitudes had small leaves which contained larger and less cells than genotypes from higher latitudes (Trewin, 2008). From the multivariate analysis, it is clear that the main component determining the leaf size in this population is the leaf number. External factors can influence cell number and area but internal factors mainly determine the size of the organs (Mizukami & Fischer, 2000). For example, mutants of *struwwekpeter* (*swp*) in *Arabidopsis* showed reduced cell number but as a compensation cell area was increased (Autran *et al.*, 2002). *ERECTA* is another gene which controls epidermal cell expansion and proliferation (Masle *et al.*, 2005) both linked with leaf expansion and the duration of the leaf expansion (Tisné *et al.*, 2008).

ERECTA was also found to play a role in stomatal density (Masle *et al.*, 2005). SD differed significantly between the two types of leaf size (large or small) on the abaxial side but not the adaxial. Leaf surface SD was higher for genotypes developing large leaves and lower for 'small leaves' genotypes. Trewin (2008) found significant differences in abaxial SD between populations in 2004 and 2006. SD was also higher on the abaxial than the adaxial side. This stomatal behaviour is common (Radoglou & Jarvis, 1990; Afas *et al.*, 2006) and sometimes no stomata are developed on the adaxial, which is called 'perfect' hypostomaty (Hardy *et al.*, 1995). The reduced SD or absence of stomata on the adaxial surface which is also the most exposed side of the leaf could be a way to avoid external stresses such as light, water or wind (Afas *et al.*, 2006).

Higher SD described in ‘large leaves’ genotypes under well-watered conditions improves gas exchange and increases photosynthetic rates (Afas *et al.*, 2006) which could then raise WUE. In fact, a positive correlation between SD and WUE has been observed in well-watered plants (Hardy *et al.*, 1995). In poplar, a negative correlation was found between $\Delta^{13}C$ and stomatal density (Dillen *et al.*, 2008) interpreted as a positive correlation between WUE and SD. Dillen *et al.* (2008) described genotypes of *P. deltoides* x *P. trichocarpa* expressing a high WUE and developing small stomata but in high numbers. This could explain the high values of WUE (low $\Delta^{13}C$) measured in genotypes from The Netherlands, Germany and North Italy and the low WUE for genotypes from Spain and Southern France grown under well-watered conditions. On the other hand, genotypes that develop many stomata on their leaves would loose more water in the event of drought and would have to rapidly close their stomata (Casson & Hetherington, 2010). Spanish genotypes might be showing an adaptation to external environments such as drought stress.

Stomatal index was measured for samples grown in the greenhouse but difference was not significant between ‘small’ and ‘large’ leaves genotypes for the abaxial and adaxial surfaces. This is because cell number and sizes were different between the two groups. Masle *et al.* (2005) observed the same lack of significance in SI between wild *Arabidopsis* and *ERECTA* mutants. The latter showed an increase in SD and cells were smaller (Masle *et al.*, 2005). Trewin (2008) also measured SI in the field experiment in Belgium and found a significant difference between populations in 2004 but not in 2006.

In conclusion, this population of *P. nigra* presents genotypic variation in many traits related to their leaves, cells, stomata and also WUE depending on their latitude of origin from which the trees were collected. Genotypes from the South of Europe such as Spain and Southern France, developed small leaves, few but large cells, low number of stomata (abaxial side) and high $\Delta^{13}C$, while genotypes from wet environments (North Italy, The Netherlands, Germany) had large leaves, small but more cells, more stomata and low $\Delta^{13}C$. From these observations, it can be hypothesized that ‘small leaves’ trees are adapted to drought. A preliminary experiment was done using those genotypes grown in a growth room, comparing the transpiration and stomatal conductance under well-watered conditions (Appendix C). No significant differences were observed but

only two replicates per genotype were used (appendix C). However further investigation is required in order to verify this hypothesis under drought stress.

Chapter 5: Adaptive mechanisms for drought tolerance identified in a European population of black poplar (*Populus nigra* L.)

Abstract

Predictions indicate that summer droughts will increase in frequency and intensity over Europe as a consequence of global climate change. Therefore the study of adaptation to drought for the ecologically and economically important genus *Populus* is essential. From previous studies, wild trees of *Populus nigra* were collected in five European countries, from Spain to The Netherlands, for an association genetics study, reflecting a wide range of rainfall and temperatures. Small leaf sizes were observed in genotypes from Spain and Southern France, possibly indicating an adaptation to drought tolerance, in order to prevent water loss. This hypothesis is tested in this chapter with extreme ('large leaves' and 'small leaves') genotypes of the *P. nigra* association population. Six genotypes from different latitudes of origin in Europe and displaying contrasting leaf phenologies and carbon isotope discrimination values ($\Delta^{13}C$) were selected for a drought experiment in controlled conditions. Carbon isotope discrimination, oxygen isotope composition, stomatal conductance, leaf area and biomass related traits were measured to identify physiological differences in large- and small-leaves genotypes in response to drought. Biomass was affected by drought stress for all genotypes with different degrees of reaction and one Spanish genotype lost mature leaves while developing new leaves and branches. Spanish and French genotypes rapidly responded to drought stress with stomatal closure and decreased in $\Delta^{13}C$ which is negatively associated with water use efficiency.

These results provide important insights into morphological and physiological variation underpinning adaptation to drought across Europe that may be valuable in future conservation and management of trees facing climate change.

5.1. Introduction

Water is a major element for the development and survival of plants including forest trees. Recently a series of summer droughts in Europe have had major effects on forest tree mortality (Solberg, 2004; Bréda *et al.*, 2006). Furthermore, reduction of rainfall is predicted in the future increasing the intensity and the frequency of summer droughts (Kundzewicz *et al.*, 2007). Climate change will be responsible for increasing drought and temperature which is likely to increase tree mortality (Broadmeadow, 2002; Bréda & Badeau, 2008; Allen *et al.*, 2010).

Black poplar (*Populus nigra*) is a riparian species that presents low resistance to drought and is widely distributed in Europe, North Africa, Central and West Asia (Vanden Broeck, 2003). It has many economical uses, including domestic plantations, for soil protection and also as a parent for breeding programs (Vanden Broeck, 2003). Ecologically, this species is utilised as an indicator of riparian woodlands (Vanden Broeck, 2003). Threats towards this species have increased due to the alteration of its environment such as human activities on river drainage and water management (Gaudet *et al.*, 2008). Understanding the natural response to drought of *P. nigra* is important seeing the danger this species is facing and will face in the future.

An association population of *Populus nigra* grown in a common garden in Belgium was used in previous studies on this species. Variation in leaf size was observed with the genotypes originating from the driest environments having small leaves (Chapter 4). This trait is considered to be an adaptation for water deficit in order to avoid water loss by stomatal closure (Chaves *et al.*, 2003). It has been proven that small leaves are associated with high Ψ_W (Radin *et al.*, 1994). Small leaf size is also a trait that has been used in breeding programs to improve drought tolerance (Levi *et al.*, 2009; Ashraf, 2010).

In this chapter, various traits related to drought tolerance are studied to identify the processes used by those trees in response to drought, including leaf size, leaf number, stomatal conductance (Kozlowski, 1982), carbon isotope discrimination and oxygen isotope composition.

Wood carbon isotope discrimination also varied with latitude of origin when grown in a common garden (Chapter 4). Studying water use efficiency (WUE) as a measure for drought resistance using carbon isotope discrimination ($\Delta^{13}C$) is commonly done on trees (Pita *et al.*, 2001; Ponton *et al.*, 2001; Brendel *et al.*, 2002; Peñuelas *et al.*, 2008) including poplar species (Ridolfi & Dreyer, 1997; Leffler & Evans, 2001; Monclus *et al.*, 2006; Voltas *et al.*, 2006; Dillen *et al.*, 2008). WUE is the ratio between net carbon assimilation (A) and water loss (E) and is negatively associated with $\Delta^{13}C$ or positively correlated with carbon isotope composition $\delta^{13}C$ (Farquhar & Richard, 1984; Farquhar *et al.*, 1989; Condon *et al.*, 2002). However, $\Delta^{13}C$ and WUE can be associated with variation in stomatal conductance or photosynthetic capacity, so high WUE can be achieved by decreasing E and/or increasing A (Leffler & Evans, 2001; Condon *et al.*, 2002).

Oxygen isotope composition ($\delta^{18}O$) or enrichment ($\Delta^{18}O$) is a relatively new technique used to give additional information to separate the effects of stomatal conductance and carbon fixation on $\Delta^{13}C$ (Barbour *et al.*, 2000; Scheidegger *et al.*, 2000; Adams & Grierson, 2001; Keitel *et al.*, 2003; Keitel *et al.*, 2006). The number of papers in the last decade using stable oxygen isotope measures has increased (Barbour & Farquhar, 2000; Barbour *et al.*, 2000; Scheidegger *et al.*, 2000; Adams & Grierson, 2001; Cernusak *et al.*, 2003; Keitel *et al.*, 2003; Sheshshayee *et al.*, 2005; Bindumadhava *et al.*, 2006; Keitel *et al.*, 2006; Cernusak *et al.*, 2007; Farquhar *et al.*, 2007; Cabrera-Bosquet *et al.*, 2009; Ramírez *et al.*, 2009). Indeed $\delta^{18}O$ is not expected to reflect changes in RubisCo activity (Farquhar *et al.*, 1998; Keitel *et al.*, 2003) thus it is dependent on stomatal conductance but independent of photosynthetic rates (Keitel *et al.*, 2003). Scheidegger et al. (2000) developed a conceptual model to interpret the link between $\delta^{18}O$ and $\delta^{13}C$ in organic matter. The main outputs of the model are that a positive correlation between $\delta^{18}O$ and $\delta^{13}C$ indicates the changes in $\delta^{13}C$ are due to stomatal reaction and photosynthetic rates are relatively unaffected and inversely for a negative correlation, while g_s and A are both affected when $\delta^{18}O$ and $\delta^{13}C$ are not correlated (Scheidegger *et al.*, 2000). This model is based on the fact that the lighter isotope ^{16}O diffuses relatively faster than ^{18}O during evaporation, leaving the heavier oxygen isotope behind (Craig & Gordon, 1965; Bindumadhava *et al.*, 2006). Thus plants with closed stomata should

have less transpiration causing an increase in the temperature of the leaf and an increase of heavy oxygen isotope enrichment (Barbour *et al.*, 2000; Farquhar *et al.*, 2007).

The aim of this experiment was to observe traits related to drought tolerance in six genotypes of *P. nigra* collected from different latitudes of origin in Europe and test the hypothesis that ‘small leaves’ genotypes are more drought-tolerant than ‘large leaves’ genotypes.

5.2. Material and Methods

5.2.1. Plant material and growth conditions

Six genotypes were selected (Table 5.1), two from the Drôme population in France and four from the extremes samples (two Spanish, one Italian and one from Netherlands).

Table 5.1: Provenance of 6 *Populus nigra* genotypes used in the experiment

Genotype	Genotype code	River population	Country
C7	Sp1	Ebro1	Spain
RIN2	Sp2	Ebro2	Spain
6A03	Fr1	Drôme6	France
6A05	Fr2	Drôme6	France
N38	It	Ticino (left side)	Italy
NL1682	NL	Netherlands	Netherlands

Cuttings were planted in January 2007 for the leaf experiment (chapter 4) and cut back on April 24th 2007 and November 24th 2007 at 10 cm from the base. They were watered daily and put in dormancy conditions (natural light, 15ºC:13ºC). In May 2008, the trees started to grow and the temperature in the greenhouse was set at 22ºC:16ºC, day:night. During the time of the experiment, photoperiod was maintained at 16h:8h, light:dark with an average photosynthetic active radiation at the top of the plants of 150 μmol m^{-2} s^{-1}. Day and night temperature varied between 19 and 22ºC and 15 and 17ºC respectively.

Each genotype had up to 10 replicates in control treatment and 10 in drought treatment, except for Fr1 which had nine replicates under control treatment. Replication number reduced after SSR marker analysis in the Spanish genotypes (details of the protocol in Appendix D) and few replicates were found belonging to other genotypes. Sp1 and Sp2 had eight and five replicates respectively under control and nine and eight replicates respectively under drought stress. The trees were positioned in the middle bench in Boldrewood greenhouse in 10 blocks containing one replicate per genotype in each treatment. On September 1st 2008, 200mL of water was added to each tree and the pots were then covered in aluminium foil to prevent water evaporation. The first mature leaf and the first emerging leaf were tagged with cotton string. Over the next month (31 days), soil moisture content was measured every morning with a Delta-T ML2x ThetaProbe connected to an HH2 moisture meter (Delta-T Devices, Cambridge, UK).

Well-watered trees (control treatment) were watered up to field capacity and drought stressed trees (drought treatment) were kept between 15-20% soil moisture (Fig. 5.1). Figures representing the soil moisture for each genotype under well-watered and drought treatments over time are also in Chapter 2.

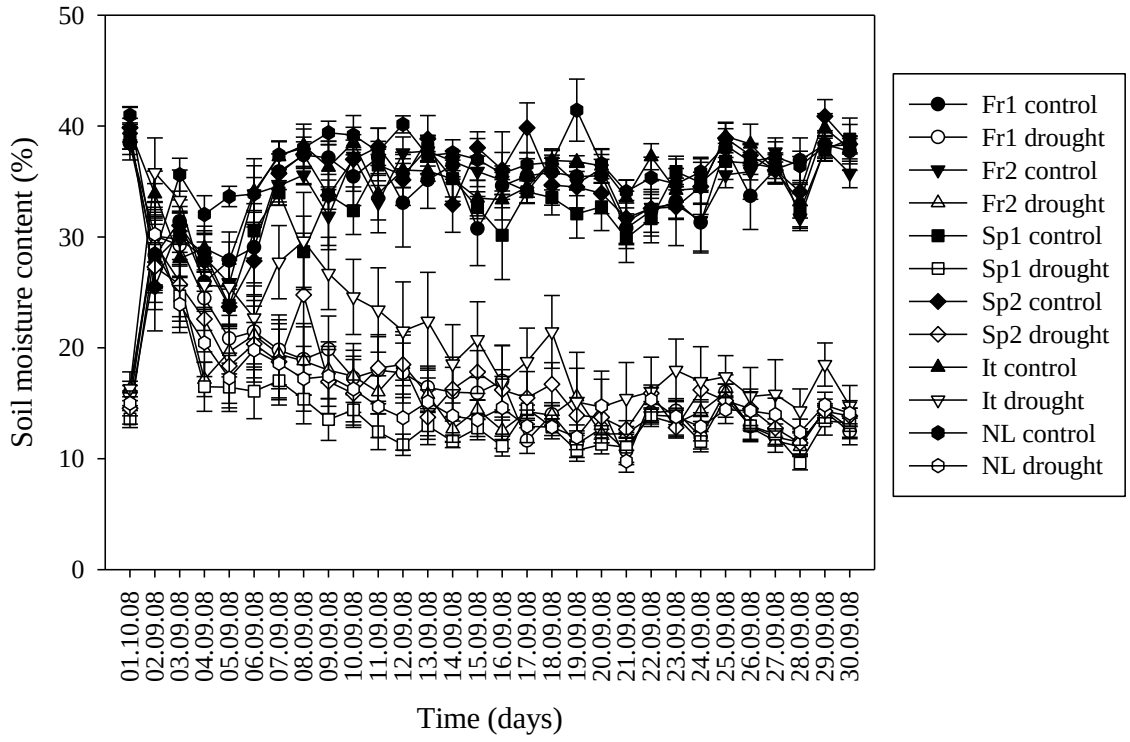


Figure 5.1: Soil moisture content (%) over time (days after drought) for each genotype. Black symbols represents well-watered (control) and white symbols are for drought treatments. Each value with bars represents the average ± standard error.

5.2.2. Measurements of biomass

Biomass measurements were conducted on September 1st 2008 (0 day after drought, 0DAD) and September 17th 2008 (16DAD) in order to examine the effect of drought over time on each genotype.

Height was measured in cm and stem diameter in mm using a digital calliper at 10 cm from the main stem base. Height and stem diameter growth was calculated by doing the difference in values between 16DAD and 0DAD.

The number of branches and leaves on the main stem were counted at 0DAD and 16DAD. The number of branches and leaves developed during the experiment was

calculated as the difference between the 16DAD and 0DAD for branch number and leaf number respectively.

Leaves newly developed (NLN) during the experiment from the tag on the first emerging leaf were also counted on September 1st 2008 and used with the number of leaves at 0DAD and 16DAD to calculate the number of fallen leaves, as senescence = $(NL_{(16DAD)}-NL_{(0DAD)})-NLN$

Specific Leaf Area (SLA) is the ratio of leaf area (cm^2) to leaf dry mass (g). Mature leaves were sampled 27 days after drought. At the end of the experiment, the third mature leaf (counting from the uppermost mature leaf) was removed at the petiole, the leaf area calculate and finally the leaves were oven dried for 48h at 80ºC and their dried weight measured.

5.2.3. Leaf area

Leaf area was measured over time every other day during the drought experiment from 1DAD until 19DAD for the first three emerging leaves (L1 being the first emerging leaf thus the oldest and L3 the youngest). Leaf measurements were taken by drawing around them on a white paper then scanned (Details on Chapter 2) and using ImageJ (Abramoff *et al.*, 2004) to calculate the leaf area.

5.2.4. Physiological measurements

Stomatal conductance (g_s) was measured on the first mature leaf tagged on 0DAD with a steady-state porometer (LI-1600; LICOR, Inc. Lincoln, Nebraska, USA) with manual data recording on 5DAD, 7DAD and 15DAD. Transpiration rates were also recorded 15 days after drought with the steady-state porometer.

A young leaf (third leaf from the top) of each tree was placed in a paper bag and oven dried for 50h at 80ºC for carbon isotope discrimination ($\Delta^{13}C$) and oxygen isotope composition ($\delta^{18}O$). Samples were ground using a ball grinder (Glen Creston ball, Retsch MM300, London, UK) and stored in a glass container. For carbon isotope discrimination, 1mg of material was weighed and placed into a 6 x 4 mm tin capsule (Ultra-clean pressed tin capsules, Elemental Microanalysis, Devon, UK). Samples were analysed using a SerCon 20-20 Stable Isotope Analyzer with ANCA-GSL Solid/Liquid

Preparation Module (SerCon, Crewe, UK). Carbon isotope composition was determined by $\delta^{13}C$ (‰) = δ_{plant} = [(R_{sample} - $R_{reference}$) / $R_{reference}$] × 1000, where R_{sample} and $R_{reference}$ are the $^{13}C/^{12}C$ ratios of the sample and the reference respectively, in VPDB (Vienna Pee Dee Belemnite) units (Scrimgeour & Robinson, 2004). Carbon isotope discrimination was calculated as $\Delta^{13}C$ (‰) = [(δ_{air} - δ_{plant})/(1+(δ_{plant} /1000)] with δ_{air} assumed to close to -8‰ (Farquhar & Richard, 1984; Monclus *et al.*, 2006).

For oxygen isotope composition, 0.2mg was weighed and placed in a 4 x 6 mm silver capsule (Pressed silver capsules, SerCon, UK). Oxygen isotope composition was measured as $\delta^{18}O_{plant}$ (‰) = (R_{plant} / $R_{reference}$) – 1, where R_{sample} and $R_{reference}$ are the $^{18}O/^{16}O$ ratios of the sample and the reference respectively, in VSMOW (Vienna Standard Mean Ocean Water) units (Farquhar *et al.*, 2007). Oxygen isotope enrichment $\Delta^{18}O$ (‰) = [($\delta^{18}O_{plant}$ - $\delta^{18}O_{source\ water}$)/(1+ $\delta^{18}O_{source\ water}$) (Cernusak *et al.*, 2003) was not calculated as $\delta^{18}O_{source\ water}$ was unknown but was considered identical with $\delta^{18}O$ as values were positive comparing to $\delta^{13}C$ values.

All the isotopes samples were analysed by the Scottish Crop Research Institute (Dundee, UK).

The percentage change of stomatal conductance, carbon isotope discrimination and oxygen isotope composition traits due to drought was calculated using the formula [(control - drought)/control] x 100 as described in Street *et al.* (2006).

5.2.5. Statistical analysis

All data were statistically analysed for genotype and treatment effect (with their interaction) using SPSS software package (SPSS, Chicago, IL, USA). Normality (Kolmogorov-Smirnov test), homogeneity and block effects were checked before performing a General Linear Model test. Data were transformed using a natural log when required to improve normality. A comparison of means was carried out between genotypes using a Student-Newman-Keuls post-hoc. A test for repeated measurements was done for leaf area over time for each leaf number. Regression between $\delta^{18}O$ / $\Delta^{13}C$ and $\delta^{18}O$ / transpiration were analysed using the Minitab software package (Minitab, State College, PA, USA).

5.3. Results

Six genotypes were selected from the association population of *P. nigra* to undergo a drought experiment in a controlled environment. Four were from the extreme genotypes selected for their extreme leaf size (Sp1, Sp2, It, NL) in order to test our hypothesis that 'small leaves' genotypes express an adaptation to water stress. The two other genotypes from France were selected to represent a wider range of temperature and precipitation. Traits were chosen because of their relation to tolerance to water deficit and to assess the effect of drought on the biomass of the trees (Table 5.2).

Natural variation in the amount of response was observed depending on the latitude of origin of the trees. Interaction between genotype and treatment was significant for carbon isotope discrimination and close to significance ($p<0.10$) for stem growth. Out of eleven main traits measured related to biomass and drought tolerance, six were significantly different in genotype and treatment (g_s 5DAD, $\Delta^{13}C$, $\delta^{18}O$, height growth, leaf development and leaf loss), one only had a significant effect in genotype (SLA) and g_s 7DAD, g_s 15DAD and stem diameter growth were only significant in treatment. Branching was the only trait which had no significance in any of the effects (genotype, treatment or their interaction).

Table 5.2: Summary of statistical results presenting the F-value and p-value for each trait using a GLM test for the main effects genotype and treatment and the interaction genotype x treatment. Bold values are significant ($p<0.05$)

Trait	Genotype		Water treatment		Genotype x Water treatment	
	F	p-value	F	p-value	F	p-value
g_s 5DAD	5.078	**<0.001**	15.860	**<0.001**	1.344	0.252
g_s 7DAD	2.168	0.064	18.271	**<0.001**	1.620	0.162
g_s 15DAD	1.469	0.207	103.092	**<0.001**	1.912	0.100
$\Delta^{13}C$	5.893	**<0.001**	7.511	**0.008**	2.567	**0.037**
$\delta^{18}O$	21.243	**<0.001**	10.933	**0.002**	1.518	0.204
Height growth	6.579	**<0.001**	37.086	**<0.001**	0.726	0.606
Stem diameter growth	2.116	0.071	14.77	**<0.001**	1.989	0.088
Branching	0.697	0.627	0.948	0.333	0.639	0.670
Leaf development	16.216	**<0.001**	24.964	**<0.001**	0.523	0.758
Fallen leaves	2.502	**0.036**	5.182	**0.025**	0.839	0.526
SLA	10.538	**<0.001**	2.977	0.088	0.923	0.470

5.3.1. Biomass measurements

Photographs were taken 20 days after the experiment started in the greenhouse and represent the main morphological variation in response to drought (Fig. 5.2). Biomass was also measured at the beginning and the end of the experiment (Table 5.3). Height growth rate decreased for all genotypes in response to drought (genotype effect $F_{5,85}$=6.6, p<0.001; treatment effect $F_{1,85}$=37.1, p<0.001) with the biggest decrease of -86% of difference between the two treatments for Italy. Fr1 and Sp2 had the smallest change between control and drought of -32% and -37% respectively. Under well-watered treatment Sp1 grew the most in terms of height growth, 13.6mm on average and Italy the least with an average of 5.2mm between the beginning and the end of the experiment.

