UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

CENTRE FOR BIOLOGICAL SCIENCES

THE GENOMICS OF PLANT RESPONSE TO ELEVATEDATMOSPHERIC CO_2 – ELUCIDATING PLASTIC AND ADAPTIVE MECHANISMS

by

Yunan Lin, BSc.

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ABSTRACT
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THE GENOMICS OF PLANT RESPONSE TO ELEVATEDATMOSPHERIC CO_2 – ELUCIDATING PLASTIC AND ADAPTIVE MECHANISMS
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The increase of carbon dioxide concentration ($[CO_2]$) is the main factor in global climate change, and the atmospheric $[CO_2]$ has risen from 280 parts per million (ppm) during the pre-industrial period to the most recently estimated figure of 400 μmol mol^{-1} due to human activities. The increase of $[CO_2]$ could potentially have a morphological, genetic and ecological effect on vegetation. *Populus* is considered as a model tree to study the autumnal senescence in response to different $[CO_2]$ for several reasons. Previous studies have identified elevated $[CO_2]$ ($e[CO_2]$) could cause delayed natural autumnal senescence on plants such as poplar and soybean. This report studied two microarrays on two *Populus* species– *Populus. x euramericana* and *Populus tremuloides* –grown under ambient and elevated $[CO_2]$ (360ppm and 550-560ppm) from POP/EUROFACE and AspenFACE and identified that $e[CO_2]$ significantly increased the antioxidative enzyme and products (anthocyanin), thus prevented oxidative stress and therefore caused delayed natural senescence.

Further study of $e[CO_2]$ effect on an evolutionary level was applied on *Plantago lanceolata*, a common grass species which has grown in a naturally high-CO_2 spring for hundreds of years. The plants from inside and outside of the spring were collected and exposed to either ambient or elevated $[CO_2]$ (380ppm and 700ppm) for a seasonal cycle. The morphological study indicated that plant biomass traits were influenced by long-term $[CO_2]$ (original site), whereas epidermal cells and stomatal traits showed more adaptation to short-term $[CO_2]$ change (elevated/ambient $[CO_2]$). The following transcriptome sequencing on the plants from inside and outside spring supported the morphological data and identified an in-sufficient Calvin cycle in spring plants' response to high $[CO_2]$. However, the significant genetic evolutionary adaption to high $[CO_2]$ failed to be detected in this experiment. Furthermore research on the genetic and genomic level was required to understand whether long-term growth in different $[CO_2]$ has a selection effect on plants. This will allow the prediction of vegetation behaviour in future atmospheric $[CO_2]$.

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DECLARATION OF AUTHORSHIP

I, **Yunan Lin**, declare that the thesis entitled:

The Genomics of Plant Response to Elevated Atmospheric CO_2 – Elucidating Plastic and Adaptive Mechanisms

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 institution, this has been clearly stated;
    - Specifically, the microarray experiment presented in Chapter 2 and 3 was carried out as part of Matthew J. Tallis PhD and post-doc work, from where I conducted a novel statistical and pathway analysis.
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
    - In particular, Richard Edwards aided with performing R analysis in the *Plantago* experiments described in Chapter 5.
- parts of this work have been published as:

Tallis MJ, Lin Y, Rogers A, Zhang J, Street NR, Miglietta F, Karnosky DF, De Angelis P, Calfapietra C, Taylor G. 2010. The transcriptome of *Populus* in elevated CO_2 reveals increased anthocyanin biosynthesis during delayed autumnal senescence. *New Phytologist* **186** (2): 415-428.