Stem growth also was lower in drought treatment ($F_{1,87}$=14.8, p<0.001) but genotype difference was not significant at the 5% level ($F_{5,87}$=2.1, p=0.071). The French genotypes had the largest difference between the two treatments around -70% change, while Sp1 and NL had a similar percentage difference of -40% while the Italy genotype increased its stem growth under drought but the standard error was as large as the averages. The Italian genotype also showed the lowest stem growth under well-watered treatment with 0.17mm on average.

Leaf formation differed between genotypes ($F_{5,91}$=16.2, p<0.001). One Spanish genotype Sp2 continued to develop around the same number of new leaves under drought (6.0 leaves) than under control (6.8 leaves) while others significantly reduced their leaf formation in response to stress ($F_{1,91}$=25.0, p<0.001). Under well-watered treatment, Sp1 developed the most new leaves during the experiment (8.88) and trees from Italy only formed 3.89 new leaves in control treatment.

Leaf senescence on the main stem increased significantly under drought ($F_{1,86}$=5.2, P=0.025) and was significantly different between genotypes ($F_{5,86}$=2.5, P=0.036). French and Spanish genotypes lost more leaves and the biggest percentage difference due to drought was for Fr1 at 156%. Trees from Italy and Netherlands did not lose more leaves under drought with an average of 6 and 8 leaves respectively.

Although Sp2 genotype lost leaves on the main stem under drought (-36.3%), Sp2 also developed more branches from 2 to 4 branches in average between the two treatments. However this trait did not show any significance in genotype ($F_{5,81}$=0.697, p=0.627) or

treatment ($F_{1,81}$=0.948, p=0.33). Netherlands and Italy did not develop any branches in response to water deficit.

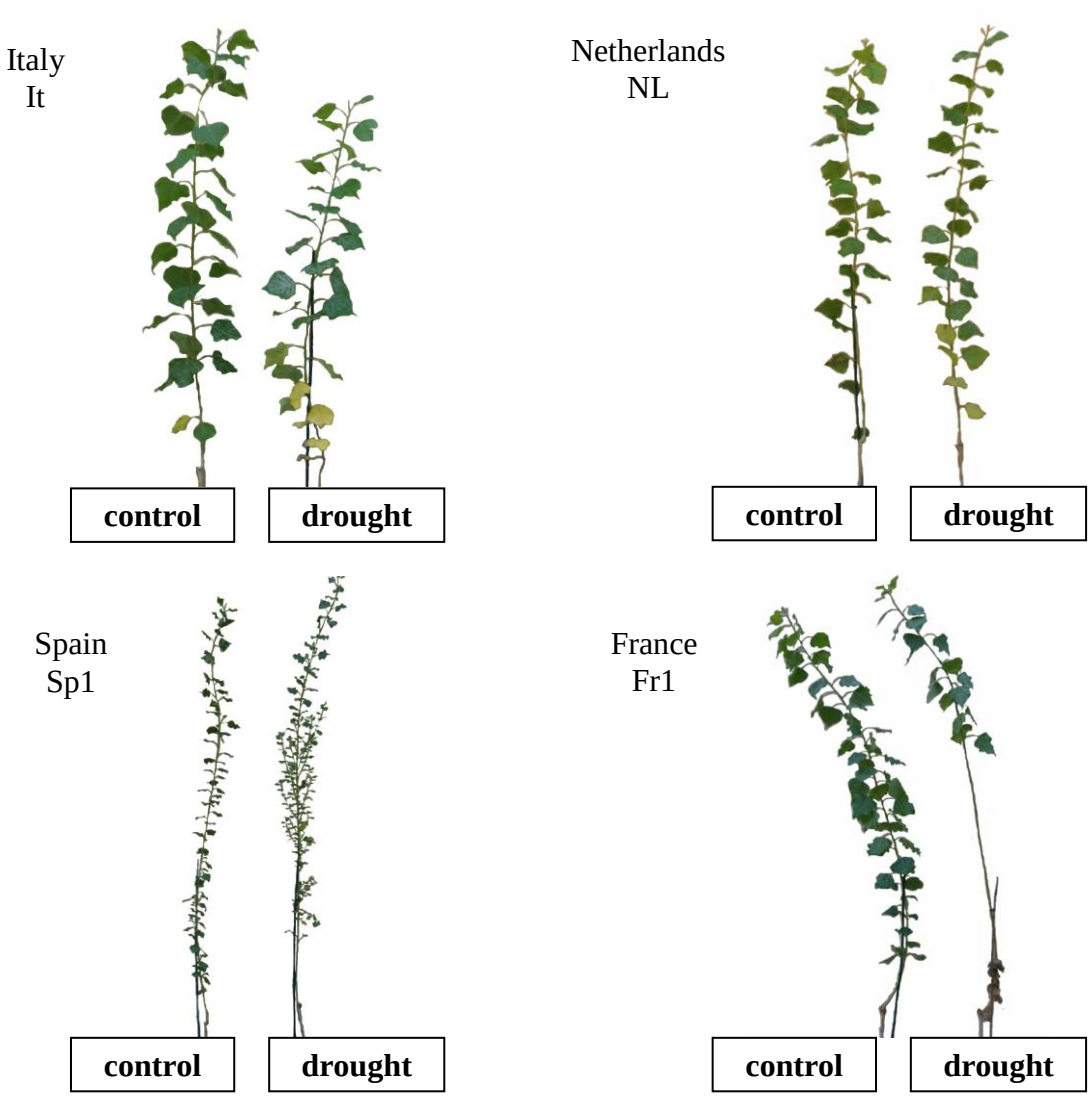


Figure 5.2: Photographic representation of the morphological effects of drought on the trees grown in the greenhouse.

Specific Leaf Area was measured at the end of the experiment and showed a genotype effect ($F_{5,87}$=10.5, p<0.001) but the effect of treatment was not significant ($F_{1,87}$=3.0, p=0.09). NL showed the highest SLA under control but also the highest percentage difference in response to drought with a reduction of 15% between the two treatments. On the other hand, Fr2 had the lowest SLA under control but had little variation between the control and stress treatments with an increase of 2.9%.

Furthermore during the experiment, one replicate of It subjected to the drought treatment died, one Fr1 and two NL trees were badly affected with the top of the main stem wilting.

Table 5.3: Summary of the biomass measurements for each genotype under well-watered (control) and drought treatments. Average ± standard error

		Height growth (mm)	Stem diameter growth (mm)	Number of leaves developed	Number of fallen leaves	Number of branches developed	SLA (cm^2/g)
Sp1	control	13.62±2.14	0.67±0.19	8.88±0.48	8.50±1.48	4.17±1.74	205.58±8.88
	drought	6.00±0.80	0.31±0.13	6.33±0.41	13.00±3.07	2.50±1.70	181.74±5.29
Sp2	control	8.8±2.41	0.67±0.15	6.8±1.02	12.20±2.06	2.00±4.00	229.09±22.97
	drought	5.56±1.21	0.36±0.10	6.00±0.65	16.62±3.62	4.00±1.81	212.49±14.30
Fr1	control	10.75±1.24	0.68±0.09	6.00±0.62	10.33±2.30	0.00±0.00	222.21±7.45
	drought	7.28±1.48	0.20±0.08	4.50±0.53	26.50±6.62	0.44±0.58	205.01±10.73
Fr2	control	8.35±1.03	1.04±0.20	5.40±0.56	6.40±1.57	3.87±4.16	185.41±5.62
	drought	3.95±0.84	0.27±0.12	3.60±0.34	10.40±3.31	0.00±0.44	190.79±8.57
It	control	5.22±1.18	0.17±0.16	3.89±0.48	6.12±1.37	6.11±5.99	243.50±15.75
	drought	0.70±0.97	0.25±0.26	2.62±0.37	6.62±1.57	0.00±0.00	251.75±15.38
NL	control	11.14±1.39	0.50±0.09	5.20±0.53	7.40±1.17	0.00±0.00	297.04±27.10
	drought	6.17±1.57	0.30±0.15	3.67±0.53	8.67±1.05	0.00±0.29	251.97±17.89

5.3.2. Leaf area

Leaf area was measured for the first three leaves emerging from 1DAD until 19DAD. Genotypes were significanty different in leaf area for all leaf numbers (Leaf 1: $F_{5,82}$=7.538, p<0.001; Leaf 2: $F_{5,54}$=6.162, p<0.001; Leaf 3: $F_{5,36}$=6.328, p<0.001). For the trees under well-watered conditions, variation was observed between the genotypes with 'small' and 'large' leaves as in Chapter 4. Sp1 and Sp2 from Spanish had the lowest leaf area (1700 mm^2 and 1000 mm^2 in average respectively) and It from Northern Italy had the highest leaf area (4700 mm^2 in average for Leaf 1). In response to water stress, all the genotypes decreased in leaf area (Leaf 1: $F_{1,82}$=21.753, p<0.001; Leaf 2: $F_{1,54}$=36.857, p<0.001; Leaf 3: $F_{1,36}$=23.689, p<0.001). Sp2 showed the least reduction of leaf area with an average of 800 mm^2 for leaf 1 under water deficit and 975 mm^2 under well-watered conditions (Fig. 5.3A).

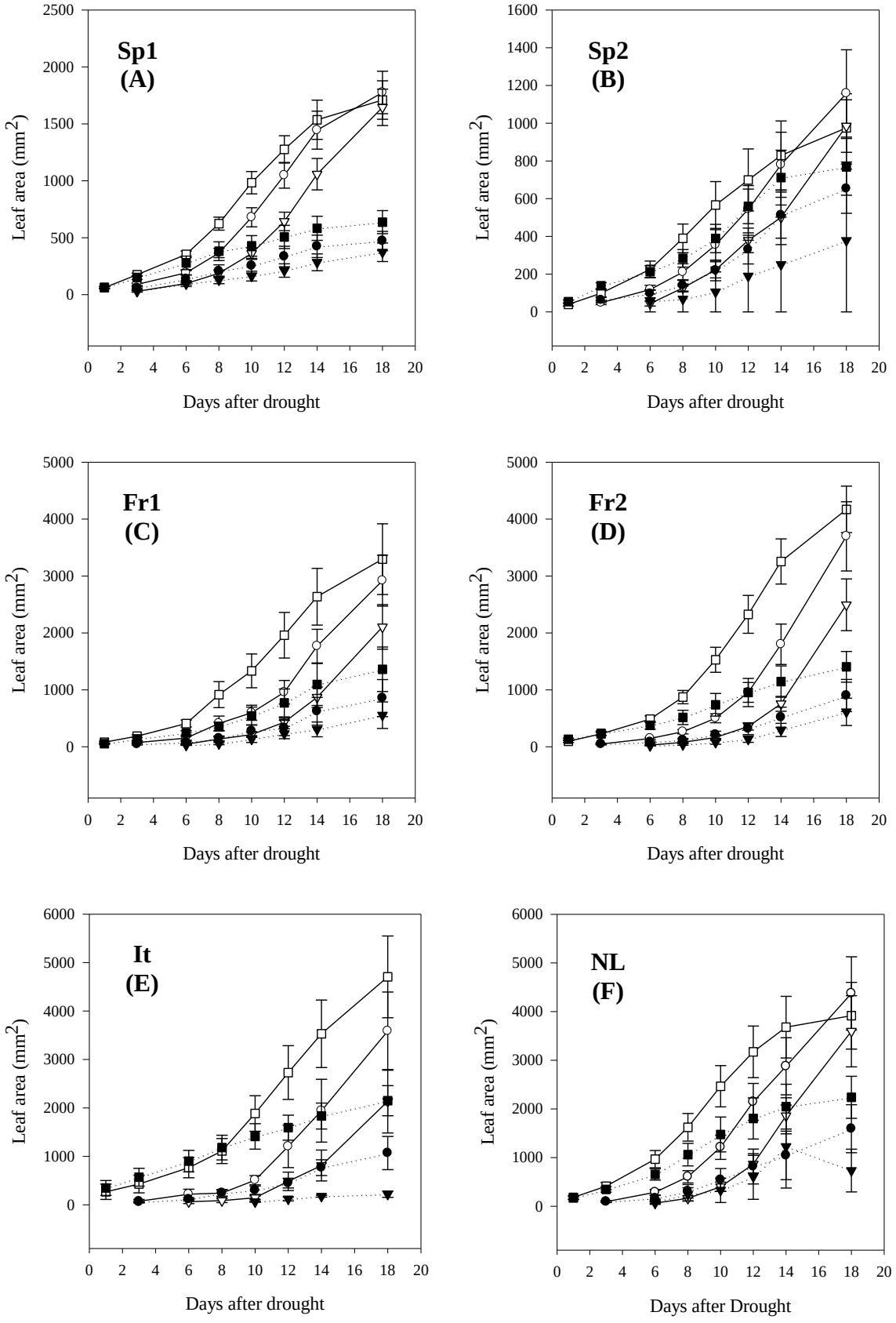


Figure 5.3: Leaf area development over time (days after drought) for the first emerging leaf (■), the second leaf emerging (●) and the third leaf emerging (▼) under well-watered conditions (full lines and white symbols) and drought stress (broken lines and black symbols) for each genotypes: Sp1 (A), Sp2 (B), Fr1 (C), Fr2 (D), It (E), NL (F)

5.3.3. Stomatal conductance

Stomatal conductance corresponds to the stomatal aperture and was measured at 5DAD, 7DAD and 15DAD (Table 5.2). Spanish and French genotypes reacted quickly to water deficit with stomatal closure (genotype $F_{5,96}$=5.1, p<0.001, treatment $F_{1,96}$=15.9, p<0.001), stomatal conductance varying between -54% and -36% change differences between conditions at 5DAD for those four genotypes (Fig. 5.4A). On the other hand, Italy only had an increase of 3.4% from 338 mmol.m^{-2}.s^{-1} to 350 mmol.m^{-2}.s^{-1} due to drought and NL varied in g_s by -17% change from 375 mmol.m^{-2}.s^{-1} to 311 mmol.m^{-2}.s^{-1}. After 7 days, although Italy showed no variation between drought and control, genotype difference was small and non-significant at 5% level ($F_{5,95}$=2.2, p=0.064) as the other genotypes (Sp1, Sp2, Fr1, Fr2, NL) closed their stomata due to drought (treatment: $F_{1,95}$=18.3, p<0.001), particularly Sp1 with a percentage difference of -51.5% from 510 mmol.m^{-2}.s^{-1} to 250 mmol.m^{-2}.s^{-1} (Fig. 5.4B). Finally 15 days after drought (Fig. 5.4C), all genotypes behaved the same ($F_{5,92}$=1.5, p=0.21) with a decrease in stomatal conductance in response to drought comparing to the control ($F_{1,92}$=103.1, p<0.001).

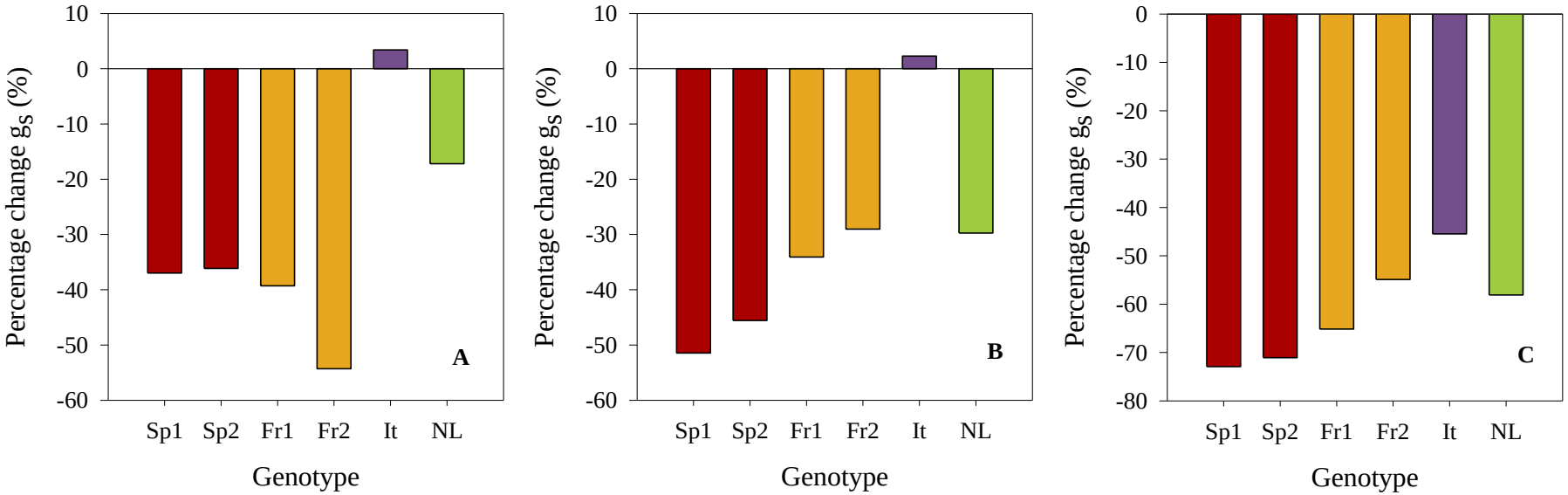


Figure 5.4: Percentage difference of stomatal conductance, 5 (A) 7 (B) and 15 (C) days after drought, using the formula [(control - drought)/control] x 100 from Street *et al.* (2006).

5.3.4. Carbon and Oxygen isotopes

Young leaves developed during the experiment were collected in order to measure carbon and oxygen isotope measurements, $\Delta^{13}C$ and $\delta^{18}O$ respectively (Fig. 5.5). $\Delta^{13}C$ showed variation between genotypes ($F_{5,58}$=5.9, p<0.001), a significance of the treatment effect ($F_{1,58}$=7.5, p=0.008) and also a significant interaction genotype x treatment ($F_{5,58}$= 2.6, p=0.037) showing that the response to drought in $\Delta^{13}C$ was different depending on the genotype. While Sp1, Sp2 and Fr1 decreased their $\Delta^{13}C$ by around 10% under drought indicating an increase in water use efficiency (WUE), varying in absolute values from 22.5‰, 24.4‰ and 22.4‰ respectively under controlled conditions. Fr2 showed no variation between treatments maintaining their values around 21.2‰ and Italy increased $\Delta^{13}C$ under drought from 23.3‰ to 24.6‰.

$\delta^{18}O$ showed significant effects of genotype ($F_{5,43}$=21.2, p<0.001) and treatment ($F_{1,43}$=10.9, p=0.002). Oxygen isotope composition increased for most genotypes in response to drought, except for Sp2, with the largest difference for Italy and Fr1 of 6.2% and 5.6% respectively.

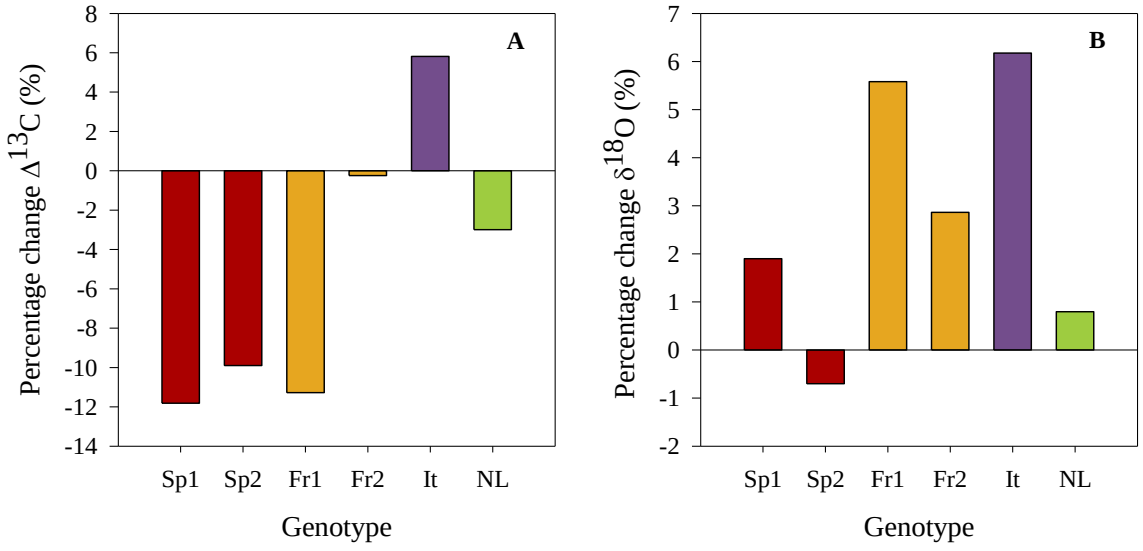


Figure 5.5: Percentage difference of carbon isotope discrimination (A) and oxygen isotope composition (B), using the formula [(control - drought)/control] x 100 from Street *et al.* (2006).

In order to understand if changes in $\Delta^{13}C$ are influenced by g_s or photosynthetic capacity, correlations between $\Delta^{13}C$ and $\delta^{18}O$ were tested for control and drought data (Fig. 5.6) and combined values ($y = -0.42x + 34.29$, $R^2 = 0.34$, $p=0.046$). The correlation for the combined values ($p<0.05$) and for the drought values were significant ($p=0.028$) and negative, the correlation for the control values were not significant ($p=0.68$).

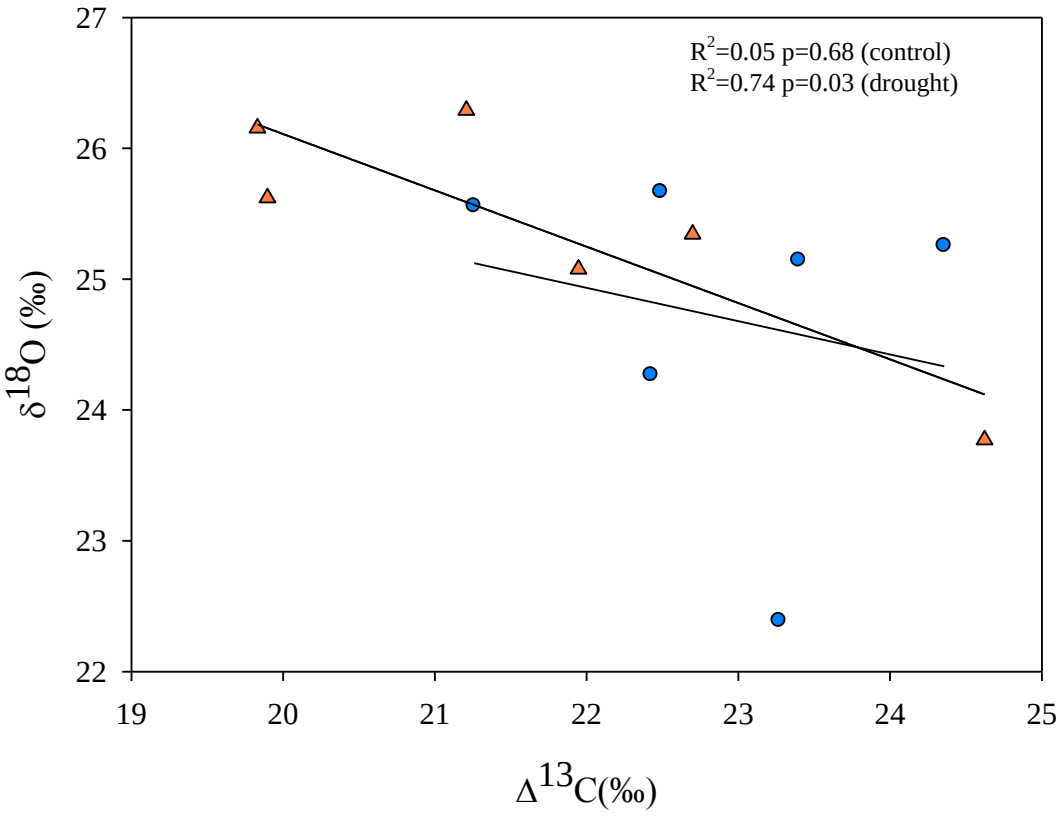


Figure 5.6: Relationship between the mean leaf $\delta^{18}O$ and leaf $\Delta^{13}C$ for control ($y = -0.25x + 30.53$, $R^2 = 0.047$, $p=0.68$) in blue and drought stress ($y = -0.43x + 34.72$, $R^2 = 0.738$, $p=0.03$) in orange

Relationships between $\delta^{18}O$ and transpiration rate were also studied and no significant correlations were observed for controlled or drought values (Fig. 5.7) as well as for the combined data ($y = -0.32x + 25.69$, $R^2 = 0.077$, $p=0.383$). On the other hand, mean $\delta^{18}O$ for the drought values was found to be close to a significant negative correlation with mean transpiration rate ($p=0.084$).

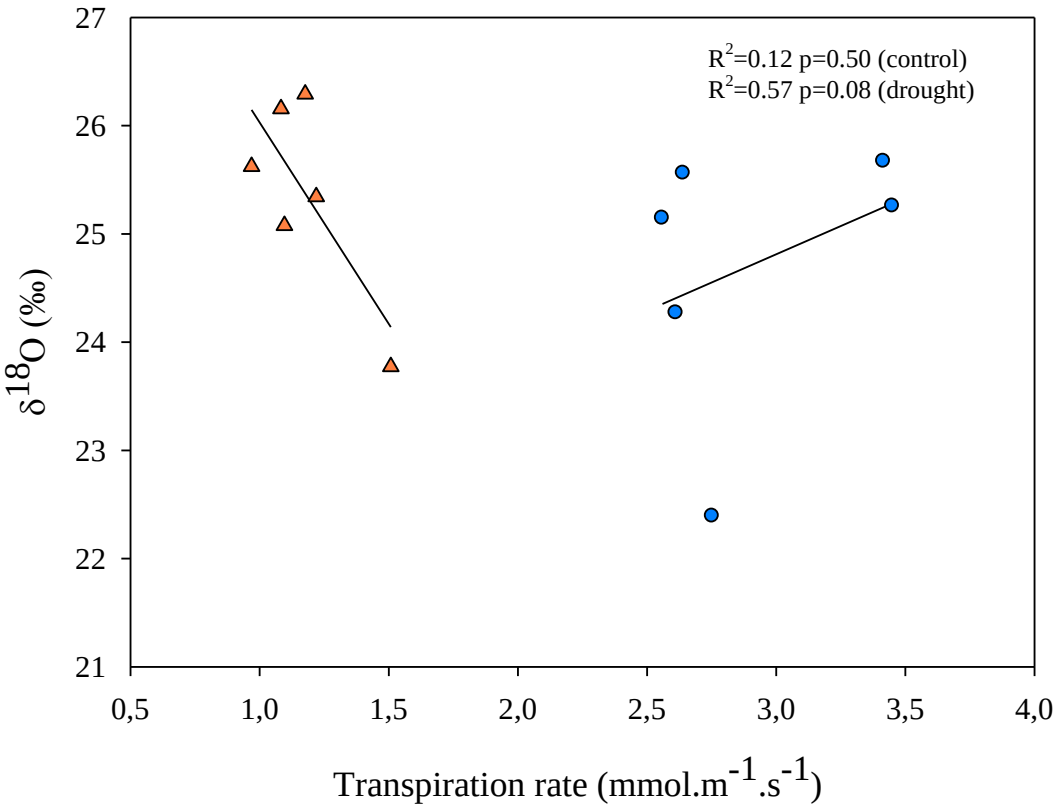


Figure 5.7: Relationship between the mean leaf $\delta^{18}O$ and transpiration rate for control ($y = 1.04x + 21.68$, $R^2 = 0.12$, p=0.499 in blue and drought stress ($y = -3.73x + 29.75$, $R^2 = 0.57$, p=0.084) in orange

5.4. Discussion

In this study, the hypothesis that "genotypes with small leaves are better adapted to drought" was tested. The response to dehydration stress was investigated morphologically and physiologically in six wild black poplars originated from four locations in Europe (Spain, France, North Italy and Netherlands).

Grown under well-watered treatment in Belgium (chapter 4), the genotypes selected for this experiment showed variation in $\Delta^{13}C$ with higher values for the 'small leaves' genotypes such as Sp1 and Sp2 (from Spain) indicating that these trees displayed lower water use efficiency. Carbon isotope discrimination is a negative indirect measurement for water use efficiency (Farquhar *et al.*, 1989). Wood $\Delta^{13}C$ can be very dependent on the environment where the tree is growing (Leavitt, 1993; Garcia-G *et al.*, 2004) which could explain high $\Delta^{13}C$ (thus low WUE) for the Spanish genotypes grown in Belgium a wetter and colder environment than their latitude of origin. To test if the 'small leaves' genotypes had greater water use efficiency in drought conditions, six genotypes were subjected to a moderate drought experiment in a greenhouse. Models predict an increase in summer droughts with longer periods of water shortage. A slow water deficit was applied by decreasing the soil moisture and then maintaining it between 15 and 20% in order to observe long-term responses and thus acclimatory and adaptive traits (Chaves *et al.*, 2003). A slow water deficit rather than an acute drought is also a better approach to reproduce field conditions (Cattivelli *et al.*, 2008).

At the level of the biomass, genotypes responded differently to drought stress. French, Italian and Dutch genotypes responded fairly similarly to water deficit: height growth and new leaf formation decreased, no branches were developed in either treatments and SLA did not change. Uniquely Fr1 and Fr2 had a high leaf loss due to drought stress while genotypes It and NL did not lose leaves in response to drought. Mature leaf senescence in response to drought allows remobilizing nutrients from the mature leaf towards younger leaves (Abreu & Munné-Bosch, 2008). It can be a necessary process in drought tolerance to reduce water loss through leaf transpiration (Chaves *et al.*, 2003; Munné-Bosch & Alegre, 2004). Survival was also reduced for the Italian genotype with

one replicate that died under soil dehydration stress and two NL were affected by drought with the top of the stem wilting.
In contrast, one Spanish genotype (Sp2) coped better with drought stress. As with the French genotypes leaf loss increased on the main stem under drought, SLA and new leaf development were not affected by drought. Leaf area for leaf 1 was less reduced by water deficit compared with other genotypes. These trees also formed more branches which had new leaves. In a preliminary experiment in the growth room, 'small leaves' genotypes also developed more branches under well-watered conditions compared with 'large leaves' genotypes (Appendix D). Branching measured in *Populus* spp. was discovered to be an important trait for stem dry weight which is then related to tree biomass (Robison & Raffa, 1998). Other studies show a similar behaviour pattern with *Populus* clones developing more sylleptic branches had high productivity (Scarascia-Mugnozza *et al.*, 1999) and biomass production (Barigah *et al.*, 1994). On the other hand, Rae *et al.* (2004) showed the same biomass yield values for two *Populus* genotypes, even though the trees developed different number of sylleptic branches but it was still recommended to use branching as a trait for breeding program.

Physiologically, although there was no significant interaction between genotype and treatment in stomatal conductance, the genotypes reacted differently in the intensity of decrease in response to water deficit. Spanish genotypes as well as French genotypes responded quickly to drought by closing their stomata only 5 and 7 days after drought while the Italian genotype did not vary in g_s. At 7DAD, Spanish genotypes had the largest percentage of change in g_s. Variation in stomatal behaviour can exist within species and was showed by Sparks & Black (1999) in four populations of *Populus trichocarpa* originating from contrasting environments. Samples from coastal and wetter locations exhibited little stomatal closure and experienced rapid xylem cavitation under drought compared to the trees from a drier environment (Sparks & Black, 1999). A similar finding was observed for two species of *Populus* each originating from different environment (Street *et al.*, 2006). *P. deltoides* rapidly closed their stomata after a drought stress and reduced their photosynthetic rate while *P. trichocarpa* originally from a wetter location showed smaller reductions in g_s and in A (Street *et al.*, 2006). Trees from The Netherlands and Northern Italy, native from a wetter environment than Spain and France, also showed lower stomatal response to drought in the early days of

the experiment. Stomatal closure is a biological process to avoid water loss in the event of drought stress but can have other physiological consequences as it can inhibit photosynthesis (Cornic, 2000).

Carbon isotope discrimination was measured to study the variation in water use efficiency (the ratio between net carbon assimilation and water loss). Genotypes reacted differently in response to drought for $\Delta^{13}C$. Spanish and French genotypes decreased $\Delta^{13}C$ showing thus an increase in WUE. This seems logical as g_s decreased rapidly under drought stress but after 15 days all genotypes showed the same pattern of stomatal closure. In consequence, Spanish and French genotypes reduced transpiration by closing their stomata but also kept photosynthetic activity high compared to the Italian genotype. When studying variation of $\delta^{13}C$ in beech planted in different sites though Europe, the highest values (thus the lowest values of $\Delta^{13}C$) were observed in the most southern location in France (Keitel *et al.*, 2006). Conversely trees of *Pinus greggi* grown in wet sites had higher $\delta^{13}C$ compared to dry sites (Garcia-G *et al.*, 2004). However this studies use the same genotypes to study the variation in carbon isotope discrimination between sites with different levels of precipitation (Garcia-G *et al.*, 2004). Monclus et al. (2005) used different genotypes of *Populus* (tolerant and non-tolerant to drought) and showed that the drought tolerant trees tended to decrease in $\Delta^{13}C$ but the inverse was observed for the non-tolerant genotypes.
Different correlations have been reported between $\Delta^{13}C$ and productivity. In poplar, productivity and $\delta^{13}C$ were not correlated and genotypes were discovered combining high WUE and productivity (Monclus *et al.*, 2005; Monclus *et al.*, 2006). Negative correlations were found in *Picea mariana* grown in water limited environments (Flanagan & Johnsen, 1995) or when discrimination in $\Delta^{13}C$ was due to carboxylation efficiency. Positive correlations between $\Delta^{13}C$ and productivity have been reported when stomatal conductance is responsible for $\Delta^{13}C$ changes (Pita *et al.*, 2001). It is thus important to distinguish between the effects of stomata and photosynthetic capacity on $\Delta^{13}C$.

In association with the studies of oxygen isotope composition ($\delta^{18}O$), the correlation of $\Delta^{13}C$ with $\delta^{18}O$ can help to determine whether variation is due to stomata or photosynthesis. The correlation between $\Delta^{13}C$ and $\delta^{18}O$ was significant for the drought

treatment and the combined control and drought treatments, reflecting an effect of stomatal conductance in the variation of $\Delta^{13}C$ (Scheidegger *et al.*, 2000) and thus WUE. A lack of correlation was present for the control data. The correlation for the drought treatment was significant (p=0.03) and it tended to be negative (R^2=0.74) demonstrating the importance of stomatal conductance in the variation of $\Delta^{13}C$. Negative associations between $\Delta^{13}C$ and $\Delta^{18}O$ were observed in other plants such as wheat (Barbour *et al.*, 2000; Cabrera-Bosquet *et al.*, 2009), cotton (Barbour & Farquhar, 2000) and also trees, for example *Eucalyptus* (Cernusak *et al.*, 2003). Oxygen enrichment is also used to study the effects of stomata or evaporation demand. If the source of variation for $\delta^{18}O$ is stomatal and not evaporation demand, transpiration should be negatively associated with $\delta^{18}O$ (Farquhar *et al.*, 2007). This was observed for our results but not significantly (Fig. 5.6), only at 10% significance for the drought treatment (R^2=0.57, p=0.08). A negative correlation was also detected in sap phloem sugar of *Eucalyptus globulus* (Cernusak *et al.*, 2003). On the other hand, Sheshshayee et al. (2005) observed a strong positive correlation between transpiration and $\Delta^{18}O$ leaf biomass in groundnut and rice plants and concluded ^{18}O enrichment in the leaf biomass was a good measure indicating transpiration rates and thus stomatal conductance. It was later described that the variation in $\Delta^{18}O$ was due to evaporative demand explaining the positive correlation between transpiration and $\Delta^{18}O$ (Farquhar *et al.*, 2007).

In conclusion, one Spanish genotype showed adaptation to water deficit compared to other genotypes grown at the same conditions. Spanish and French genotypes both reduced stomatal conductance after only 5 days of moderate drought stress. Furthermore, the factors affecting $\Delta^{13}C$ and thus WUE were shown to involve stomatal control.

Screening for variation in wild plants from diverse geographic locations under drought stress is a very useful strategy to discover genotypes naturally adapted to water deficit. Similar studies have been done with other plants such as *Arabidopsis* (Bouchabke *et al.*, 2008). Finding drought tolerant genotypes can subsequently be used to identify and to study drought-related genes (Kurahashi *et al.*, 2009).

Chapter 6: Genomic plasticity in response to drought in a natural population of back poplar (*Populus nigra* L.)

Abstract

A drought experiment was conducted in the greenhouse in the UK focusing on 6 genotypes of black poplar showing extreme variation in leaf size and $\Delta^{13}C$, from Spain, France, The Netherlands and Northern Italy. Morphologically and physiologically variations in response to water deficit were observed (Chapter 5). Gene expression using microarrays and real-time PCR were then studied and revealed differences in response to drought between genotypes. In particular, in comparing the transcriptome of trees originating from Spain, with those from Northern Italy, it was apparent that the gene-level responses to drought differed significantly such that 3167 transcripts were differentially expressed in response to drought in the Italian genotypes while only 649 transcripts were differentially expressed in response to drought in the Spanish trees. These findings highlighted several GO (Gene Ontology) categories that differed most between treatments and this included genes identified for stomatal formation and patterning such as *ERECTA, TOO MANY MOUTHS, MUTE* and *SPEECHLESS*. Gene expression differences were also studied between the two genotypes in well-watered conditions and showed 754 transcripts with a higher expression by Sp2 and 852 transcripts with a higher expression by It when compared with Sp2. The Spanish genotype had more osmoprotectant and repair transcripts highly expressed under well-watered conditions than the Italian genotype which could be linked to a possible drought adaptation. The analysis of the microarrays enabled us to identify candidate genes which were used for real-time PCR for four genotypes from Spain, France, the Netherlands and Italy. The significance of these findings for genomic adaptations to drought in poplar is considered.

6.1. Introduction

Populus is widely accepted to be a model tree for numerous reasons. It is a fast-growing forest tree, its genome is fully sequenced and it is an important genus economically and ecologically (Taylor, 2002; Tuskan *et al.*, 2004; Tuskan *et al.*, 2006; Jansson & Douglas, 2007). Poplars are also sensitive to drought as they are more abundant in riparian environments and they demand a high quantity of water for optimal development (Dreyer *et al.*, 2004; Street *et al.*, 2006). However, variation in response to water deficit has been observed between genotypes of *Populus* (Marron *et al.*, 2002; Monclus *et al.*, 2006; Street *et al.*, 2006; Huang *et al.*, 2009a; Regier *et al.*, 2009; Cocozza *et al.*, 2010).

Drought is one of the main stresses affecting plant growth. Climate change will also be an issue in the future and is predicted to become responsible for increased water shortage and decreased soil moisture for the development and growth of plants. An understanding of the adaptation to drought is needed in plants using genomic tools and develop crops which can tolerate water stress. Complex pathways and numerous genes are involved in the adaptation to drought (Bray, 1997; Shinozaki & Yamaguchi-Shinozaki, 2007). Genomic technologies address the issue of multigenic response to drought, in contrast to single gene studies that can limit the focus to the interaction between genes and the different pathways involved in drought response and adaptation.

In the past fifteen years, microarrays have enabled the discovery of genes of interest related to a particular condition, such as stress, pathogen or pest attack. Contrary to northern blots and real-time qPCR which are able to study only one or a few genes at a time, microarrays allows the study of the expression of thousands of genes simultaneously. Microarrays are also a useful technology to discover unknown genes expressed during a particular process that were not predicted prior to the experiment from any formulation of hypothesis (Richmond & Somerville, 2000).
There are two main types of arrays: DNA-fragment-based and oligonucleotide-based microarrays. They are different due to the nature of the DNA which is placed on the arrays (Richmond & Somerville, 2000). DNA-based arrays have DNA fragments deposed robotically onto glass slides while the oligonucleotide-based arrays are oligonucleotides synthesised *in situ* on the arrays (Wu *et al.*, 2001). Affymetrix GeneChip (Affymetrix, Santa Clara, USA) are oligonucleotide microarrays. Each

nucleotide fragment is located in a probe cell and the target fragment (RNA or DNA) which is biotin-labelled hybridises to the fragment in the probe (Affymetrix, 2009). The fluorescence of the labelled fragments which is hybridised is quantified for each probe to give an estimation of gene expression (Kathiresan *et al.*, 2006).
The first paper using microarrays was published in 1995 measuring the expression of 45 *Arabidopsis thaliana* genes in different tissues (leaf and root), in mutant and wild type plants (Schena *et al.*, 1995). The first Affymetrix microarray for *Arabidopsis* contained 8000 genes (Zhu & Wang, 2000) while today it contains 24000 genes. The poplar GeneChip in Affymetrix has 56,000 transcripts and gene predictions.

Microarrays are a technology that have been extensively utilised in the study of gene expression in response to water stress for numerous plants such as *Arabidopsis* (Seki *et al.*, 2001; Seki *et al.*, 2002), rice (Rabbani *et al.*, 2003), barley (Neslihan Ozturk *et al.*, 2002; Tommasini *et al.*, 2008), cotton (Payton *et al.*, 2010), maize (Hayano-Kanashiro *et al.*, 2009), wheat (Way *et al.*, 2005) and loblolly pine (Watkinson *et al.*, 2003). Gene expression in drought stress was also studied with microarray analysis for different species of *Populus*, for example *P. deltoides* and *P. trichocarpa* (Street *et al.*, 2006), *P. euphratica* (Bogeat-Triboulot *et al.*, 2007), *Populus* clones of *P. deltoides* x *P. nigra* and *P. nigra* x *P. maximowiczii* (Wilkins *et al.*, 2009).

In this study, we used 6 genotypes of *P. nigra* originating from 4 different European countries showing extreme variation in leaf size and in carbon isotope discrimination when grown in well-watered conditions. From the analysis of morphological and ecophysiological measurements in *P. nigra*, natural variation in response to drought stress was also observed between genotypes of different latitudes of origin (Chapter 5). Leaf samples were collected 20 days after a moderate drought stress for microarrays and real-time qPCR analysis. Direct comparisons between the transcriptome of extreme genotypes in well-watered and drought conditions provide insight into the genomic pathways induced during water stress.
The objective of this research was to observe the natural variation in gene expression between two genotypes of *P. nigra* from contrasting origins in Europe under a moderate drought experiment but also comparing the control plants grown under well-watered conditions.

The aims of this chapter were to:

1) Study the variation in response to moderate water stress between potentially drought sensitive (It) and drought tolerant (Sp2) genotypes.

2) Study the possible natural adaptation to water deficit of a Spanish genotype under well-watered condition compared to a North Italian genotype.

3) Identify candidate genes related to the adaptation to drought and understand their role in enabling survival in environments with reduced water input.

6.2. Material and Methods

6.2.1. Plant material and experimental design

Plant material was cuttings of *P. nigra* grown in the greenhouse at the University of Southampton. Six genotypes were used for the moderate drought experiment Each genotype had up to 10 replicates in control treatment and 10 in drought treatment, except for Fr1 which had nine replicates under control treatment. Replication number reduced after SSR marker analysis in the Spanish genotypes (details of the protocol in Appendix D) and few replicates were found belonging to other genotypes. Sp1 and Sp2 had eight and five replicates respectively under control and nine and eight replicates respectively under drought stress. The trees were positioned in the middle bench in Boldrewood greenhouse in 10 blocks containing one replicate per genotype in each treatment. On September 1st 2008, 200mL of water was added to each tree and the pots were then covered in aluminium foil to prevent water evaporation. Over the next month (31 days), soil moisture content was measured every morning with a Delta-T ML2x ThetaProbe connected to an HH2 moisture metre (Delta-T Devices, Cambridge, UK). Well-watered trees (control treatment) were watered up to field capacity and drought stress trees (drought treatment) were kept between 15 and 20% soil moisture. During the time of the experiment, photoperiod was maintained 16h:8h, light:dark with an average photosynthetic active radiation at the top of the plants of 150 μmol m^{-2} s^{-1}. Day and night temperatures varied between 19 and 22ºC, and 15 and 17ºC respectively. Details of the experimental design are in the Chapters 2 and 5. The leaf collection for RNA extraction was done 20 days after drought from the same experiment carried out in Chapter 5.

6.2.2. Microarray and qPCR analysis

The young leaves were sampled for all the replicates of the six genotypes, placed in an aluminium bag, flash frozen in liquid nitrogen and stored at -80°C. The samples of young leaves were considered as the first two unfurled leaves, because for some trees the first emerging leaf was too small for RNA extraction. RNA was extracted using the CTAB protocol (details in chapter 2).

6.2.2.1. Microarray analysis

Two genotypes from Spain and Italy were selected for microarray analysis. These genotypes were selected as the most extreme responses to drought with the Spanish genotype (Sp2) showing a better adaptation to water stress, and the drought-sensitive genotype from North Italy (It). Three biological replicates of RNA samples for both well-watered and drought treatments per genotype were sent to NASC Affymetrix Services (Nottingham, UK) for Affymetrix microarrays analysis using GeneChip Poplar Genome Array (Santa Clara, USA). Each replicate was hybridised with a single chip. Affymetrix .CELs files were imported into GeneSpring (Agilent Technologies, Santa Clara, USA) to apply a robust multi-array average (RMA) to each chip. Normalisation per chip was applied using three positive control genes: *UBQ11* (grail3.0064002701), *TUA5* (estExt_fgenesh4_pm.C_LG_III0736) and *ACT2* (estExt_fgenesh4_kg.C_LG_I0082). Genes were selected from Brunner *et al.* (2004). A normalisation per gene was also performed to the median. Data analysis was done with a Volcano Plot ($p<0.05$, 2-fold change) in order to identify genes commonly and differentially expressed for each genotype in response to drought and a comparison between genotypes was also performed under well-watered conditions (Fig. 6.1).

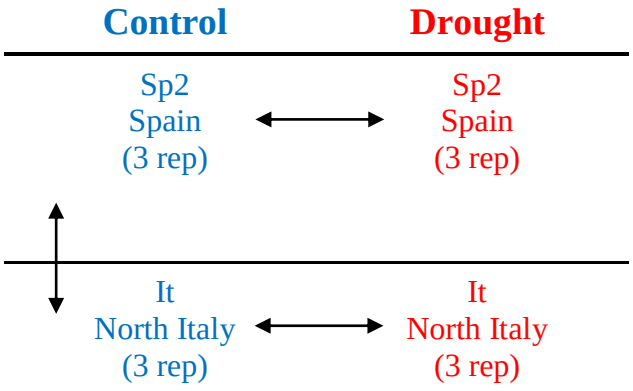


Figure 6.1: Diagram of the microarray experimental design

6.2.2.2. Real-time qPCR

RNA was also extracted using the CTAB protocol for a French genotype (Fr2) and a Dutch genotype (NL). RNA of the 4 genotypes at both conditions (drought and well-watered) was used for Real-Time qPCR on candidate genes discovered from the microarrays analysis. RT-PCR technique was utilised to confirm variation observed in gene expression between Sp1 and It from the microarrays analysis for 4 genotypes used during the drought experiment (details in Chapter 2).

Forward and reverse primers were designed specifically for each candidate gene (Table 6.1). The reference gene *YLS8* was selected from Czechowski *et al.* (2005). RNA samples were treated with the Turbo DNA-*free* kit (Ambion, Austin, USA) to remove genomic DNA, following manufacturer's instructions. Reverse transcription of 5µg of RNA to cDNA was performed using the ImProm-II Reverse Transcription kit (Promega UK, Southampton, UK) following manufacturer's instructions.