Signed:

Date:

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ABBREVIATIONS

Abbreviation	Definition
[CO2]	Carbon dioxide concentration
°C	Degree Celsius
$^1O_2$	Singlet oxygen
$^3O_2$	Triplet oxygen
A	Photosynthetic carbon uptake
a[CO_2]	Ambient atmospheric carbon dioxide concentration
A'	Diurnal photosynthetic carbon assimilation
ABA	Abscisic acid
ABP	Auxin binding protein
ACC	1-aminocyclopropane-1-carboxylate
AFLP	Amplified fragment length polymorphism
AGI	Arabidopsis genome initiative
AGP	Arabinogalactan protein
AHK3	ARABIDOPSIS HISTIDINE KINASE 3 gene
AMOVA	Analysis of molecular variance
ANOVA	Analysis of variance
AOS	Active oxygen species
APX	Ascorbate peroxidase
Ar	Argon
A_{sat}	Light-saturated CO_2 uptake
ATP	Adenosine triphosphate
bHLH	Basic helix loop helix protein
bp	Base pair
BRs	Brassinosteroid
C	Carbon
Ca	Calcium
CAD	Cinnamyl alcohol dehydrogenase
CAM	Crassulacean acid metabolism
CAT	Catalase
CCR	Cinnamoyl CoA reductase

cDNA	Complementary DNA
CE	Control environment
CESA	Cellulose synthase
CH_4	Methane
CHS	Chalcone synthase
C_i	Intercellular carbon dioxide concentration
CO_2	Carbon dioxide
CS	Cell Size
CSL	Cellulose synthase-like
Cu^{2+}	Copper ion
CV	Coefficient of variation
DEPC	Diethylpyrocarbonate
df	Degree of freedom
DFR	Dihydroflavonol 4-reductase
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
Dr	Doctor
DRE	Dehydration-response element
DRF	Dehydration-responsive family
e[CO_2]	Elevated atmospheric carbon dioxide concentration
ECS	Epidermal cell size
EDTA	Ethylenediaminetetraacetic acid
EIFs	Eukaryotic translation initiation factors
ERFs	Ethylene response factors
ESTs	Expressed sequence tags
FACE	Free-air carbon dioxide enrichment
FBA	Fructose-bisphosphate aldolase
FDR	False discovery rate
Fe^{3+}	Iron ion
g	Gravity
G proteins	Guanine nucleotide-binding proteins
GA	Gibberellin acid
Gb	Giga base pairs

g_{cmax}	Maximum stomatal conductance of CO_2
GEO	Gene expression omnibus
GHG	Greenhouse gas
GLM	Generalized linear models
GLR	Glutamate receptor
GMC	Guard mother cell
GO	Gene ontology
GolS	Galactinol synthase
GPx	Glutathione peroxidase
g_s	Stomatal conductance
Gton	Gigaton
H_2	Hydrogen air
H_2O	Water
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulphide
HCl	Hydrochloric acid
HCO_3^-	Bicarbonate
He	Helium
HIC	High carbon dioxide gene
HO·	Hydroxyl radical
HT1	High leaf temperature gene
IAA	Indole-3-acetic acid
IGA	Istituto di Genomica Applicata
Inv-A	Alkaline/ neutral invertase A
IPCC	Intergovernmental panel on climate change
ITE	Instantaneous transpiration efficiency
JA	Jasmonic acid
JGI	Joint genome institute
J_{max}	Maximum rate of electron transport driving RuBP regeneration
kb	Kilo base pairs
LAI	leaf area index
LDOX	Leucoanthocyanidin dioxgenase
LG	Linkage group

Lhcb	Chlorophyll a/b binding protein
LHC-I	Photosystem I light harvesting complex
LHC-II	Photosystem II light harvesting complex
LRXs	Leucine-rich repeat/extension family proteins
m	Meter
M	Molar
Ma	Megaannum (1Ma = 10^6 year)
MAPLE	Microevolutionary adaptation of plants to elevated CO_2
Mb	Mega base pairs
MeJA	Methyl Jasmonate
MeOH	Methanol
mg	Microgram
MIAME	Minimum information about a microarray experiment
ml	Microliter
mM	Millimole
mm	Millimetre
Mn^{2+}	Manganese ion
mRNA	Messenger RNA
MS	Mean of squares
Mya	Million years ago
Myr	Million year
N	Nitrogen
N_2	Nitrogen air
N_2O	Nitrous oxide
NaCl	Sodium chloride
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
$NADP^+$	2-oxoaldehyde dehydrogenase
NASC	Nottingham Arabidopsis stock centre
NDH	Chloroplast NAD(P)H dehydrogenase
ng	Nanogram
NGS	Next generation sequencing
nm	Nanometre
No.	Number

NSF	National science foundation
O_2	Oxygen
O_2^-	Superoxide
O_3	Ozone
Oct	October
Oligo	Oligonucleotide
PAGE	Parametric analysis of gene set enrichment
PAL	Phenylalanine ammonia lyase
PAP	*PRODUCTION OF ANTHOCYANIN PIGMENT* gene
PCA	Principal component analysis
PCD	Programmed cell death
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
pH	Percent hydrogen
P_i	Inorganic phosphate
PICME	Platform for integrated clone management
PME	Pectin methylesterases
PNUE	Photosynthetic nitrogen use efficiency
ppm	Parts per million by volume in the atmosphere
Prof.	Professor
Prx	Peroxiredoxin
PS I	Photosystem centre subunit I
PS II	Photosystem centre subunit II
QTL	Quantitative Traits Loci
RAPD	Random amplified polymorphic DNA
RILs	Recombinant inbred lines
RISC	RNA-induced silencing complex
RMA	Robust multi-array analysis
RNA	Ribonucleic acid
RNAi	RNA interference
RNA-seq	Transcriptome sequencing
ROS	Reactive oxygen species
RPKM	Reads per kilobase of exon model per million mapped reads

rpm	Revolutions per minute
RT-qPCR	Reverse transcriptase polymerase chain reaction
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP	Ribulose-a,5-bisphosphate
S	Stomatal size
SA	Salicylic acid
SAG	Senescence associated gene
SAUR	Small auxin up RNA
SD	Stomatal density
Sept	September
SI	Stomatal index
SLA	Specific leaf area
SNPs	Single nucleotide polymorphism
SOD	Superoxide dismutase
SS	Sum of squares
SUS	Sucrose synthase
TAIR	The arabidopsis information resource
TCA	Tricarboxylic acid cycle
TF	Transcription factor
T_m	Annealing temperature
Trx	Thioredoxin
UDP-Glucose	Uridine diphosphate glucose
UK	United Kingdom
UPGMA	Unweighted pair group method with arithmetic mean
USA	United States of America
USDA	United States department of agriculture
USP	Universal stress protein
UV	Ultraviolet
v	Volume
V_{cmax}	Maximum rate of RuBP carboxylation
w	Weight
WUE	Water use efficiency
XET	Xyloglucan endotransglycosylase

XTH	Xyloglucan endotransglycosylase/hydrolase
Zn^{2+}	Zinc ion
β-Ffase	Beta-fructofuranosidase

Chapter1: General introduction

1.0 Overview

The concentration of atmospheric carbon dioxide (CO_2)has increased rapidly over the past decades from 260 ppm to 400 μmol mol^{-1} today (Kunzig, 2013). This rapid increase is thought to be the cause of climate changes which have influenced human and ecosystem functioning. Plants, which are central to the carbon cycling that occurs between the atmosphere and soil, are responsive to increased [CO_2]. Plants are able to acclimate to climate change by physiological, biochemical and gene expression changes (acclimation or phenotypic plasticity) or genetic change (adaptation, evolution). Much research at physiological level has identified acclimation to elevated [CO_2] by putting plants into different concentrations of CO_2, but whether there will be evolutionary adaptation after long-term exposure to elevated [CO_2] is still unclear.

Plants have shown phenotypic acclimation to increased [CO_2] and one such change is delayed autumnal senescence. Where a response to $CO_2$is apparent, it is important to study the underlying molecular and genetic mechanisms to understand the regulation within plants in response to high [CO_2].

This chapter reviews the important physiological changes observed when plants were exposed to elevated [CO_2] and the molecular changes which have been identified so far. The important senescence phenomena are also reviewed in this chapter. The different genomic techniques for studying transcriptome change under different [CO_2] are reported, as is the appearance and consequence of second generation sequencing technologies and how they might be utilised in Plant Environmental Physiology.