Table 6.1: Forward and Reverse primers for each candidate genes (5' to 3')

Gene name	**Forward primer (5' to 3')**	**Reverse primer (5' to 3')**
SPEECHLESS	GCCCATTAGCCCAAGAAC	GGGCCTGAGAATTTAACC
ATHVA22A	GGTCATTCCATACTTCAGTGG	GTATCGGTCATCCTCATCATCG
IP3	GAAAGAAATGAACTCGGACC	AGTCACATGGTCACCAGTGC
ERECTA	GAATTGTCCCTCCATGAGC	TGCTCGATACTGCTCTGC
YLS8	GATTGATCTTGGAACTGG	GGAGTAGTCTTTCGGAGC

cDNA was diluted 1:5 in DEPC-treated water. qPCR reaction was composed of 5µL 2X Precision-SY Master Mix (PrimerDesign Ltd, UK), 5pmol forward and reverse primers and 25ng diluted cDNA. Plates were run on a Chrom4 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, USA). Reactions were incubated at 95°C for 10 minutes and then 40 cycles of 15 seconds at 95°C, 1 minute at 60°C and plate read. An incubation at 72°C for 10 minutes followed. A melting curve was then performed from 60°C to 95°C with a read every 0.2°C and 1 second hold, in order to check for primer dimers, DNA contamination and secondary products. Values were exported with the software Opticon Monitor 3.1 (Bio-Rad Laboratories, Hercules, USA).

Amplification efficiency was measured from the equation in Liu & Saint (2002):

$E = (R_{n,A} / R_{n,B})$ ^ $[1/C_{T,A}-C_{T,B})] + 1$

where $R_{n,A}$ and $R_{n,B}$ are R_n at arbitrary thresholds A and B in an individual curve, respectively, and $C_{T,A}$ and $C_{T,B}$ are the threshold cycles at these arbitrary thresholds (Liu & Saint, 2002).

Ratios were calculated as $E^{(control - drought)_{target}} / E^{(control - drought)_{reference}}$

6.3. Results

6.3.1. Microarrays

Normalized data from the twelve arrays were obtained from GeneSpring (Fig. 6.2 and appendix CD3) from a volcano plot (Appendix CD4) and analysed by comparing the change in gene expression between control and drought conditions for each genotype (fold change = average drought / average control) and also the differences between both genotypes under control conditions (fold change = average control Sp2 / average control It).

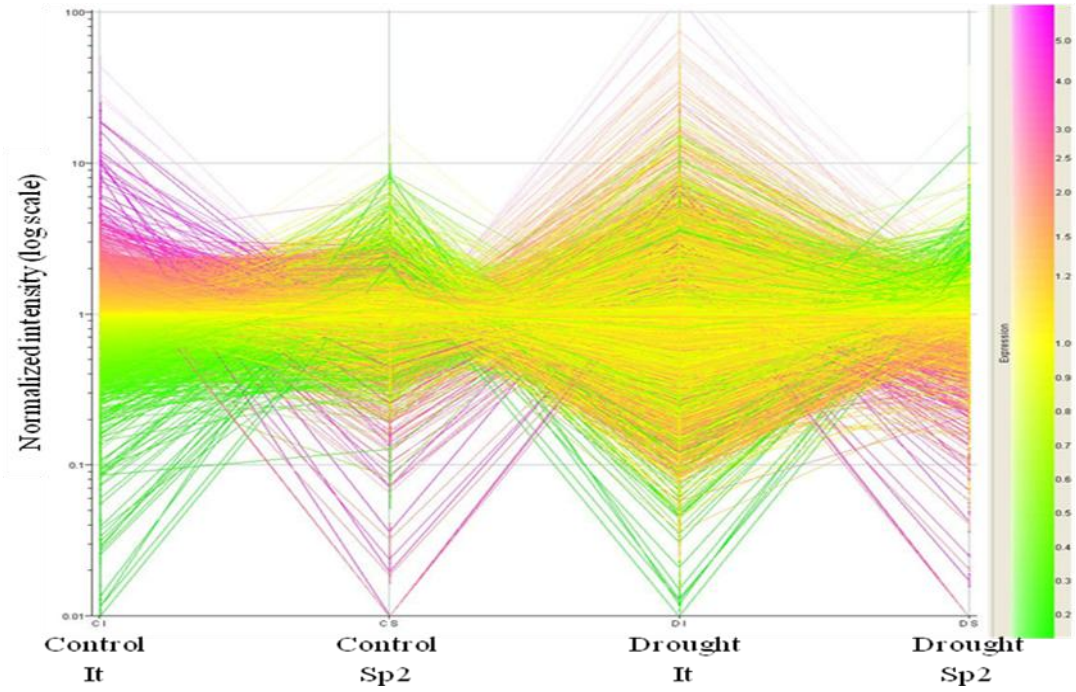


Figure 6.2: Normalized expression ratios of the Affymetrix microarrays (Santa Clara, USA) for each genotype under control and drought conditions using the averages of the three biological replicates on the first two unfurled young leaves. Each colored line represents a single probe or transcript.

6.3.1.1 Genes expressed in response to drought

The Northern Italian genotype It, modified their gene expression of 3167 transcripts in response to drought, including 1048 two-fold up (Fig. 6.3) and 2119 two-fold down regulated (Fig. 6.4). For the Spanish genotype Sp2, the number of transcripts significantly different between control and drought conditions was lower than the Italian genotype, with a total of 649 transcripts in response to drought, 232 two-fold up (Fig.

6.3) and 417 transcripts were two-fold down regulated (Fig.6.4). Only 37 probes were commonly up regulated between the two genotypes and 230 were commonly down regulated.

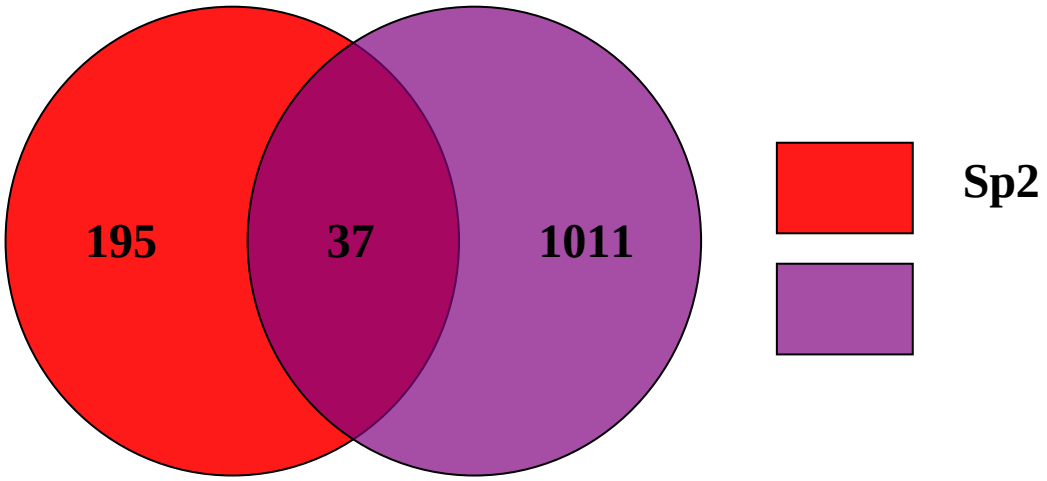


Figure 6.3: Venn diagram representing the Affymetrix ID probes which were two-fold up regulated in response to moderate drought - differentially expressed between the Spanish Sp2 (red) and the Italian It (purple) genotypes and commonly expressed between the two genotypes. Numbers in the circle overlap indicate the number of transcripts common to both genotypes and numbers outside the overlap indicate the number of transcripts exclusive to the genotype indicated.

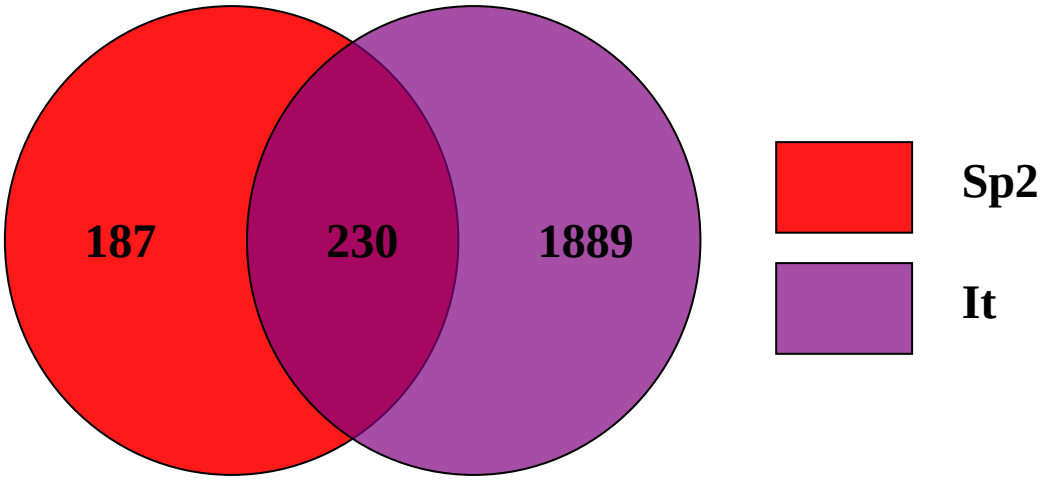


Figure 6.4: Venn diagram representing the Affymetrix ID probes which were two-fold down regulated in response to moderate drought - differentially expressed between the Spanish Sp2 (red) and the Italian It (purple) genotypes and commonly expressed between the two genotypes. Numbers in the circle overlap indicate the number of transcripts common to both genotypes and numbers outside the overlap indicate the number of transcripts exclusive to the genotype indicated.

Using the Poparray website (http://aspendb.uga.edu/poparray), probe annotations were obtained for each transcript with their corresponding *Populus* gene model, *Arabidopsis* target name, *Arabidopsis* description, GO (Gene Ontology) biological process target Name, GO cellular component target name and GO molecular function target name. The annotations were studied for each genotype in response to drought stress, either commonly or differentially expressed between the two genotypes. The transcripts were grouped in different categories depending on their annotations. The drought category contained transcripts which were mainly up regulated for Sp2 while for It they were both up and down regulated. Osmoprotectant and stomata were two categories which had more transcripts expressed by the Italian genotype than the Spanish one.

The categories represented with the highest number of up regulated transcript numbers for the Italian genotype were other stresses (salt, cold, heat and oxidative stresses), growth and second messengers while they were drought, growth and other stresses for the Spanish genotype (Table 6.2 and details in the Appendix CD5).
For the down regulated transcripts, categories with the highest transcripts were growth, repair and other stresses for It and repair, other stresses and ABA for Sp2. The two genotypes had no transcripts commonly expressed for ABA, drought and photosynthesis but shared transcripts in the categories of growth, second messengers and other stresses (Table 6.2).
The common transcripts were mainly down regulated rather than up regulated. Growth, other stress and second messengers were the categories with the largest transcripts down regulated by both genotypes. The stomata category also contained two transcripts commonly down regulated which were both *ERECTA* gene which is linked to stomatal development and transpiration efficiency (Masle *et al.*, 2005).

Table 6.2: Number of transcripts in each category related to water stress and growth expressed by the Italian (It), the Spanish (Sp2) or both genotypes in response to drought

	UP			DOWN		
			Common			Common
Categories	**Italy**	**Spain**	**Italy - Spain**	**Italy**	**Spain**	**Italy - Spain**
ABA	14	2	0	13	5	0
Degradation	12	0	0	25	0	1
Detoxification	9	1	1	9	3	0
Drought	19	14	0	23	2	0
Growth	26	12	2	74	3	6
Growth inhibition	7	1	1	12	1	1
Osmoprotectant	22	1	3	26	1	1
Other stress	31	9	1	35	5	7
Photosynthesis	4	0	0	0	2	0
Repair	19	2	1	48	7	3
Second messengers	27	2	1	25	2	7
Stomata	4	0	0	13	1	2
Transcription factor	22	2	0	13	2	6
Wax	1	1	0	0	0	0

Pathway analysis

The software Mapman (Thimm *et al.*, 2004) was used to study the variation of gene expression in pathways in response to drought between the two genotypes. An overview of all the different bins was first studied with their significance and then three pathways were focused on metabolism, cellular response and biotic stress.

The significance of the bins was performed using the Wilcoxon Rank Sum Test with a Benjamini Hochberg correction. The genes were categorised depending on their gene ontology in bins and sub bins. The bins were then tested to predict a difference in behaviour of gene expression compared to the other bins. Only one bin for the Spanish genotype (Table 6.3) was close to significance which was the bin of Cell ($p= 0.0524$). The Italian genotype had five main bins and nine sub-bins which were significant ($p<0.05$) including DNA, RNA, stress, transport and minor CHO metabolism (Table 6.3).

Table 6.3: Description of the significant bins and sub-bins from the microarrays transcripts list in response to drought for the Spanish and the Italian genotypes. The probability (p-value) was calculated using a Wilcoxon Sum of Rank test with a Benjamini Hochberg correction in MapMan (Thimm *et al.*, 2004).

Genotype	Bins and sub-bins	Description	Number of genes	p-value
It				
	28	DNA	51	0.000069
	28.1	DNA.synthesis/chromatin structure	46	0.000069
	20.1	Stress.biotic	40	0.000146
	20.1.7	Stress.biotic.PR-proteins	21	0.000244
	30.2.17	Signalling.receptor kinases.DUF 26	32	0.000927
	27	RNA	205	0.001391
	27.3	RNA.regulation of transcription	181	0.001439
	31.3	Cell.cycle	19	0.003736
	20	Stress	59	0.005183
	30.2.3	Signalling.receptor kinases.leucine rich repeat III	15	0.008419
	34	Transport	91	0.008419
	26.4	Misc.beta 1,3 glucan hydrolases	21	0.034951
	30.2	Signalling.receptor kinases	96	0.037742
	3	Minor CHO metabolism	13	0.042266
Sp2				
	31	Cell	16	0.052461

The transcripts in the photosynthesis bin were mainly up regulated for the Italian genotype and down regulated for the Spanish one. This can be observed in Fig. 6.5 for the light reactions and the Calvin cycle.

Minor CHO metabolism pathway showed variation in gene expression between the Italian and Spanish genotypes. For It, the transcripts were up and down regulated for the raffinose, trehalose and callose metabolisms (Fig. 6.5a). For Sp2, they were mainly down regulated for the metabolism of raffinose and trehalose (Fig. 6.5b).

The cell wall pathway had transcripts up regulated for Sp2 in the categories of synthesis, modification and degradation (Fig. 6.5b) while the Italian genotype had transcripts both up and down regulated for the same categories (Fig. 6.5a).

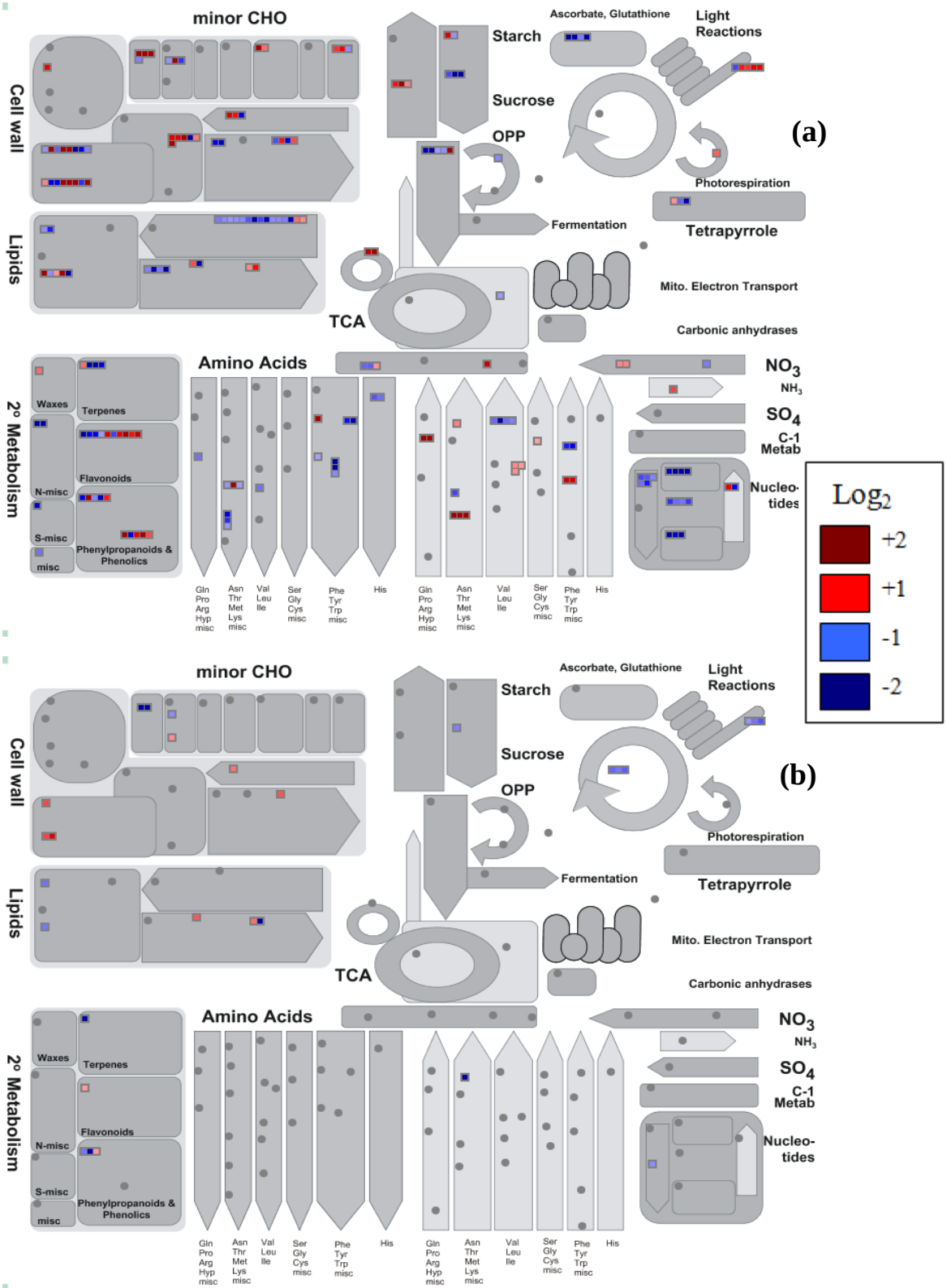


Figure 6.5: Metabolism overview pathway representation for the Italian **(a)** and the Spanish **(b)** genotypes transcripts in response to drought. Each square represents a transcript from the microarray analysis, the blue squares are down regulated and the red squares are up regulated.

For both genotypes, the secondary metabolism pathway had transcripts up and down regulated (Fig. 6.5 and 6.7). Only the Italian genotype had a transcript in the wax sub-bin which was up regulated (Fig. 6.5a). The gene ontology of this transcript was related to wax biosynthetic process.

The bin related to cell contained transcripts that were mainly down regulated for both genotypes, including cell division and cell cycle (Fig. 6.6).

The development bin showed transcripts mainly down regulated for the Italian genotype (Fig. 6.6a) including storage proteins and up regulated for the Spanish one (Fig. 6.6b). Although transcripts in this bin were mainly down regulated for It, two NAC domain transcription factors were up regulated. For Sp2, two up regulated transcripts were a senescence-associated protein-related and *RD26* (Response to Desiccation 26).

The stress bin was particularly studied and was divided in biotic and abiotic stresses. For both genotypes, transcripts in the biotic stress sub-bin were up and down regulated although they contained more up regulated transcripts than down regulated (Fig. 6.6). For the Italian genotypes in the biotic stress categories, many transcripts were genes of defense, particularly PR-proteins, and were up regulated (Fig. 6.7a). PR-proteins are pathogenesis-related proteins and water stress is a factor that can induce those proteins (Edreva, 2005). The abiotic stress sub-bin (Fig. 6.6) was also divided into categories: heat, cold, drought/salt, touch/wounding, light and miscellaneous. The heat category had transcripts related to heat shock proteins and they were both up and down regulated for the Italian genotype (Fig. 6.6a) and contained two up and one down regulated transcripts for the Spanish genotype (Fig. 6.6b). Only It had transcripts in the drought/salt category and they were mostly dehydration-responsive family proteins. Four were down regulated and two were up regulated (Fig. 6.6a). Finally the miscellaneous category had transcripts that were down regulated for the Italian genotype (Fig. 6.6a) and up regulated for the Spanish genotype (Fig. 6.6b). The latter had a miscellaneous transcript which was a *RD2* (Responsive to Desiccation 2).

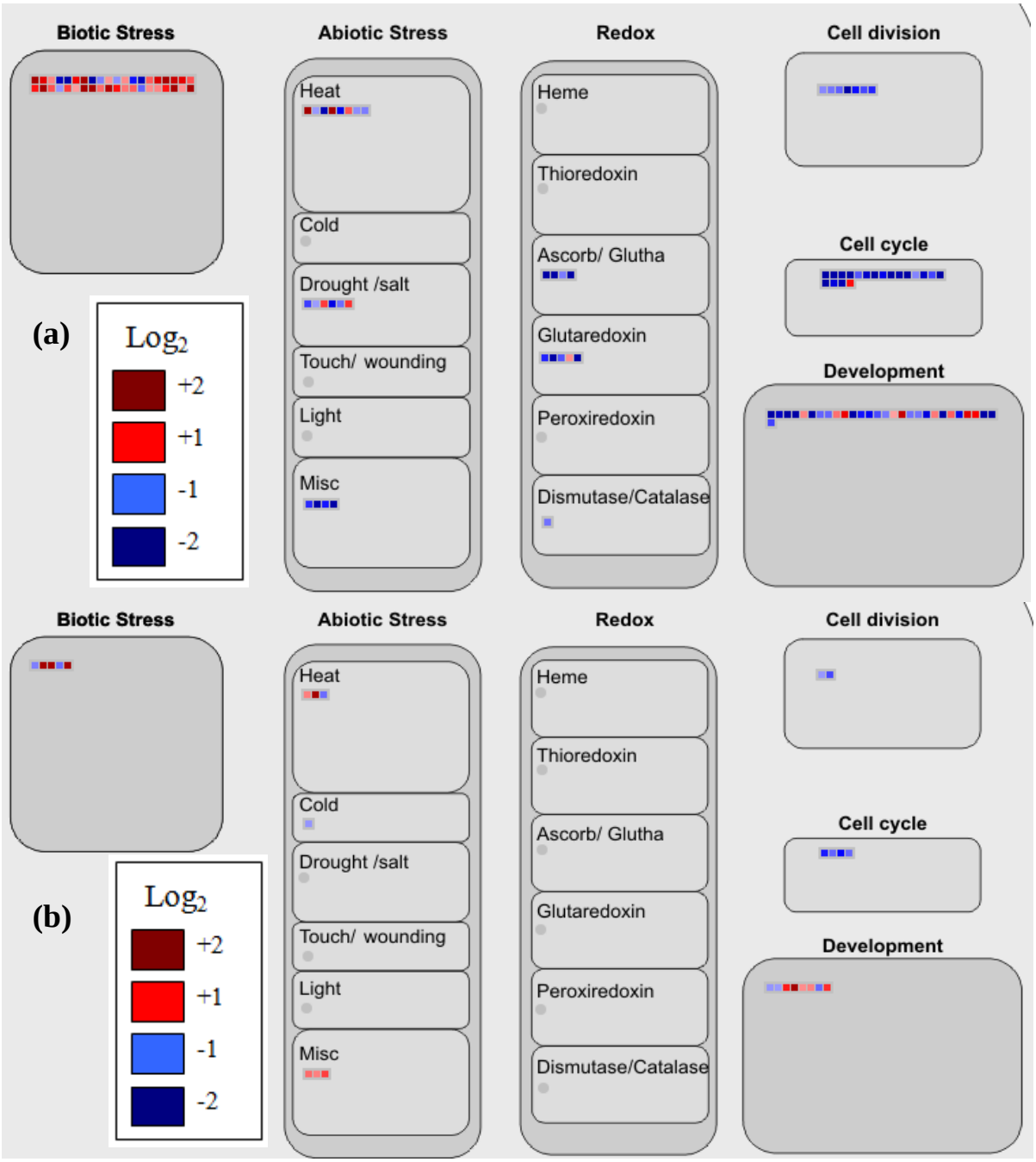


Figure 6.6: Cellular response overview pathway representation for the Italian **(a)** and the Spanish **(b)** genotypes transcripts in response to drought. Each square represents a transcript from the microarray analysis, the blue squares are down regulated and the red squares are up regulated.

The signalling bin contained many more transcripts for the Italian genotype than the Spanish but they were for both genotypes up and down regulated (Fig. 6.7). For It, transcripts related to calcium and leucine rich signalling were up and down regulated and g-proteins were down regulated. For Sp2 receptor kinases and g-proteins were mainly up regulated while calcium signalling transcripts were down regulated.
The RNA bin showed variation in gene expression between the two genotypes. The transcripts for the Italian genotype were mainly down regulated while the ones for the Spanish genotype were both up and down regulated. The transcription factors (TF) related to stress were described in the biotic stress pathway (Fig. 6.7). For the Italian genotype, one heat shock TF was up regulated, MYB transcripts were up and down regulated, *ERF* which correspond to *AP2/EREBP* transcription factors were down regulated and *WRKY* domain was mainly up regulated (Fig. 6.7a). Sp2 had one *MYB* transcription factor which was down regulated and another *AP2/EREBP* up regulated (Fig. 6.7b).

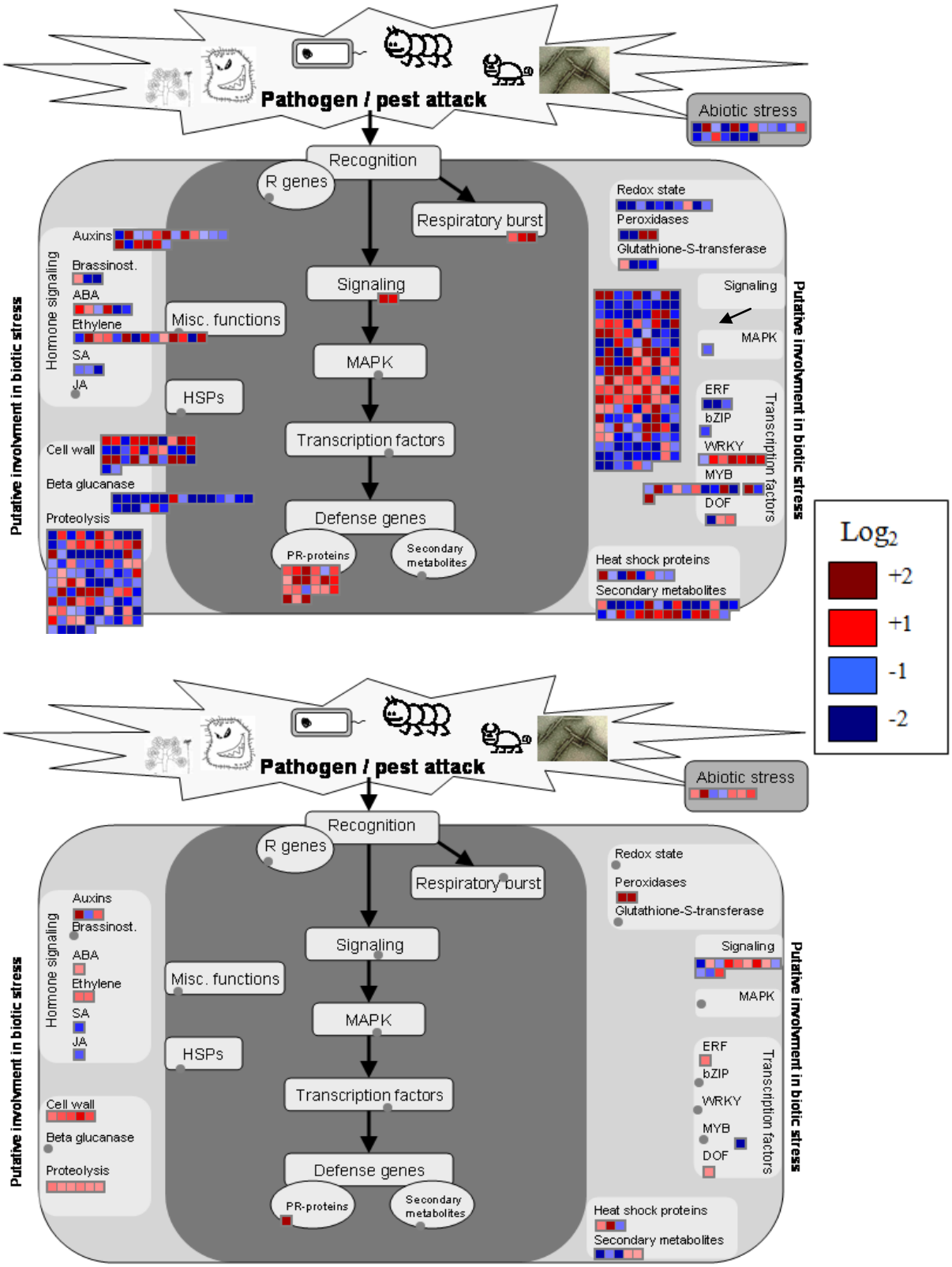


Figure 6.7: Biotic stress pathway representation for the Italian **(a)** and the Spanish **(b)** genotypes transcripts in response to drought. Each square represents a transcript from the microarray analysis, the blue squares are down regulated and the red squares are up regulated.

The Italian and the Spanish genotypes showed variation in the gene expression of hormone metabolism (Fig. 6.7). For It, transcripts for auxin, brassinosteroid, ABA and ethylene were up and down regulated, except for salicylic acid degradation which was down regulated (Fig. 6.7a). For Sp2, auxin transcripts were up and down regulated but ABA responsive-activated and ethylene degradation were up regulated and salicylic acid degradation and jasmonic acid degradation were down regulated (Fig. 6.7b).
For the ABA metabolism pathway in Mapman (Thimm *et al.*, 2004), only the Italian genotype showed two transcripts with variation in gene expression in response to drought. Both transcripts were up regulated and involved in the synthesis of 9-cis epoxycarotenoid dioxygenase (Fig. 6.8). Qin & Zeevaart (1999) showed that the 9-*cis*-epoxycarotenoid cleavage catalyzed by NCED is the rate-limiting step in the drought-induced ABA biosynthesis pathway.

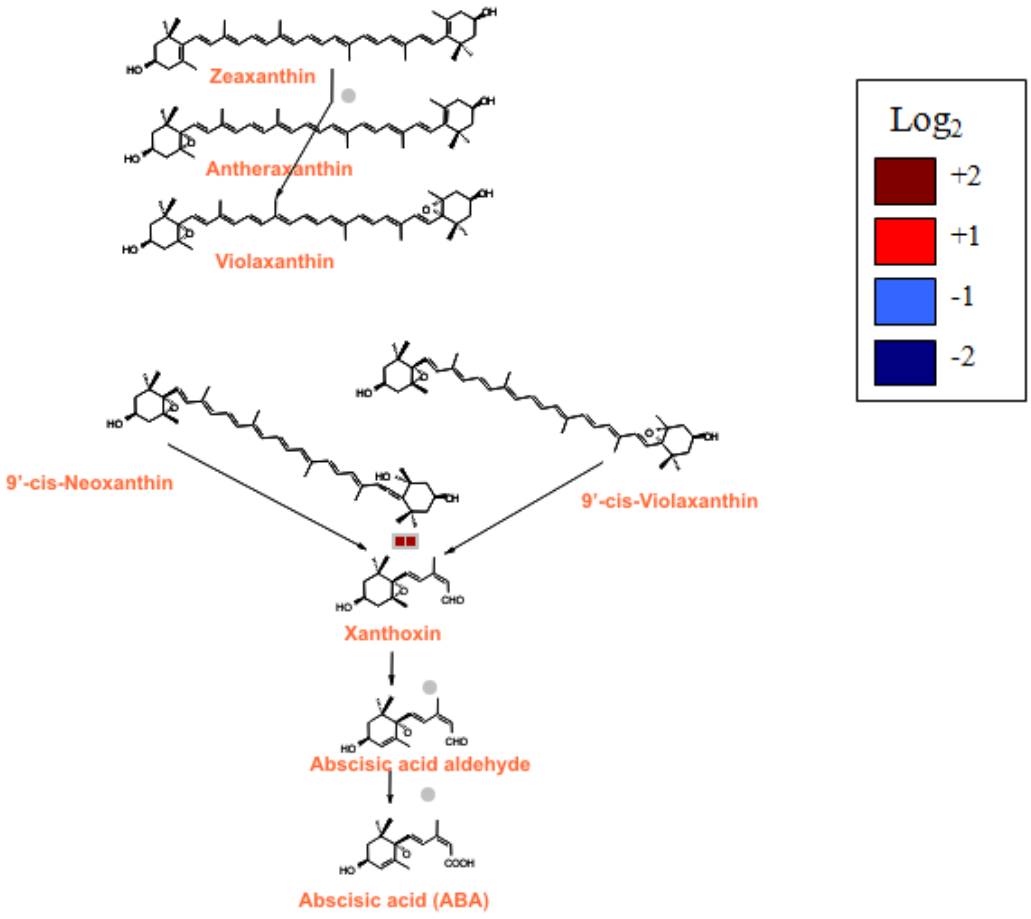


Figure 6.8: ABA metabolism stress pathway representation for the Italian genotype transcripts in response to drought. Each square represents a transcript from the microarray analysis, the blue squares are down regulated and the red squares are up regulated.

6.3.1.2. Differential gene expression under well-watered conditions

A comparison in gene expression between the two genotypes was also done under well-watered conditions. 754 positive values of $\log_2$ gene expression had their expression higher in Sp2 compared to It, and 852 negative values were less expressed in Sp2 compared to It, i.e. higher expression in It compared to Sp2.

Looking at the annotations of the transcripts, variation in the category of gene expressed was observed under well-watered conditions between the Italian and Spanish genotypes (Table 6.4 and details in the Appendix CD6). Number of genes related to growth was higher in It (41 genes) than Sp2 (18 genes). ABA, degradation, drought and other stresses were also categories where the genes were more highly expressed in It than in Sp2. Osmoprotectant and repair were two categories which had a higher number of genes more highly expressed in the Spanish genotype (15 and 17 genes respectively of each category) than the North Italian one (10 and 9 genes respectively).

Genes related to water stress were highly expressed in the Spanish genotype compared to the Italian genotype which included heat shock proteins, second messengers such as *IP3* (Inositol 1,3,4-triphosphate 5/6-kinase 4) and protein kinases (e.g. *MAPKK9*: Mitogen-activated protein kinase kinase kinase 9). Genes related to drought were highly expressed in It than Sp2 were for example *ATHVA22A* (ABA and stress-inducible protein), *ABA2* (ABA deficient 2), *ERD15 (Early Response to Dehydration 15)* and *RD21* (Responsive to Dehydration 21).

Table 6.4: Number of transcripts in each category related to water stress and growth expressed under well-watered conditions between Sp2 and It

Categories	More expressed in Sp2 compared to It	More expressed in It compared to Sp2
ABA	5	9
Degradation	9	14
Detoxification	8	11
Drought	7	13
Growth	18	41
Growth inhibition	2	4
Osmoprotectant	15	10
Other stresses	11	18
Photosynthesis	2	3
Repair	17	9
Second messengers	6	7
Transcription factors	5	9
Wax	0	1

Pathway analysis was also done using the software Mapman (Thimm *et al.*, 2004). No bins or sub-bins were significant from a Wilcoxon Rank Sum Test with a Benjamini Hochberg correction.

The metabolism pathway showed a higher gene expression of a wax gene and most lipid genes for It compared to Sp2 in well-watered conditions (Fig. 6.9). In the second metabolism pathway, Sp2 had more genes highly expressed compared to It in phenylpropanoids and lignin biosynthesis (Fig. 6.9).

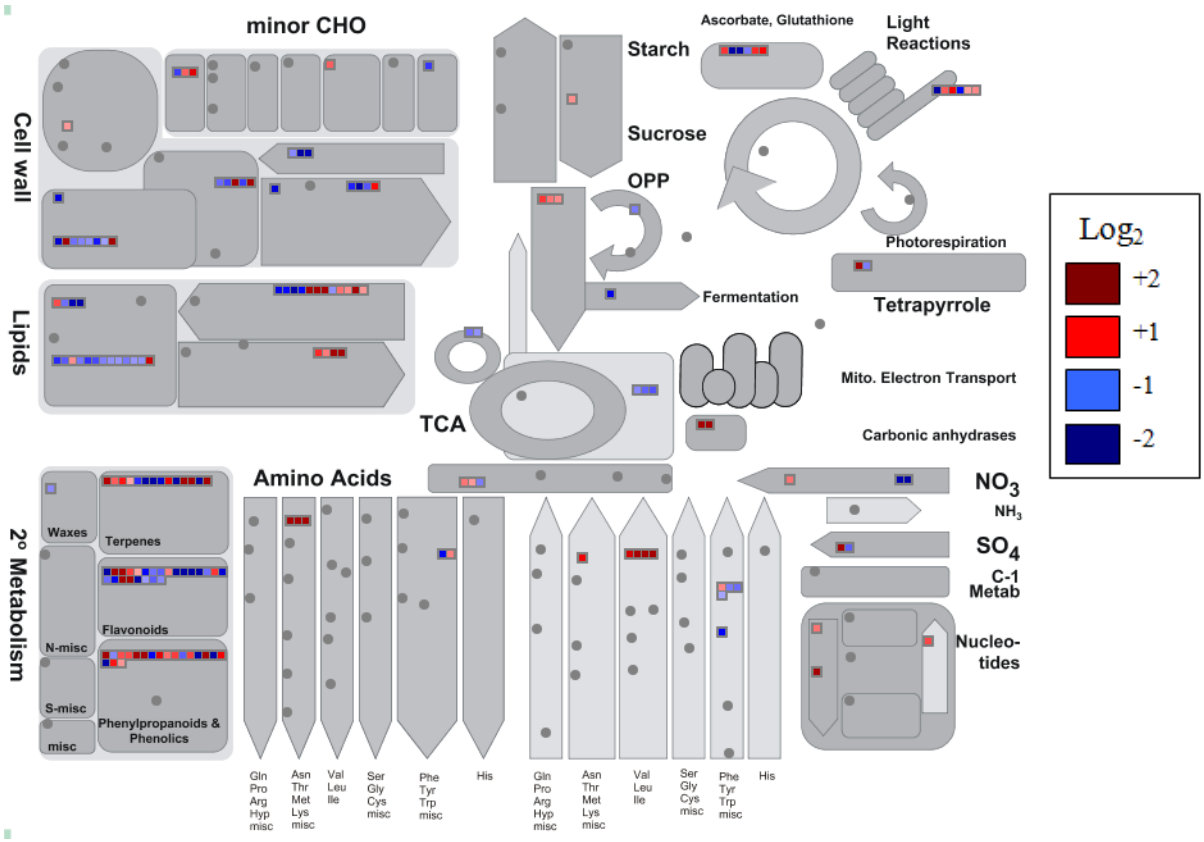


Figure 6.9: Metabolism overview pathway representation for the well-watered transcripts. Each square represents a transcript from the microarray analysis, the blue squares are less expressed in the Spanish genotype and the red squares are more expressed in the Spanish genotype compared to the Italian genotype.

The cellular response overview pathway showed genes related to heat were more expressed in the Spanish genotype (Fig. 6.10), including a heat shock protein with an expression fold change of 10.34 in Sp2 compared to It. Genes related to cold and drought were more highly expressed in It than Sp2 including two *USP* (Universal Stress Protein) and *ERD15* (Early Response to Drought 15).

The biotic stress pathway also showed differences in gene expression for the plant hormones between the two genotypes under well-watered conditions (Fig. 6.11). Auxin and ABA genes had a lower expression in Sp2 than It including a *CCD1* gene (Carotenoid Cleavage Dioxygenase). Transcripts related to brassinosteroid and jasmonate were also less expressed in Sp2. Salicylic acid and ethylene were mainly showing a high expression in the Spanish genotype than the Italian one.

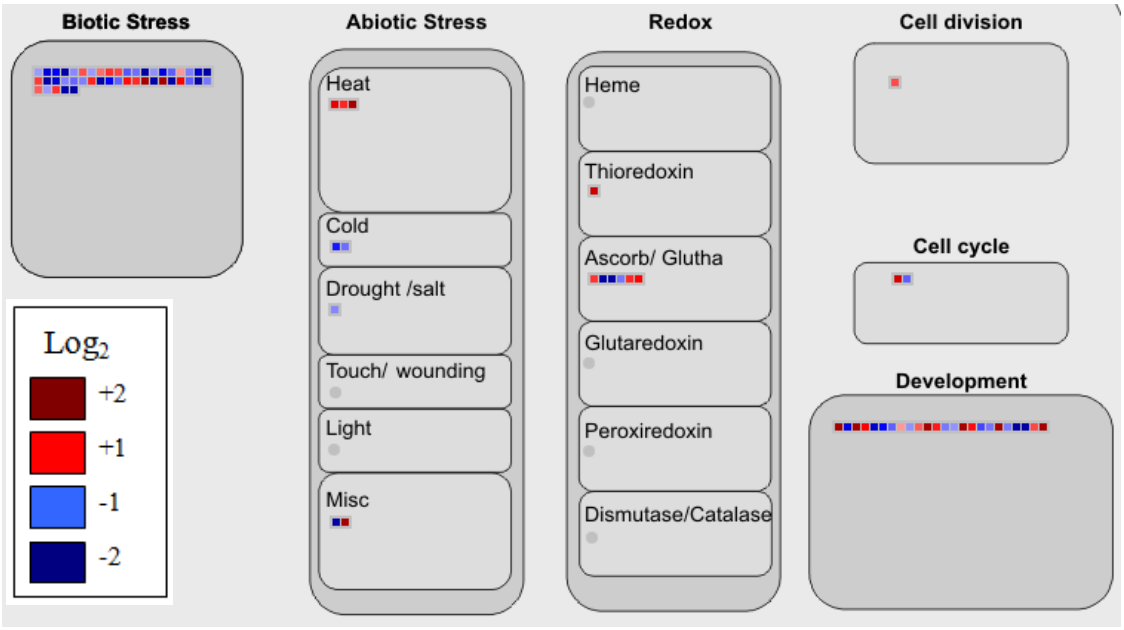


Figure 6.10: Cellular response overview pathway representation for the well-watered transcripts. Each square represents a transcript from the microarray analysis, the blue squares are less expressed in the Spanish genotype and the red squares are more expressed in the Spanish genotype compared to the Italian genotype.

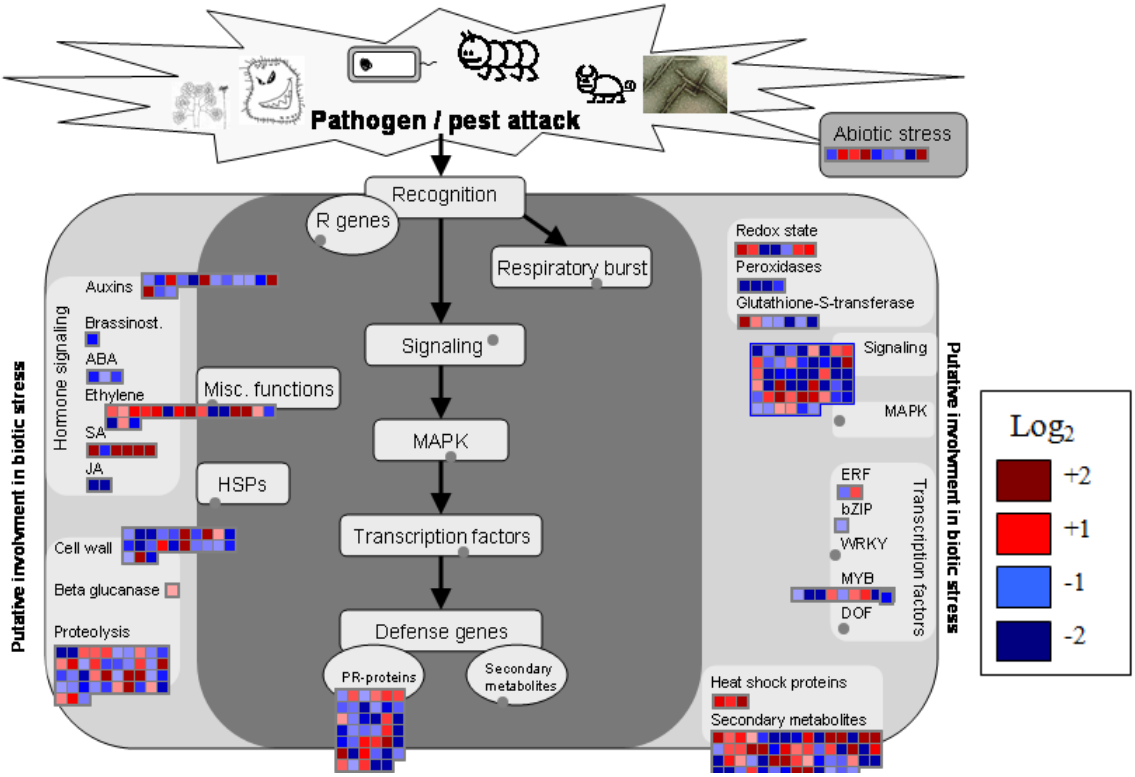


Figure 6.11: Biotic stress pathway representation for the well-watered transcripts. Each square represents a transcript from the microarray analysis, the blue squares are less expressed in the Spanish genotype and the red squares are more expressed in the Spanish genotype compared to the Italian genotype.

6.3.2. Candidate genes and Real-time qPCR

From the microarrays analysis, six candidate genes (Table 6.5) were selected to perform real-time PCR on the genotypes used for the microarrays (Sp2 and It) but also on two other genotypes, a French (Fr2) and a Dutch (NL), under well-watered and drought conditions.

The selected genes were related to drought including *RD26*, stomata (*ERECTA* and *SPEECHLESS*), ABA (*ATHVA22A* and *CCD1*) and signalling (*IP3*). *SPEECHLESS* was down regulated in response to drought in the Italian genotype. It is essential for the asymmetric divisions that establish the stomatal lineage. *ATHVA22A* is a protein induced by ABA and stress and was more expressed in the Italian genotype under well-watered conditions. *IP3* was more expressed in Sp2 compared to It under well-watered conditions. It is a secondary messenger involved in stress signalling transduction. *RD26* is a NAC transcription factor related to ABA-mediated response to drought. This gene was up regulated in response to drought only for the Spanish genotype. The gene *CCD1* encodes a protein for 9-*cis*-epoxycarotenoid dioxygenase involved in the production of ABA and was up regulated in response to drought for the Italian genotype. Finally the *ERECTA* gene was down regulated in response to drought for both genotypes. It is involved in the cell division generating stomatal complexes and was identified as a quantitative trait locus for transpiration efficiency (Masle *et al.*, 2005).