1.1 The effect of elevated atmospheric CO_2 on the environment

Climate change, including increased average global temperature, rising sea level and more frequent extreme weather events, has caused significant effects on human lifestyle and health. The main driver of nearly all climate change is increased greenhouse gas (GHG) emissions. GHGs act as a partial blanket preventing the reflection of long-wave radiation from the Earth's surface and thus leading to enhanced surface temperature and further associated climate change, *i.e*., sea level rise(Edenhofer *et al.*, 2011). The main components of GHGs are CO_2, methane (CH_4) and nitrous oxide (N_2O), of which the largest component is CO_2 whose annual emissions have grown by 80% from 1970 to 2004 (IPCC, 2007)due to human activity including industry and deforestation (Houghton, 2004). The increased [CO_2] has resulted in climate change that has exacerbated environmental risks for human lifestyle, involving increased drought (caused by increasing global average temperature) and floods (caused by rising sea levels) (Patz *et al.*, 2005). Increased [CO_2] can benefit plant photosynthesis, hence potentially balancing the effect of temperature increases which can causes a reduction in photosynthesis(Thuiller, 2007). This paper also showed that e[CO_2] had a greater effect on improving the growth of plants which were experiencing water limitation compared to plants grown in normal conditions..

Carbon is moved between the atmosphere, land and ocean to keep the earth's climate stable, *i.e*., not too cold nor too hot (Figure 1.1.1). Land and oceans are major sinks for CO_2 emissions (57% of each year's CO_2 emissions on average), whereas the rest stays in the atmosphere, according to the records between 1959 and 2008 (Le Quéré *et al.*, 2009). Along with the increased CO_2 emissions, a greater amount of CO_2 is being dissolved in the oceans leading to ocean acidification and reducing the ocean's pH level and causing dangers for marine species which have low CO_2 tolerance (Ferrari *et al.*, 2011).

Another severe casualty of increased [CO_2] induced climate change is biodiversity throughout the world, and this has been brought to the public's attention by recent

research. The research of Sala *et al.* (2000) on biodiversity change showed that land use change is the major factors that had been driving biodiversity change, followed by increased atmospheric CO_2 concentration. Other factors driving biodiversity change mentioned in this paper were climate change, nitrogen deposition and biotic exchange (non-native species invasion). Species have been continuously fighting to adapt to climate change throughout history, however, due to anthropogenic pressures, there are concerns that the rapid pace of current climate change will be unprecedented for many species (Thuiller *et al.*, 2011). Thomas *et al.* (2004) point out that 15% ~ 37% of 1,103 animal and plant species they studied in this research are likely to become to extinct by 2050 on the basis of mid-range climate-warming scenarios, as a result of the consequence of higher GHG emission. The known details of plant response to increasing [CO_2] will be discussed in detail, from morphological and genetic aspects, in the following sections.