Table 6.5: Information on the six candidate genes from the microarray analysis, gene description and position on the *Populus* genome

Fold change	Log_2	Affymetrix Probe ID	Gene Model	Annotation from *Arabidopsis*	*Arabidopsis* Description	Gene expression	LG	Position	Name
0.23	-2.09	PtpAffx.210224.1.S1_at	fgenesh4_pm.C_LG_XII000063	Encodes a basic helix-loop-helix (bHLH) transcription factor that is necessary and sufficient for the asymmetric divisions that establish the stomatal lineage in *Arabidopsis thaliana*. Expression of SPCH in young epidermal cells allows these cells to make asymmetric divisions.	SPCH (SPEECHLESS)	Response to drought Unique in It	XII	1099259-1101920	*SPEECHLESS*
0.18	-2.49	Ptp.4751.1.S1_at	estExt_fgenesh4_pg.C_LG_XII0676	Part of the AtHVA22a family. Protein expression is ABA- and stress-inducible.	ATHVA22A (*Arabidopsis thaliana* HVA22 homologue A)	Well-watered conditions More expressed in It	XII	8377019-8380204	*ATHVA22A*
2.67	1.42	PtpAffx.69528.1.A1_at	gw1.VII.365.1	inositol 1,3,4-trisphosphate 5/6-kinase 4 (AtITPK4)	ATITPK4 (INOSITOL 1,3,4-TRISPHOSPHATE 5/6-KINASE 4)	Well-watered conditions More expressed in Sp2	VII	172038-175212	*IP3*
5.9	2.56	PtpAffx.4325.1.S1_at	estExt_Genewise1_v1.C_LG_XI3994	Encodes a NAC transcription factor induced in response to desiccation. It is localized to the nucleus and acts as a transcriptional activator in ABA-mediated dehydration response.	RD26 (RESPONSIVE TO DESSICATION 26); transcription factor	Response to drought Unique in Sp2	XI	11881054-11882792	*RD26*
3.08	1.62	PtpAffx.200858.1.S1_at	estExt_Genewise1_v1.C_LG_I1784	Encodes a protein with 9-*cis*-epoxycarotenoid dioxygenase activity	CCD1 (CAROTENOID CLEAVAGE DIOXYGENASE 1)	Response to drought Unique in It	I	19247841-19257657	*CCD1*

Log_2 It	Log_2 Sp2	Affymetrix Probe ID	Gene Model	Annotation from *Arabidopsis*	*Arabidopsis* Description	Gene expression	LG	position	Name
-2.55	-1.05	PtpAffx.206636.1.S1_at	fgenesh4_pg.C_LG_VI001481	Involved in specification of organs originating from the shoot apical meristem. ER has been identified as a quantitative trait locus for transpiration efficiency by influencing epidermal and mesophyll development, stomatal density and porosity of leaves. Together with ERL1 and ERL2, ER governs the initial decision of protodermal cells to either divide proliferatively to produce pavement cells or divide asymmetrically to generate stomatal complexes.	ER (ERECTA)	Response to drought Common in It and Sp2	VI	14628776-14635942	*ERECTA*

Real-time qPCR data were obtained for the genes *SPEECHLESS*, *ATHVA22A*, *IP3* and *ERECTA*. No unique primer pair was found for the candidate genes *RD26* and *CCD1* as the melting curve revealed.

For each gene, a comparison was done between microarrays and real-time PCR expression values for each genotype in response to drought. Similar pattern of expression was observed between the two methods (Fig. 6.12), except for the gene *IP3* for the Spanish genotype which was up regulated for the microarray but down regulated for real-time qPCR (Fig. 6.12b).

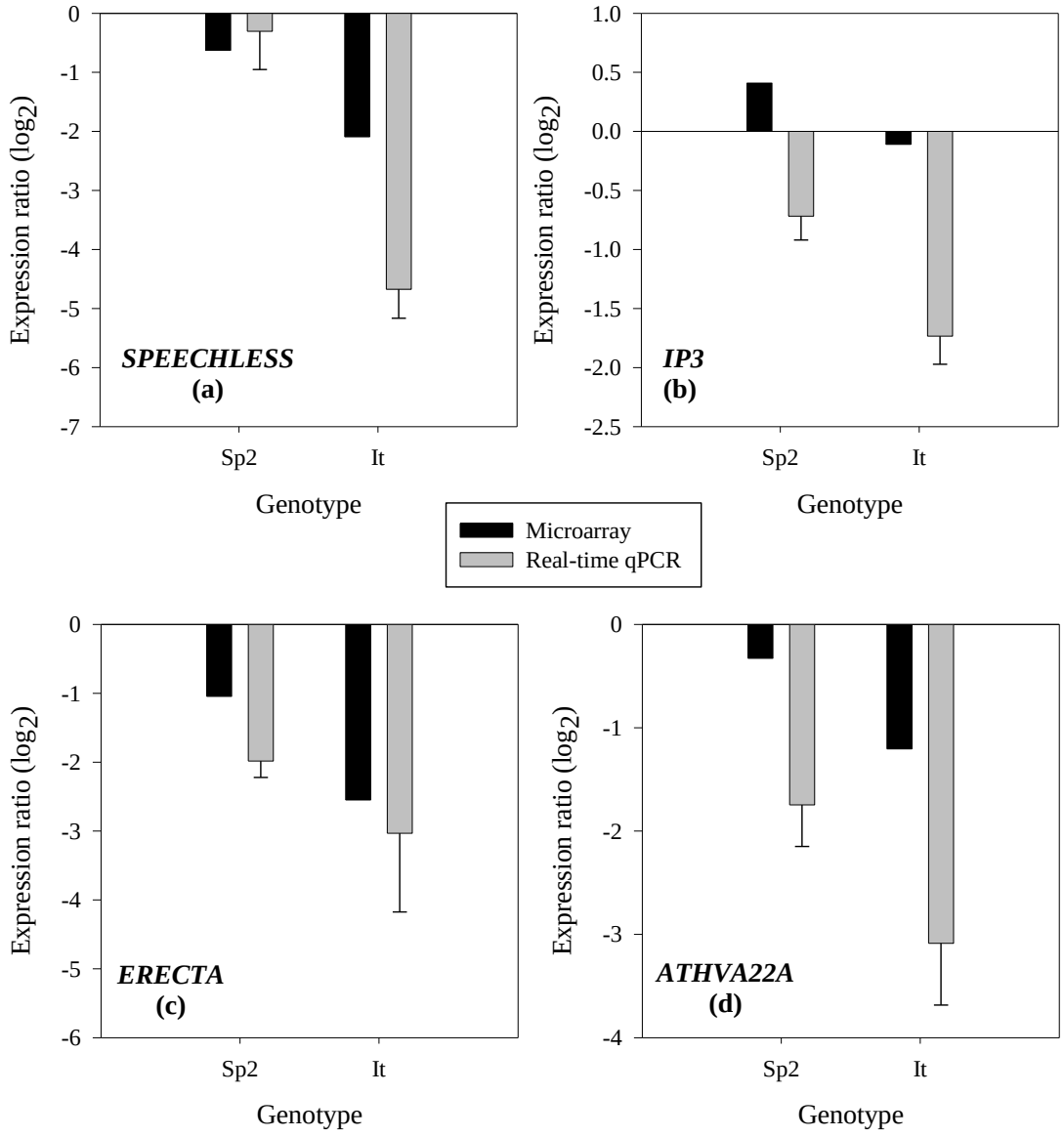


Figure 6.12: Comparison of expression values (log_2) between microarray analysis and real-time PCR per genotype in response to drought for four candidate genes: *SPEECHLESS* (a), *IP3* (b), *ERECTA* (c), *ATHVA22A* (d)

Measurements of gene expression were also conducted using real-time qPCR for two other genotypes and compared to Sp2 and It. Real-time qPCR values were expressed in response to drought for each genotype (Fig. 6.13). *SPEECHLESS*, *IP3* and *ATHVA22A* expression ratios were all significantly different between genotypes in response to drought (*SPEECHLESS*: $F_{3,32}$=9.311, p<0.001; *IP3*: $F_{3,32}$=11.629, p<0.001; *ATHVA22A*: $F_{3,32}$=16.219, p<0.001).

SPEECHLESS was down regulated in response to drought for all genotypes (Fig. 6.13a), particularly the Italian genotype (log_2 -4.7) compared to the Spanish genotype (log_2 -0.3) and Fr2 and NL which had quite similar ratios (log_2 -1.7 and -2.3 respectively).

Expression ratios for *IP3* were up and down in response to drought depending on the genotype (Fig. 6.13b). Sp2 and NL had similar values (log_2 -0.7 and -0.6 respectively), It had the lowest value (log_2 -1.7) while Fr2 had a positive expression ratio (log_2 0.5).

The expression ratios in response to drought for the *ERECTA* gene were all down regulated (Fig. 6.13c) with no significant different between genotypes ($F_{3,32}$=0.845, p=0.48).

The *ATHVA22A* gene was up and down regulated in response to drought depending on the genotypes (Fig. 6.13c). By S-N-K post-hoc testing, Fr2 and NL ratios were not statistically different, although Fr2 expression ratio was positive (log_2 0.8) and the ratio for NL was negative (log_2 -0.3). The values for Sp2 and It were down regulated and also different between each others and with the two other genotypes (log2 -1.7 and -3.1 respectively).

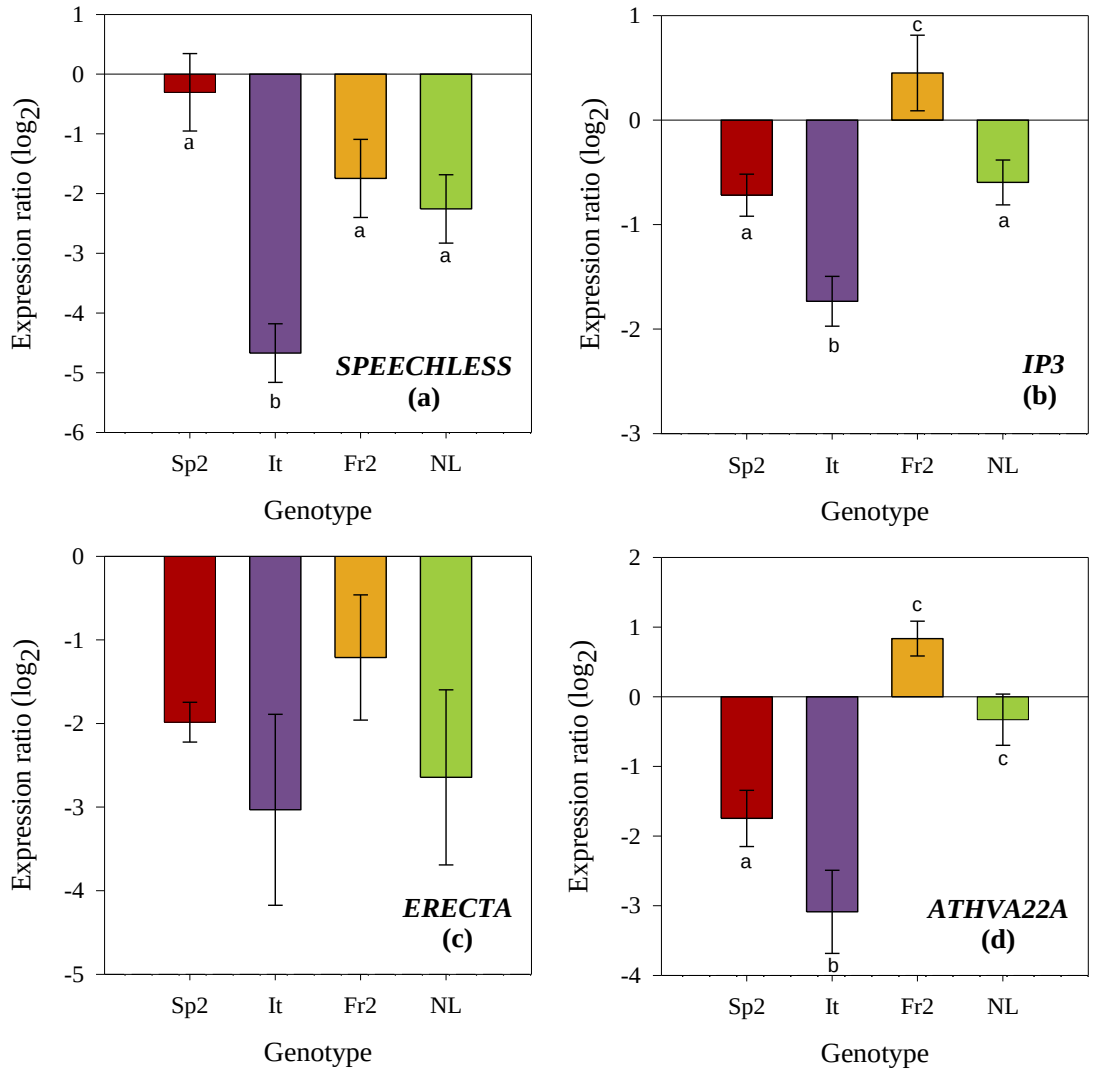
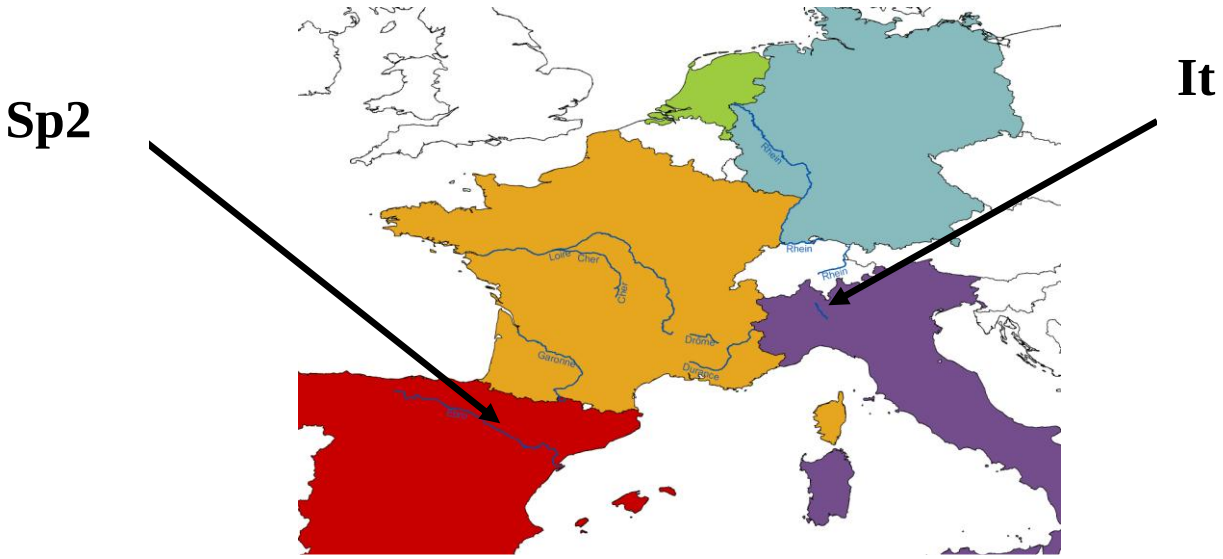


Figure 6.13: Expression ratio (log_2) from real-time qPCR in response to drought per genotype for each candidate gene: *SPEECHLESS* (a), *IP3* (b), *ERECTA* (c) and *ATHVA22A* (d). Same letter indicate no significant difference at 5% level, Student-Newman Keuls post-hoc testing. Each value with bars represents the average ± standard error.

6.4. Discussion

The aims of this chapter were to identify natural variation in gene expression between extreme genotypes of *P. nigra*, originating from different environments, in relation to the adaptation to water dificit. A genomic study was conducted in both well-watered and moderate drought stress conditions, using microarray analysis and real-time qPCR. The Italian genotype was selected because of its latitude of origin (Fig. 6.14), as the precipitations were higher than the Spanish genotype but the annual mean and the extreme month temperatures were comparable between the two environments (Fig. 6.14). Similar observations were also done when examining seasonal precipitations and temperatures. Variations in genetic expression were then assumed only water related and not temperature related.



Climatics from the location of origin (www.worldclim.org/bioclim.htm)	**Sp2**	**It**
Mean annual temperature (°C)	13.7	13
Max temperature of warmest month (°C)	29.5	29
Min temperature of coolest month (°C)	1.3	-1
Mean annual precipitation (mm)	365	982
Precipitation of the wettest month (mm)	53	122
Precipitation of the driest month (mm)	17	55

Figure 6.14: Location on a European map and climatic data related to the two genotypes used for the microarray analysis. Data were collected by Jennifer DeWoody from www.worldclim.org/bioclim.htm

A genomic analysis was done at two levels for those genotypes: their individual response to a moderate drought stress and also their natural differences under well-watered conditions. The microarrays were analysed using the annotations for each transcripts from PopArray (http://aspendb.uga.edu/poparray) and by studying the pathway in the software MapMan (Thimm *et al.*, 2004). Recently, the latter has been widely used in plants studies, for example in salt stress (Gruber *et al.*, 2009), in elevated CO_2 (Tallis *et al.*, 2010), and also in drought stress (Giraud *et al.*, 2008; Degenkolbe *et al.*, 2009; Hayano-Kanashiro *et al.*, 2009; Evers *et al.*, 2010; Payton *et al.*, 2010).
For this experiment, a progressive and moderate drought was applied and soil moisture was maintained between 15 and 20% in order to observe adaptive responses as opposed to shock responses when water is completely withheld. Water potential was also measured during this experiment using a pressure bomb (Appendix E) and displayed no significant differences between treatment ($F_{1,46}$=0.21, p=0.649) and genotypes ($F_{5,46}$=0.615, p=0.689) showing the moderate stress felt by those trees. In the fields and for most crops, plants will experience a slow soil drying rather than an acute and fast drought stress (Cattivelli *et al.*, 2008). A long-term experiment may also reveal drought tolerance mechanisms (Degenkolbe *et al.*, 2009).

6.4.1. Genes expressed in response to water deficit

The number of transcripts expressed in response to water deficit was very high for the Italian genotype compared to the Spanish one. 3167 transcripts were differentially expressed in response to drought in the Italian genotype while only 649 transcripts were differentially expressed in response to water deficit in the Spanish trees. Although it might be expected for the supposedly drought tolerant plant to express more genes in response to drought, similar findings were observed in rice. For example, Degenkolbe *et al*. (2009) used two genotypes of rice – a drought tolerant and a drought sensitive – to study their genomic response to a moderate and long-term water stress. The drought tolerant genotype had less genes drought regulated than the sensitive genotype (Degenkolbe *et al.*, 2009). However, opposite results have also been observed in drought stress with a higher number of genes induced by water stress in the tolerant genotype than the sensitive one in rice (Hazen *et al.*, 2005) and also in *Arabidopsis* under cold stress (Hannah *et al.*, 2006). A possible reason for the Italian genotype of *P.*

nigra to express more genes compared to Sp2 in response to drought could be due to the damage water stress induces in It while the Spanish genotype is less affected. Indeed the number of transcripts related to repair and osmoprectant was extremely higher in response to drought for It than for Sp2 (Table 6.2). Similar conclusions were drawn for salt-stressed rice cultivars (Walia *et al.*, 2005; Walia *et al.*, 2007) and drought stressed rice genotypes (Degenkolbe *et al.*, 2009).

Sp2 and It also only had 267 transcripts in common in response to water deficit out of the 3816 transcripts expressed in total by both genotypes. Natural differences are shown between the two water stressed trees. When comparing two genotypes of potatoes, Schafleitner *et al.* (2007) observed only 186 up regulated and 77 down regulated genes in common while 1713 genes were expressed in total in response to drought.

The microarray analysis thus focused on the differences in gene expression between the two black poplars from Spain and Northern Italy. The gene annotations and pathway analysis revealed large variation in response to water deficit between Sp2 and It such as photosynthetic genes, minor CHO metabolism, cell wall, wax biosynthesis, stress related genes, signalling, transcription factors and ABA metabolism.

6.4.1.1. Stomata

Genes related to the formation of stomata were expressed in response to water deficit. Stomata are pores flanked by two symmetrically opposed guard cells on aerial organs, particularly on leaves. They regulate gas and water-vapour exchange between the plants and the atmosphere (MacAlister *et al.*, 2007) and prevent water loss by stomatal closure. They are evenly distributed on the epidermis of the leaf (Nadeau & Sack, 2002) and are produced by asymmetric and symmetric cell divisions (Bergmann & Sack, 2007). The formation and distribution of stomata are well documented (Barton, 2007; Gray, 2007; Pillitteri *et al.*, 2007; Bergmann & Sack, 2007; Casson & Hetherington, 2010). However, little is known on the environmental control of their formation (Bergmann & Sack, 2007; Casson & Hetherington, 2010). Genes regulating the development of stomata have been discovered in response to light (Casson *et al.*, 2009) and CO_2 (Gray *et al.*, 2000).

Stomatal genes were down-regulated in response to water deficit for the Italian genotype, including *TMM (TOO MANY MOUTHS), ERECTA, ERL1 (ERECTA-LIKE 1), SPEECHLESS* and *MUTE*. *TMM* encodes a putative cell-surface receptor and *ERECTA*

is a family receptor-like kinase and a MAP-kinase kinase kinase. They negatively regulate the production of stomata and play an important signalling role in the distribution of stomata (Yang & Sack, 1995; Geisler *et al.*, 2000; MacAlister *et al.*, 2007). Mutants *tmm* have more stomata than wild type and they are arranged in clusters (Yang & Sack, 1995; Geisler *et al.*, 2000). Some of the mutants also have their pore formation stopped and others have smaller pores in the clusters (Yang & Sack, 1995).

SPEECHLESS and *MUTE* are basic helix-loop-helix (bHLH) transcription factors (Lampard & Bergmann, 2007; MacAlister *et al.*, 2007; Pillitteri *et al.*, 2007). They are necessary for the asymmetric cell divisions establishing stomatal development. *SPEECHLESS* initiate stomatal lineage by promoting the transition for protodermal cell to MMC (Meristemoid Mother Cell) (Barton, 2007; Gray, 2007; Pillitteri *et al.*, 2007). Then *MUTE* is required for the transition from MMC to GMC (Guard Mother Cell) which will develop to mature guard cell pair (Barton, 2007; Pillitteri *et al.*, 2007). Null mutants *speechless* do not produce meristemoid, guard mother cell or stomata (Gray, 2007). Meristemoids of null mutants *mute* do not transit into stomata (Pillitteri *et al.*, 2007). Genetic stomatal patterning was disrupted in *P. nigra* in response to drought particularly for the Italian genotype with five genes related to stomatal development down regulated while the Spanish genotype only had the *ERECTA* gene down regulated. The Italian genotype down regulated *SPEECHLESS* and *MUTE* genes in response to water deficit, suggesting they reduced their formation of stomata. The down regulation of *ERECTA, ERL1* and *TMM* for It and of *ERECTA* for Sp2 implies the formation of stomatal clusters in response to water stress.

6.4.1.2. Chlororespiration

Differences in gene expression between the two genotypes in response to water deficit were also observed for genes related to light reactions. For example *PIFI (POST-ILLUMINATION CHLOROPHYLL FLUORESCENCE INCREASE)* and *IM (IMMUTANS)* were up regulated in the Italian genotype while the Spanish genotype had genes related to light reactions and to the calvin cycle down regulated. *PIFI* and *IMMUTANS* might protect plants from environmental stresses such as high light, heat and drought (Rumeau *et al.*, 2007; Wang & Portis, 2007). *PIFI* is co-expressed with *NDH* genes and together have an important role in chlororespiration (Wang & Portis,

2007; Suorsa *et al.*, 2009). Chlororespiration is the respiratory electron transport from NAD(P)H to plastoquinone and to O_2 (Wang & Portis, 2007; Nixon, 2000). The NDH complex might act as an emergency electron sink for photosynthetic electron flow by stromal reductant oxidation and prevent the formation of ROS (Reactive Oxygen Species) in the stroma (Nixon, 2000). *IMMUTANS* also act as an emergency electron sink in order to prevent plastoquinone pool overreduction and damages of PS II (Nixon, 2000; Suorsa *et al.*, 2009). Mutants *immutans* develop leaves with green- and white-sectors which contain abnormal and some normal chloroplasts in the white-sectors (Wu *et al.*, 1999). In *P. deltoides* and *P. trichocarpa*, similar up regulation of early light-inducible proteins was observed in response to acute drought (Street *et al.*, 2006).

6.4.1.3. Pathogenesis-related proteins

The expression of transcription factors in response to water shortage differed between Sp2 and It for PR-proteins (Pathogenesis-related proteins). They were mainly up regulated in response to drought in the Italian genotype. Chitinase and β-1,3-glucane are examples of PR-proteins (Kauffmann *et al.*, 1987; Legrand *et al.*, 1987). They play an important role in plant defence against pathogens but also in plant adaptation to environmental stresses (Edreva, 2005). It was observed in lupine roots that 16 kDa polypeptides related to PR-10 proteins were accumulated in responses to abiotic stresses including osmotic and salt stresses (Przymusinski *et al.*, 2004). PR-10 was also activated in a Cu/Zn tolerant clone of birch (*Betula pendula*) in response to a high level of copper ions (Utriainen *et al.*, 2002).

6.4.1.4. Transcription factors

Under water stress, transcription factors play an important role in the induction and the control of expression of genes related to drought. The initiation of transcription factors permits to activate the transcription of their genes (Yamaguchi-Shinozaki & Shinozaki, 2005). They are activated by ABA-dependent, by ABA-independent and also by both pathways.

The Italian genotype had more transcription factors related to drought activated (up and down) by water deficit than the Spanish genotype. This included *ERF/AP2* transcription

factor family (*ETHYLENE RESPONSE FACTOR / APETALA2*) such as *ANT (AINTEGUMENTA), CRF1* and *CRF2 (CYTOKININ RESPONSE FACTORS),* which were down regulated and *WRKY* transcription factors (*WRKY21, WRKY47, WRKY50, WRKY51,WRKY70*) which were up regulated, except for one transcript (*WRKY27*). *ANT* controls cell division during ovule development (Klucher *et al.*, 1996), plant organ cell number and organ size during shoot development (Mizukami & Fischer, 2000) and its expression was found in floral and vegetative tissue (Klucher *et al.*, 1996). Null mutants *ant1* have a reduced number of cell number and reduced leaf size (Mizukami & Fischer, 2000). *WRKY* is a large family of transcription factors and is induced by numerous factors including wounding, pathogen and abiotic stresses (Ülker & Somssich, 2004; Rushton *et al.*, 2010). In tobacco, a *WRKY* transcription factor was induced during a combination of drought and cold stresses (Rizhsky *et al.*, 2002). Zou *et al.* (2004) suggested that a *WRKY* gene, isolated from *Larrea tridentat (*a xerophytic evergreen C3 shrub), controlled the expression of ABA-related genes (Zou *et al.*, 2004).

In response to water deficit, Sp2 had an *ERF* transcription factor (*ETHYLENE RESPONSE FACTOR*) up regulated, a *MYB* transcription factor down regulated, and *RD26* which encodes a NAC transcription factor was up regulated (above 5 fold change). *RD26* is induced by stresses such as water stress, high salinity, cold and ABA treatments (Yamaguchi-Shinozaki *et al.*, 1992; Fujita *et al.*, 2004; Benedict *et al.*, 2006). Gene expression of mutants overexpressing *RD26* was studied by microarray analysis and revealed the up regulation of many ABA- and drought-responsive genes under drought stress in this mutant (Fujita *et al.*, 2004).

6.4.2. Differential gene expression under well-watered conditions

Natural gene variation between Sp2 and It was also studied under well-watered conditions. The Spanish genotype expressed more genes related to repair and osmoprotectants than the Italian genotype. Heat-shock proteins such as *HEAT SHOCK PROTEIN 18.2* (expression fold above 10) and a heat shock protein binding called *GFA2* (*GAMETOPHYTIC FACTOR 2*) and ubiquitins are examples of gene related to repair which were more expressed by Sp2 under well-watered conditions. They prevent aggregation of non-native proteins (Wang *et al.*, 2004). Genes related to glucose, sucrose synthetase, amine oxidase and fructose-1,6-bisphosphatase were more

expressed by Sp2 than It under well-watered conditions and are considered as osmoprotectants. Their roles are numerous, scavenging reactive oxygen species (Chaves *et al.*, 2003), increasing desiccation tolerance (Pelah *et al.*, 1997) or protecting macromolecules and membranes (Shinozaki & Yamaguchi-Shinozaki, 1997). Sp2 also had a higher expression in a glutathione transferase (AtGSTU19) compared to It (above 5 fold change) which is an anti-oxidant activated to eliminate ROS, superoxide radicals and H_2O_2. AtGSTU19 has its expression induced by different stresses such as drought, oxidative stress and high doses of auxin and cytokinin (Wagner *et al.*, 2002). The Spanish genotype is originally from a dry environment compared to North Italy which is typically moist. The gene expression by the Spanish genotype under well-watered conditions of osmoprotectants, proteins of repair and detoxification could be the expression of an adaptation to drought.

A second messenger was also more expressed by Sp2 than It under well-watered conditions which was an *ATITPK4 (INOSITOL 1,3,4-TRISPHOSPHATE 5/6-KINASE 4)*. It was used for real-time PCR confirmation of microarrays. IP_3 are second messengers for signalling transduction during stresses and are induced by a ABA-binding to receptor, to release Ca^{2+} into the cytoplasm through calcium-sensitive channels on the tonoplast and generate stomatal closure (Gilroy *et al.*, 1990).

The Italian genotype had a higher expression compared to the Spanish genotype for genes related to stress under well-watered conditions such as *RD21 (RESPONSIVE TO DEHYDRATION 21), ERD15 (EARLY RESPONSE TO DEHYDRATION 15)* and two *USP (UNIVERSAL STRESS PROTEIN). RD21* is a cysteine proteinase and it was observed that it was induced by salt stress but not by ABA, cold or heat stresses (Koizumi *et al.*, 1993). *ERD15* encodes a hydrophilic protein lacking Cys residues that is expressed in response to drought stress (Kiyosue *et al.*, 1994b), light stress and treatment with plant-growth-promoting rhizobacteria (*Paenibacillus polymyxa*) which demonstrates a possible co-regulation of biotic and abiotic stress responses (Timmusk & Wagner, 1999). *ERD15* also negatively regulates ABA responsiveness and the ABA transduction signalling, as overexpressed mutants were less tolerant to drought or cold (Kariola *et al.*, 2006). The two *USP* encoded were both adenine nucleotide alpha hydrolases-like superfamily proteins and are involved in responses to stresses such as cold.

Genes related to ABA were generally more up regulated in It than Sp2 under well-watered conditions such as *ABA2* and *ATHVA22A.* The latter was used for real-time qPCR confirmation of the microarray results. Its expression is induced by ABA and stress. *HVA22* was first isolated from barley, *Hordeum vulgare* L., and was suggested to be a regulatory protein (Shen *et al.,* 1993) and also play an important role of cell protection from damages under stresses (Shen *et al.*, 2001). AtHVA22A is regulated by ABA, cold, dehydration and salt stresses (Chen *et al.*, 2002a).

6.4.3. Candidate genes and real-time qPCR

There are two ways to validate microarray results: *in silico* analysis and laboratory-based analysis (Chuaqui *et al.*, 2002). The *in silico* analysis compares the microarray analysis with databases of gene expression and the literature available on the candidate genes while laboratory-based analysis is the verification of gene expression ratios using the same samples than the ones for microarrays (Chuaqui *et al.*, 2002). Real-time PCR and northern blots are examples for laboratory-based analysis. Real-time PCR methods quantify the RNA levels of candidate genes. From the microarray analysis of *P. nigra*, six genes were chosen for confirmation by real-time qPCR and further analysis with the original two genotypes and also two more from France and The Netherlands. Real-time PCR results were obtained for four candidate genes (Fig. 6.12).
Overall, the expression values from the microarrays were confirmed by real-time qPCR except for one genotype of one gene. Sp2 showed an up regulation for *IP3* in the microarray in response to water deficit while it was down regulated in the real-time PCR results. Although real-time PCR is commonly used to confirm microarray analysis, results do not always match (Chuaqui *et al.*, 2002; Morey *et al.*, 2006). The quality of RNA, sample preparation and primer designing methods can for example affect the results of real-time PCR and microarrays (Morey *et al.*, 2006).
Real-time PCR method was also used to quantify the expression ratios for two more genotypes (Fr1 from France and NL from The Netherlands) in response to water deficit for the four candidate genes: *SPEECHLESS*, *IP3*, *ERECTA* and *ATHVA22A.*

This research was conducted to study the genomic adaptation to drought in *Populus nigra*. The combination of microarray analysis and real-time PCR was utilized in this chapter to investigate the gene expression under well-watered and in response to water

deficit of two genotypes of black poplar from contrasting European environments: Sp2 a Spanish genotype from a dry environment and It an Italian genotype from a wetter environment. The Spanish genotype altered expression of fewer genes in response to drought when compared to the Italian genotype which could be explained by stronger damages affecting the Italian genotype. This could explain the increase in expression of pathogenesis-related genes under drought by the Italian genotype. The latter also modified the expression of stomata developmental and patterning genes. Direct measurements of changes in their stomatal number in response to water deficit would be necessary to confirm the hypothesis. Transcription factors were differentially expressed between the Spanish and Italian genotypes. For example, Sp2 up regulated *RD26* a NAC transcription factor which is responsible for inducing numerous ABA- and drought-responsive genes. Finally, the microarray analysis was also performed between the two genotypes under well-watered conditions and revealed the Spanish genotype had more genes expressed related to repair, osmoprotection and detoxification than the Italian. This could be an adaptation to drought expressed by Sp2 under well-watered conditions in order to prevent damages in the event of water stress.

Chapter 7: General discussion

Water is essential for plant growth, especially for a water-demanding crop such as *Populus* (Dreyer *et al.*, 2004). Besides global climate is changing and models tend to predict an increase in drought periods, particularly in the summer (IPCC, 2001; Broadmeadow, 2002). Plant adaptation to water stress is very complex and can combine different strategies including escape, avoidance and tolerance (Chaves *et al.*, 2003). *Populus* is important ecologically and economically. It provides materials such as timber and is a source for bioenergy. Poplar species also act in ecological processes such as carbon sequestration and nutrient cycling (Taylor, 2002) and preserve biodiversity (Harfouche *et al.*, 2011). It is therefore essential to understand the adaptation to drought stress in an important genus such as *Populus*.
This study focused on the natural variation, both inter-specific (*P. deltoides* and *P. trichocarpa*) and intra-specific (*P. nigra*), in the phenotypic and genetic responses of *Populus* to water deficit. Combining various methods of analysis, including microarrays, QTL, real-time qPCR, stomatal conductance and carbon isotope discrimination, this research provides a complete picture of the response to water deficit in this genus.

7.1. Overview

A mapping population was used in Chapter 3 to study the genetics linked to adaptive traits related to water use efficiency. Family 331 is a F_2 population (Wu *et al.*, 1997; Rae *et al.*, 2009) obtained from a cross between two siblings of a F_1 family called Family 53 (Bradshaw & Stettler, 1993) which was obtained from a cross between two species, *Populus trichocarpa* and *Populus deltoides.* They are originally from different environments; *Populus trichocarpa* (93-968) native to the floodplains (Rae *et al.*, 2004) of North West America, and *Populus deltoides* (ILL-129) from an area in the North East of America with less annual rainfall than the West.

The mapping population was used to discover quantitative trait loci (QTL) and to quantify phenotypic plasticity in water use efficiency using measurements of carbon isotope composition and stomatal conductance. QTL colocated with QTL from the literature such as osmotic potential (Tschaplinski *et al.*, 2006), abscised leaves, chlorophyll and carotenoid contents (Street *et al.*, 2006), biomass and SLA (Rae *et al.*, 2006; Rae *et al.*, 2009). Combining our QTL with those from the literature enabled us to

define three QTL hotspots which were studied for candidate genes related to water use efficiency. These genes included a universal stress protein (*USP*), an *AP2* transcription factor, the *ERECTA* gene and a Late Embryogenesis Abundant (*LEA1*) gene. However, the list of genes within the three QTL hotspots was over 2500. By exploiting microarray data from the literature which studied the genetic response to drought of the two clones of the same mapping population (Street *et al.*, 2006), the number of genes within the QTL hotspots was reduced from over 2500 to 26. The reduced list included genes induced by drought, signalling genes and genes involved in protein turnover such as chaperones and ubiquitins. Combining QTL and microarray data can also give more significance to candidate genes (Kathiresan *et al.*, 2006). In the literature, examples of combining QTL and microarray analysis are provided for drought related candidate genes. Degenkolbe *et al.* (2009) compared the gene expression under drought of two rice cultivars, one sensitive and one tolerant to drought. From 236 genes with a significant Genotype x Environment interaction, less than half were positioned within published drought stress QTL in the Gramene Database (Degenkolbe *et al.*, 2009). Candidate genes were also found from microarray analysis of rice within QTL of osmotic adjustment (Hazen *et al.*, 2005).

Assessment of water use efficiency related traits revealed differences between the two species in drought response with a better stomatal response by ILL-129 (*P. deltoides*) and a limited ability of 93-968 (*P. trichocarpa*) to close their stomata in response to soil drying and to exogenous ABA. Similar observations were seen by other researches (Schulte & Hinckley, 1987a; Schulte & Hinckley, 1987b; Schulte *et al.*, 1987; Hinckley *et al.*, 1989; Kim *et al.*, 2008). However *P. trichocarpa* revealed a better water use efficiency demonstrated by a higher carbon isotope composition ($\delta^{13}C$) compared to *P. deltoides*. Various reasons could explain this observation including a high stomatal conductance in *P. trichocarpa* responsible for better photosynthetic rates, differences in leaf internal conductance between the two species (Warren & Adams, 2006) or precondition to drought in *P. trichocarpa* modifying stomatal response in young emerging leaves (Schulte *et al.*, 1987).

Populus nigra is a temperate tree typically found along European rivers but also widely distributed in North Africa and Central and West Asia (Vanden Broeck, 2003). This species of poplar is near extinction in Europe (Muller *et al.*, 2002) and is threatened by loss of its habitat due to human perturbations such as river drainage and water management (Gaudet *et al.*, 2008). A population of 500 genotypes of wild black poplar was collected from five European countries: Spain, France, Italy, Germany and The Netherlands. Their latitude of origin represented a wide range of contrasting environments in precipitation and temperature. This population was planted in Belgium for a common garden experiment. Variation in leaf area was observed in a previous study with larger leaves developed by North Italian, German and Dutch genotypes and smaller leaves by Spanish genotypes, when grown in the Belgian common garden (Trewin, 2008).

This observation was confirmed with an experiment conducted in the greenhouse at the University of Southampton using sixteen genotypes showing extreme differences in leaf size: eight 'small leaves' genotypes from Spain and the South of France and eight 'large leaves' genotypes from Northern Italy, Germany and The Netherlands. The 'small leaves' genotypes had few large cells and a low stomatal density while the 'large leaves' genotypes developed many small cells and had a high stomatal density. From these observations, it was hypothesised that the 'small leaves' genotypes from a drier environment (Spain and Southern France) were expressing a drought adaptation with fewer stomata and smaller leaf area in order to prevent water loss (Chaves *et al.*, 2003). Small leaf size is also a trait that has been used in breeding programs to improve drought tolerance (Levi *et al.*, 2009; Ashraf, 2010).

An experiment was then conducted to investigate the phenotypic and genetic differences in the response to soil drying by those extreme genotypes in leaf size (Chapters 5 and 6). After a slow and moderate water deficit (between 15 and 20 % soil moisture), all the genotypes decreased in height growth, stem diameter growth and leaf area compared to the trees in controlled conditions. Variation in response to drought between the genotypes was observed in traits such as branch numbers, new leaf development and leaf senescence. French and Spanish genotypes lost mature leaves under water stress in a process of drought avoidance in order to reduce water loss (Chaves *et al.*, 2003; Munné-Bosch & Alegre, 2004) and also to remobilize nutrients to younger leaves

(Abreu & Munné-Bosch, 2008). Furthermore, Sp2 (Spanish genotype) coped better under water deficit than other genotypes by developing branches and maintaining the formation of new leaves under drought. This genotype also showed a rapid reduction in stomatal conductance after 5 days of drought while the stomatal conductance of all the genotypes was reduced 15 days after drought. *P. deltoides* which is originally from a drier location compared to *P. trichocarpa* also rapidly closed their stomata in response to drought (Chapter 3). In four populations of *P. trichocarpa* originally from contrasting environments, Sparks & Black (1999) also observed a lower stomatal closure in response to drought for the genotypes from wetter areas compared with trees from drier areas.

In well-watered conditions, hence same condition of growth in the common garden in Belgium, the whole population of *P. nigra* showed significant variation of wood and leaf carbon isotope discrimination ($\Delta^{13}C$) depending on their latitude of origin. High values were displayed by trees from drier environments such as Spanish and Southern French genotypes indicating low water use efficiency (WUE) while trees originally from wet areas (Northern Italy, Germany and The Netherlands) showed low $\Delta^{13}C$ values and so a high WUE (Chapter 4). However, when grown under water stress, the Spanish genotypes displayed a decrease in $\Delta^{13}C$ while the Italian genotype increased in $\Delta^{13}C$ thus reducing their WUE (Chapter 5). As observed in the literature, plants vary in $\Delta^{13}C$ with their environment such as precipitation and soil moisture, with a tendency to reduce with drought (Ferrio *et al.*, 2003; Otieno *et al.*, 2005; Ridolfi & Dreyer, 1997). Genotypic variation in $\Delta^{13}C$ has also been observed in *Populus nigra* in response to drought with a significant interaction effect between genotypes and treatment ($F_{5,58}$= 2.6, p=0.037). Monclus *et al.* (2006) also noticed genotypic variation in $\Delta^{13}C$ under drought. They studied $\Delta^{13}C$ in 29 genotypes of *Populus deltoides* x *Populus nigra* and found 23 genotypes tending to decrease $\Delta^{13}C$ in response to drought, whereas six genotypes tended to increase in $\Delta^{13}C$ (Monclus *et al.*, 2006).

Gene expression also varied in response to drought between extreme genotypes (Chapter 6). Microarray technique focused on two genotypes from Spain and Northern Italy. Their latitude of origin contrasted mainly in precipitation. Their response to water deficit and their differences in well-watered conditions were studied using Affymetrix

GeneChip Poplar Genome Array (Santa Clara, USA). The main observations were the high number of genes expressed by the Italian genotype under drought compared to the Spanish genotype. This could be due to the high level of stress sensed by the Italian trees compared to the Spanish ones (Walia *et al.*, 2005; Walia *et al.*, 2007; Degenkolbe *et al.*, 2009). Indeed, during the experiment, one Italian replicate died due to soil drying (Chapter 5). In addition the expression of osmoprotectant genes and genes related to repair was higher for the Italian genotype in response to drought. Other variation was observed in the transcriptome between the two genotypes such as chlororespiration, transcription factors, pathogenesis-related proteins. Genes for the development and patterning of stomata were also differently expressed under drought. The control of stomata developmental genes is observed for the first time to our knowledge in response to drought stress. For example, *SPEECHLESS* and *MUTE* were down regulated by the Italian genotype which would suggest a reduction of stomatal formation (Barton, 2007; Gray, 2007; Pillitteri *et al.*, 2007) under drought stress. The consequence of this would result in a decrease in water loss as the number of stomata would be reduced on the emerging leaves.

Under well-watered conditions, the two genotypes showed differences in relation to drought adaptation, including the expression of transcription factors and ABA-related genes. The Spanish genotype also had a higher expression for repair, detoxification and osmoprotectants such as glucose, amine oxidase and fructose-1,6-biphosphatase. This could be the expression of a natural adaptation to water stress by the Spanish genotype compared to the Italian in order to survive periods of drought such as summer periods. The Italian genotype had a higher expression compared to the Spanish genotype for genes related to stress under well-watered conditions such as *RD21*, *ERD15* and two *USP* genes as well as ABA-related genes such as *ATHVA22A.*

Another technique to study the gene expression was real-time qPCR (Chapter 6) in order to confirm the results of microarray analysis as well as obtaining the expression values of two more genotypes from France and The Netherlands for four candidate genes. Combining different techniques for genomic studies provides more significance to the results. From the analysis of the transcriptomics, the aim was to define a suitable list of candidate genes related to the adaptation to drought in *Populus nigra*.

7.2. Water Use Efficiency and Stomatal Conductance QTL in *Populus*

Genotypes of *P. deltoides* and *P. trichocarpa* were collected from different environments in the United States of America and crossed to create a F_2 mapping population (Family 331) (Bradshaw & Stettler, 1993; Wu *et al.*, 1997). Interesting regions (QTL) of the *Populus* genome were discovered and linked to water use efficiency and stomatal conductance. Thousands of genes related to these traits were found within the QTL of interest but by combining microarray analysis of *P. deltoides* and *P. trichocarpa*, fewer candidate genes from the microarray analysis were listed within the QTL of interest. A concept derived from QTL which combines genetics (marker genotypes) and genomics (gene expression) is the study of expression QTL or eQTL (Fig 7.1) and is referred to as "genetical genomics" (Jansen & Nap, 2001; de Koning & Haley, 2005). eQTL are QTL that specifically influence the level of expression for a gene that may be linked to a phenotypic trait (Tuberosa & Salvi, 2006). Their study is used to understand the regulation of genes of interest (Huang *et al.*, 2009b) by *cis*-acting (locally) or *trans*-acting (at a distance) elements (Brem *et al.*, 2002; Hansen *et al.*, 2008). The level of gene expression is used as a quantitative trait (Schadt *et al.*, 2003) and measured by real-time PCR or microarrays in each individual within a segregating population such as Family 331 used in Chapter 3. eQTL could be discovered by focusing on candidate genes found from the analysis of QTL and microarrays of this mapping population.

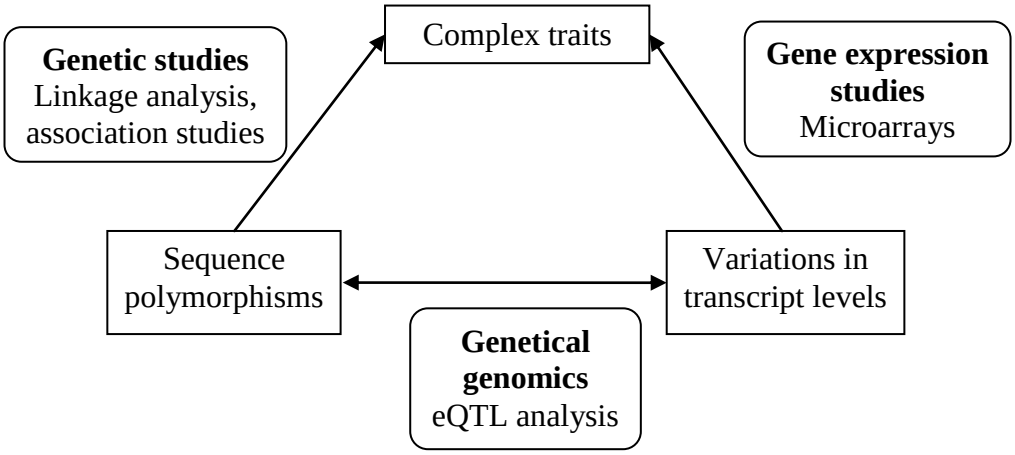


Figure 7.1: Diagram of the interaction between genetics and genomics with eQTL, modified from Li & Burmeister (2005).

7.3. Natural variation in carbon isotope discrimination in the association population of *P. nigra*

Wood carbon isotope discrimination ($\Delta^{13}C$) was measured for the 497 genotypes of the *P. nigra* association population (Chapter 4). In a common garden experiment in Belgium, significant variation was observed in $\Delta^{13}C$ depending on the latitude of origin of the genotypes. Genotypes from drier environments such as Spain and Southern France displayed high $\Delta^{13}C$ in well-watered conditions whereas the $\Delta^{13}C$ of genotypes from wetter regions (North Italy, Germany) was low. Using the natural population of black poplar, association studies could be done using the wood carbon isotope discrimination data measured for all the genotypes in this population in particular focusing on the candidate genes from the microarray analysis (Chapter 6). The advantages of using association studies rely on the natural genetic variation observed in association population (Gonzalez-Martinez *et al.*, 2008) and the reduced costs and time consumption needed for the creation of a mapping population (Abdurakhmonov & Abdukarimov, 2008; Grattapaglia *et al.*, 2009). Association studies permit the association between phenotypic traits and genes. This technique is applied to large random mating population such as the population of *P. nigra* (Chapter 4) and is based on the study of linkage disequilibrium which is the non-random association of alleles at different loci (Morton, 2005; Abdurakhmonov & Abdukarimov, 2008). Association

genetics have been previously studied for carbon isotope discrimination on *Pinus taeda* focusing on 46 SNPs from 41 candidate genes (Gonzalez-Martinez *et al.*, 2008). Two genes were associated with $\Delta^{13}C$: *DHN-1* and *LP5-like*. Using next-generation sequencing could enable the discovery of thousands of SNPs in genotypes of *P. nigra* which could be used for association studies in this population.

Next-generation sequencing refers to the recent technologies capable of generating millions of short DNA sequence reads in a single run (Mardis, 2008; Reis-Filho, 2009) and is relative cheap compared with more traditional sequencing techniques (Reis-Filho, 2009). Commercially available techniques are Roche/454, Solexa/Illumina and AB SOLiD (see reviews: Mardis, 2008; Morozova & Marra, 2008; Varshney *et al.*, 2009; Deschamps & Campbell, 2010). Applications involve whole genome sequencing either aligned to a reference genome or *de novo* without a reference genome (Morozova & Marra, 2008; Varshney *et al.*, 2009), studying the genetic variation of individuals with the discovery of Single Nucleotide Polymorphisms or SNPs (Deschamps & Campbell, 2010), sequencing of RNA (Wang *et al.*, 2010) and profiling of noncoding RNA (Mardis, 2008; Morozova & Marra, 2008).

7.4. Gene regulation in stomatal formation

Stomata are important in plants regulating gas and water vapour exchanges with the atmosphere. Stomatal formation and patterning is well known but little is known about the environmental control of these processes. Genes regulating the development of stomata have been discovered in response to light (Casson *et al.*, 2009) and CO_2 (Gray *et al.*, 2000). To our knowledge, it is the first time that changes in gene expression of stomatal formation have been observed in response to drought. In Chapter 6, a number of genes related to the development and patterning of stomata such as *SPEECHLESS*, *MUTE* and *TOO MANY MOUTHS* responded to water deficit by modifying their expression in the Italian genotype of *P. nigra*. The Italian genotype (It) down regulated *SPEECHLESS* and *MUTE* genes in response to water deficit, suggesting a reduction in stomata formation. With a limited number of stomata, water loss would then be limited. The down regulation of *ERECTA, ERL1* and *TMM* for It and of *ERECTA* for the Spanish genotype (Sp2) implies the formation of stomatal clusters in response to water stress. In order to confirm this observation another drought experiment on those

genotypes of *P. nigra* would be required, studying the changes in stomatal formation under drought stress. *Arabidopsis* mutants and poplar transformation are possible approaches to study those genes. Genetic engineering by transformation can achieve tolerance to abiotic stress such as drought in plants but also can confirm the role of a gene or pathway as a proof of concept (Vinocur & Altman, 2005; Bohnert *et al.*, 2006). In *Arabidopsis*, mutants are commonly used in the study of a gene expression by increasing the expression of a gene (knock-on) or/and by repressing (knock-out) or substantially reducing (knock-down or RNAi) the expression of a gene (Krysan *et al.*, 1999; Umezawa *et al.*, 2006).

7.6. Root systems and xylem cavitation

Few traits which could explain the drought adaptation of a plant by avoiding water dehydration are related to their potential to maximise their extraction of water, such as deep rooting systems, large roots, low vulnerability to xylem cavitation (Jackson *et al.*, 2000; Chaves *et al.*, 2003; Dreyer *et al.*, 2004). Conducting a study on root systems and xylem cavitation on individuals in this population of *P. nigra* could provide more information on the variation in their adaptation to drought stress. A study on root transcriptomics would also be very informative because roots and leaves do not express the same genes in response to drought. For example, Payton *et al.* (2010) observed only 173 stress-responsive genes expressed by both leaf and root tissues whereas they identified 1491 genes exclusive to the leaf and 852 exclusive to the root tissues. In *Populus euphratica*, genes regulated in roots were mainly repressed under drought while transcripts in the leaves were increased (Bogeat-Triboulot *et al.*, 2007).

7.5. Conclusion

Although *Populus* is considered as a drought sensitive tree, genotype variation was observed in this thesis for traits related to drought tolerance and responses to water deficit such as leaf development, stomatal conductance and water use efficiency. The main objective of this research was to study natural variation in *Populus* in response to drought stress between species (*P. deltoides* and *P. trichocarpa*) and within species (*P. nigra*) in order to understand water stress adaptation. In future climates, in many regions

of the world, precipitation will decrease while human population will increase, hence water will become scarcer. Developing plants that can grow with less water is a necessity for crop management using breeding strategies and genetic engineering.
In this thesis, research was conducted in two populations of *Populus* using different techniques in genetics, genomics and physiology to obtain a complete picture of this genus' response and adaptation to drought. *Populus* is an important genus ecologically and economically and is also a target tree for bioenergy crops. This research focused on carbon isotope discrimination and leaf size as they have proven to be applicable adaptive traits for breeding strategies for drought tolerance (Condon *et al.*, 2004; Richards, 2006; Hochman *et al.*, 2009; Levi *et al.*, 2009; Ashraf, 2010). Candidate genes were discovered linked to water use efficiency and water deficit adaptation. They could thus be instrumental in future breeding programs and tree genetic engineering for future climates and ensuring the conservation of *Populus nigra*, a species threaten of extinction in Europe.

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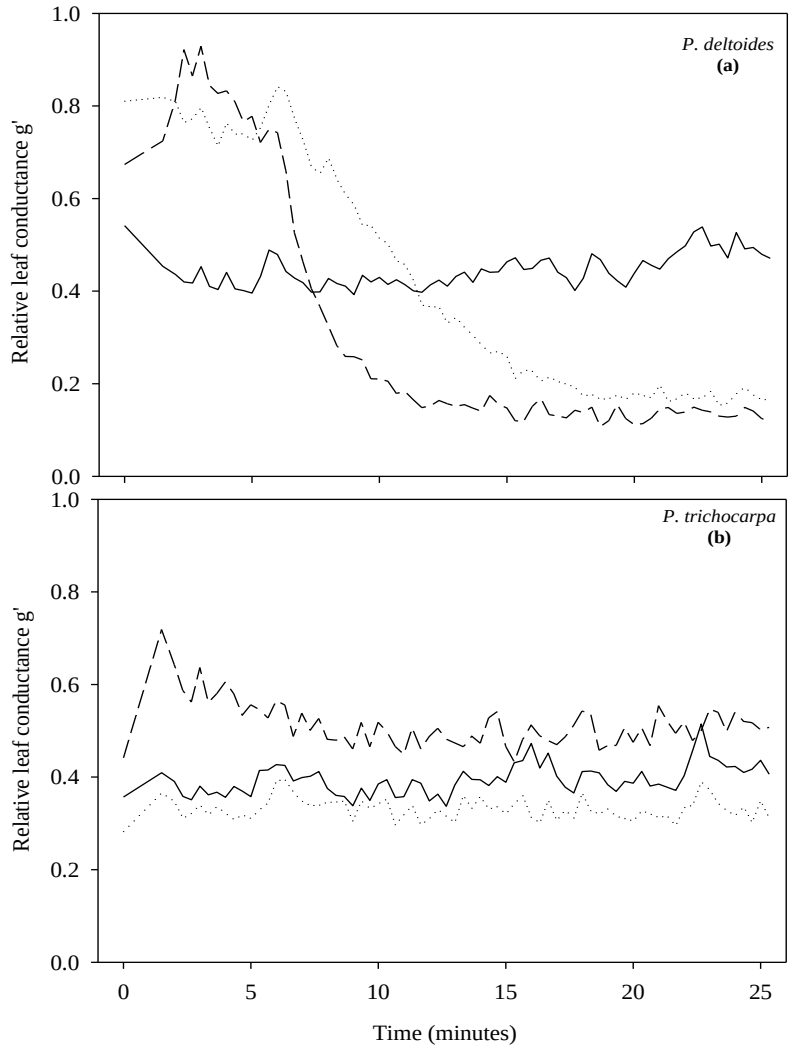
Appendices

Appendix A: Details of QTL list from figure 3 with the name of the traits, the name of the QTL used on the figure, the LG, the treatment or the location for each QTL and the reference from the literature

Trait	Name QTL	LG	Treatment or site	Reference
Osmotic potential at full turgor	osmotic_potential_Tschaplinski	VI		Tschaplinski *et al.*, 2006
$\delta^{13}C$	C13_leaves_SRC	VI	SRC	Rae *et al*., 2009
Cell area	cell_area_eCO2	VI	Elevated CO2	Rae *et al.*, 2006
Height-4	height_SRC4	VI		Rae *et al*., 2009
Height	height_r2O3	VI	Response to O3	Street *et al*., 2010
Leaf number (30d)	leaf_number_r2O330d	VI	Response to O3	Street *et al.*, 2010
Leaf abscission (30d)	abscised_leaves_O3_30d	VI	O3	Street *et al*., 2010
Leaf area absolute expansion rate	absolute_expansion_drought	VI	Drought	Street *et al.*, 2006
Leaf area 16 Days After Drought	leaf_area_droughtcontrol16d	VI	Drought	Street *et al*., 2006
$\delta^{13}C$	C13_leaves_UK	VI	UK	
Leaf number (70d)	leaf_number_O370d	VI	O3	Street *et al*., 2010
Leaf area (late season)	leaf_area_late_seaon_r2CO2	VI	Response to CO2	Rae *et al.*, 2006
Number of leaves	leaf_number_aCO2	VI	Ambient	Rae *et al.*, 2006
Biomass-1	biomass_SRC1	X		Rae *et al*., 2009
Height	height_O3control	X	Control	Street *et al.*, 2010
Leaf number (30d)	leaf_number_O330d	X	O3	Street *et al.*, 2010
$\delta^{13}C$	C13_leaves_SRC	X	SRC	Rae *et al.*, 2009
Carotenoid content	carotenoid_drought	X	Drought	Street *et al.*, 2006
Chlorophyll content	chlorophyll_content_O3	X	O3	Street *et al.*, 2010
Number of leaves	leaf_number_SRC	X		Rae *et al.*, 2009
Cell area	cell_area_aCO2	X	Ambient	Rae *et al.*, 2006
Biomass	biomass_SRC	X		Rae *et al*., 2009

Specific leaf area	SLA_aCO2	X	Ambient	Rae *et al.*, 2006
Leaf area absolute expansion rate	absolute_expansion_droughtcontrol	X	Control	Street *et al*., 2006
HT-1	height_SRC1	X		Rae *et al*., 2009
Leaf area (late season)	leaf_area_late_seaon_eCO2	X	Elevated CO2	Rae *et al.*, 2006
Specific leaf area	SLA_eCO2	X	Elevated CO2	Rae *et al*., 2006
$\delta^{13}C$	C13_leaves_UK	X	UK	
Leaf abscission (30d)	abscised_leaves_O3_30d	X	O3	Street *et al*., 2010
Specific leaf area	SLA_SRC	X		Rae *et al*., 2009
$\delta^{13}C$	C13_leaves_Italy	X	Italy	
$\delta^{13}C$	C13_leaves_Plasticity	X		
$\delta^{13}C$	C13_leaves_UK	XVI	UK	
$\delta^{13}C$	C13_leaves_Plasticity	XVI		
Leaf width to length ratio	leaf_width:length_eCO2	XVI	Elevated CO2	Rae *et al.*, 2006
g_s	stomatal_conductance_UK	XVI	UK	

Appendix B: Relative conductance (g') calculated from leaf and reference temperatures in mature leaves of **(a)** *P. deltoides* and **(b)** *P. trichocarpa* in control condition (solid lines), ABA of 10-4M solution (broken lines) and excised condition (dotted lines). A representative example is shown for each treatment and species.



Appendix C: Initial experiment in controlled room

The first experiment of this PhD was done in a controlled environment in a growth room at the University of Southampton. The 16 "extremes" genotypes of the *P. nigra* population were used. Two replicates of each genotype were planted in the greenhouse then moved to the growth rooms. Various physiological measurements were done, including height, number of branches and leaves, stem diameter, stomatal conductance, transpiration and chlorophyll content.

As height was measured, this experiment showed the "small leaves" trees were taller than the "large leaves" trees (Fig. A1; $p_{1,30}<0.05$). This demonstrates the rapid growth of the small genotypes as the experiment was recorded only four months after the hard wood cutting were planted in pots but precedent measurements showed after longer period that the "large" genotypes to be the highest trees (Harriet Trewin, personal communication).
The "large leaves" genotypes did not branch while the "small leaves" had a high number of branches ($P_{1,30}<0.01$), with an average of 7 branches for each tree. As the height, small genotypes start developing quicker than the large genotypes.

Stomatal conductance and transpiration were also measured but did not show any significant difference between the "small" and "large" genotypes (Fig. C1; $P_{1,30}>0.05$). As the trees were grown in a controlled environment with no light or temperature variation during the day, it could explain the non-difference between the two types of genotype. Besides, depending on the time during the day or night, genotypes might show variation that was not noticed in this experiment, which will need to be done in the future.

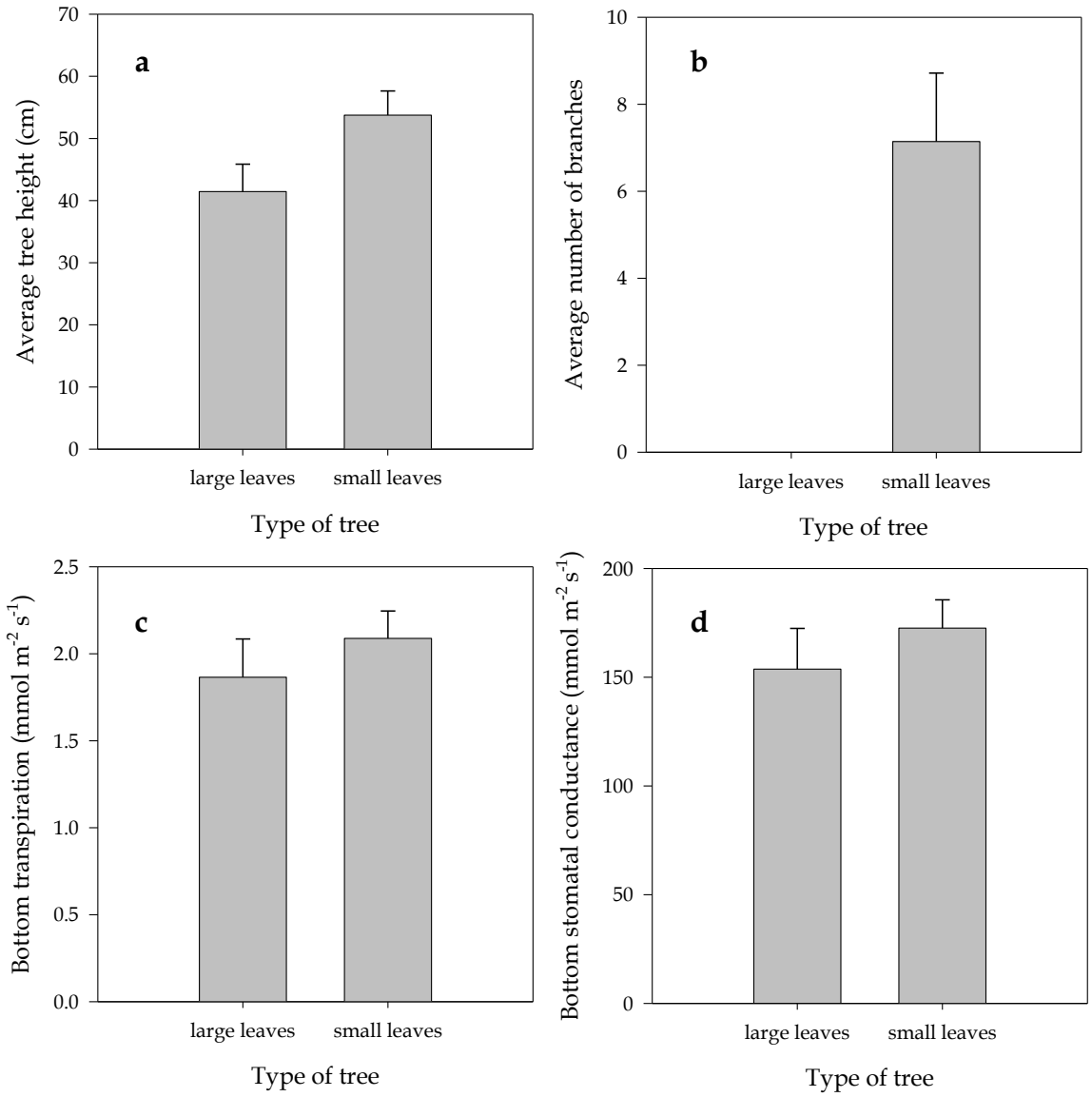


Figure C1: Physiological measurements done on 16 "extremes" genotypes of *P. nigra*, the small and leaves trees; height (**a**), number of branches (**b**), bottom leaf transpiration (**c**) and bottom leaf stomatal conductance (**d**). Each value with bars represents the average ± standard error.

Appendix D: SSR protocol

Mature leaves were collected in June 2009 from each tree used during the drought experiment, flash frozen in liquid nitrogen and stored at -80°C until further analysis. Leaf grinding was performed in liquid nitrogen using mortar and pestle and the ground material of fine powder was storedfor DNA extraction.

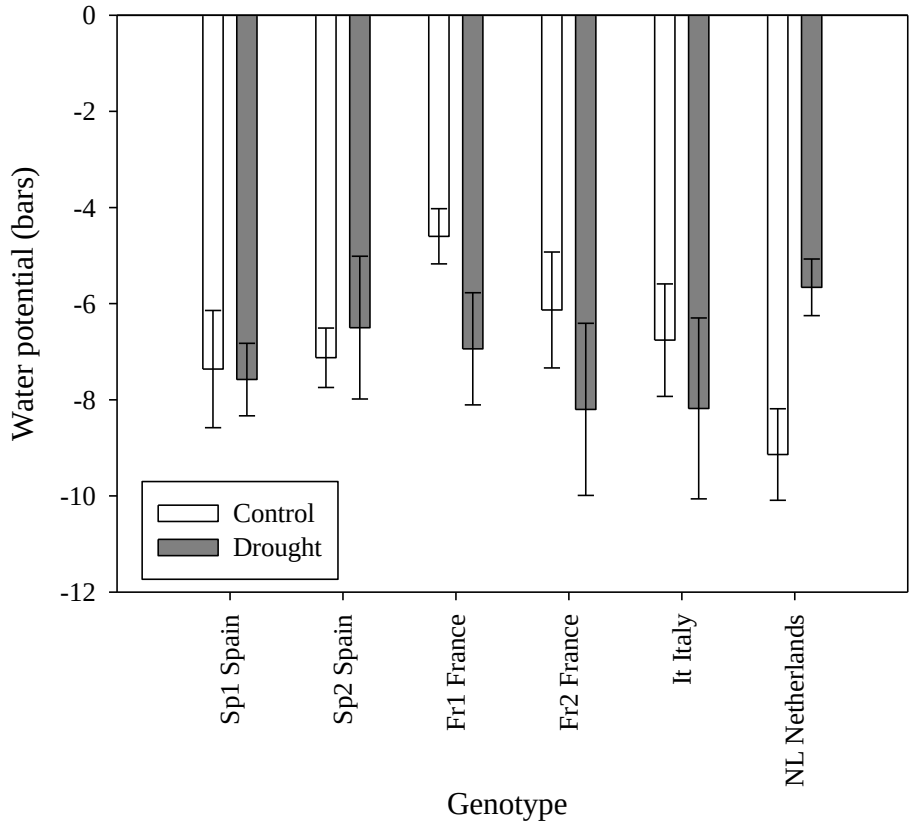
DNA was isolated using the Qiagen DNEasy Mini kit (Crawley, UK) following the manufacturer's instructions. DNA quality and concentration were assessed with a spectrophotometer (NanoDrop® ND-1000 Spectrophotometer, Thermo Fisher Scientific, Wilmington, USA).

The SSR protocol and primers (Table D1) were provided by Jennifer DeWoody (DeWoody, 2011). The forward primer was modified, incorporating a standard M13 allowing incorporation of a fluorescent M13 primer during amplification, and the reverse primer was also modified with a 3' addition of 5' –GTTCTT-3' in order to prevent the non-template addition of dATP by *Taq* polymerase (V. Jorge, personal communication). Amplification products were visualized using a 5' fluorescent label for analysis on ABI capillary electrophoresis systems. Each locus was amplified by polymerase chain reaction (PCR) of 95°C for 15 minutes, followed by 42 cycles of 94°C for 20 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. Each reaction contained 1X reaction buffer, 2 nmol of dNTP, 0.5 pmol of forward primer, 5 pmol of reverse primer, 5 pmol of fluorescently-labelled M13 primer, 0.2 units of Qiagen HotStar Taq DNA polymerase, 2.5 nM of $MgCl_2$ in a total volume of 10 µL. Capillary electrophoresis was carried out on an ABI3730 (Applied Biosystems, California, USA) systems by GeneService Limited (Nottingham, UK). Results were analyzed and scores assigned using Peak Scanner v. 1.0 (Applied Biosystems, California, USA).

Table D1: Six primer sequences – forward and reverse

Locus	**Forward (5' to 3')**	**Reverse (5' to 3')**
PMGC_2525	CGAGTCACAAGCTCCCAATAG	GCAGGCTGTCCTATCTGCG
ORPM_451	ATGGACGTTCTTGGCATCTC	TTGCCTCGCACACTACTGAC
WPMS_14	CAGCCGCAGCCACTGAGAAATC	GCCTGCTGAGAAGACTGCCTTGAC
WPMS_18	CTTCACATAGGACATAGCAGCATC	CACCAGAGTCATCACCAGTTATTG
WPMS_20	GTGCGCACATCTATGACTATCG	ATCTTGTAATTCTCCGGGCATCT

Appendix E: Water potential in leaves of *Populus nigra* in response to water deficit and under well-watered conditions. Each value with bars represents the average ± standard error.



Appendices on CD

CD1) A complete list of genes contained within the QTL hotpots with the protein ID, the gene model name, the position on the linkage group, the description, the EuKaryotic Orthologous Groups (KOG ID, class and description) and Gene Ontology (GO) categories

CD2) Video of leaf temperature of mature leaves of *P. deltoides* and *P. trichocarpa* under three different treatments: leaves in distilled water (left), in ABA 10^{-4} M solution (middle) and with petiole excised simulating acute dehydration (right). The right-hand scale shows leaf temperature in ºC. A grease spot was applied to each leaf as a dry reference and pieces of wet filter paper were used as a wet reference surface.

CD3) Complete list of genes from the microarray normalized data of *P. nigra* 2008 with the probe name and normalized expression values for each array

CD4) List of genes from the microarrays in response to drought and under well-watered conditions between Sp2 and It, two genotypes of *P. nigra*, with the probe name, the normalized expression values for each array, the averages, the log2 values, the *Populus* gene model name, the *Arabidopsis* name and description and the Gene Ontology categories (Biological, Cellular and Molecular). Worsheet 1 represents the transcripts expressed by the Italian genotype (It) in response to drought. Worsheet 2 represents the transcripts expressed by the Spanish genotype (Sp2) in response to drought. Worsheet 3 represents the transcripts differentially expressed under well-watered conditions between It and Sp2.

CD5) List of candidate genes in different categories from microarrays in response to drought for Sp2 and It, two genotypes of *P. nigra*, with the log2 values, the probe name, the *Populus* gene model name, the *Arabidopsis* name and description, Gene Ontology categories (Biological, Cellular and Molecular) and the keywork of the category. Worsheet 1 represents the transcripts only expressed by the Italian genotype (It) in response to drought. Worsheet 2 represents the transcripts only expressed by the

Spanish genotype (Sp2) in response to drought. Worksheet 3 represents the transcripts commonly expressed by It and Sp2 in response to drought.

CD6) List of candidate genes in categories from microarrays under well-watered between Sp2 and It, with the log2 values, the probe name, the *Populus* gene model name, the *Arabidopsis* name and description, Gene Ontology categories (Biological, Cellular and Molecular) and the keywork of the category.