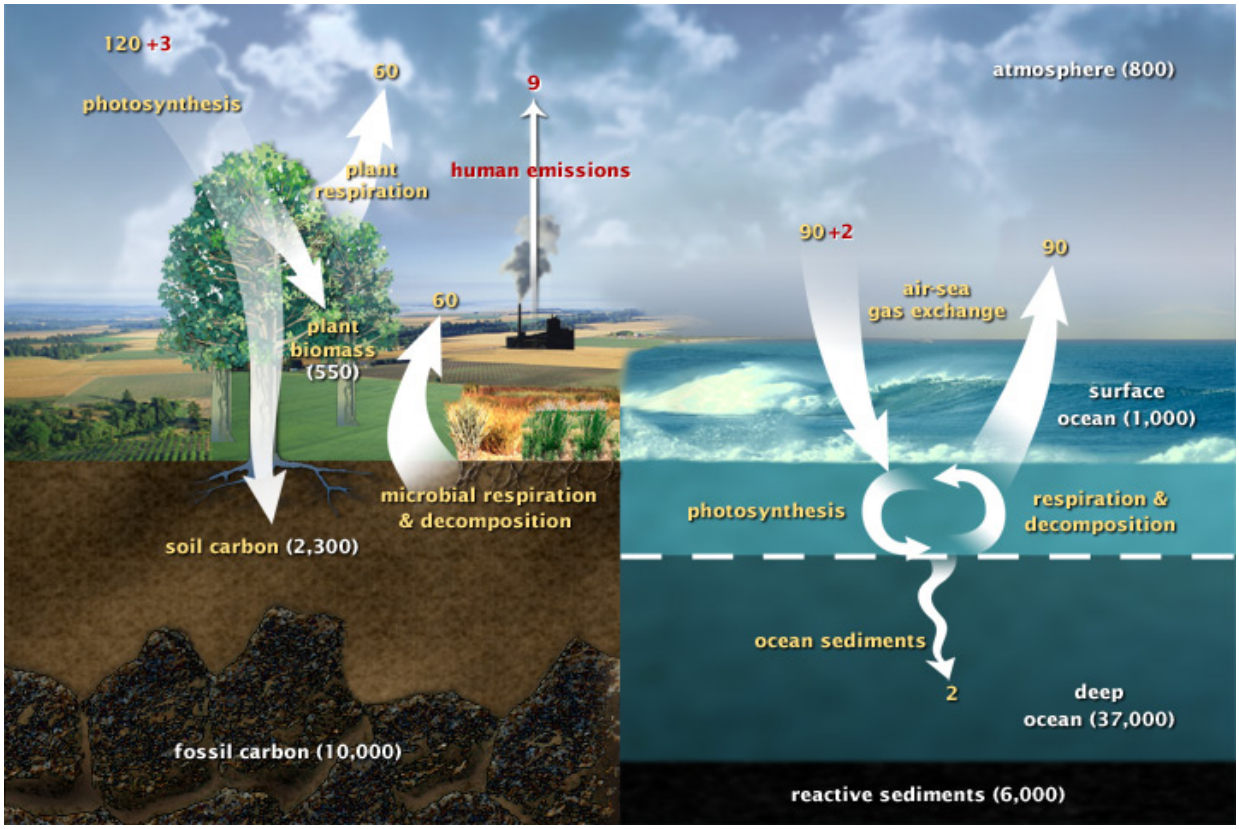


Figure 1.1.1 Carbon cycle between atmosphere, land and oceans. Yellow numbers are natural fluxes and red are human contributions of carbon per year. White numbers indicate carbon storage. All numbers are in gigaton (Gton, equals to 10^9). Figure was taken from NASA website (2011). http://earthobservatory.nasa.gov/Features/CarbonCycle/).

1.2 Plant acclimation in response to increased [CO_2]

1.2.1 Physiology traits of plant response to e[CO_2]

Numerous studies have investigated plant physiological or morphological responses to elevated atmospheric [CO_2] over the past decade (Gunderson *et al.*, 1993; Baxter *et al.*, 1994; Herrick & Thomas, 2003; Ainsworth *et al.*, 2006; Casson & Gray, 2008; Crous *et al.*, 2008), however, the responses are varied depending on species (Figure 1.2.1). The C_3 plants' photosynthesis, dark respiration and stomatal conductance were reported to respond rapidly to a [CO_2] increase (Drake *et al.*, 1997; Long *et al.*, 2004). Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation rate increased and the oxygenation of ribulose-1,5-bisphosphate (RubP) was relatively inhibited when plants were grown in e[CO_2], therefore, photosynthesis rate increased as well as photosynthetic carbon uptake (*A*)(Ainsworth & Long, 2005). It has been identified that *A* was generally stimulated more than 46% for trees, followed by grasses, shrubs, legumes and then C_3 crops that accumulated the least stimulation in *A* (13%) under e[CO_2] compared to a[CO_2] (Leakey *et al.*, 2009a), and this difference is because species or functional groups in which photosynthetic capacity is limited by Rubisco (trees and grasses) potentially stimulated higher *A* than groups that are limited by RubP (legumes, shrubs and non-leguminous C_3 crops). Light saturated leaf photosynthetic rate (A_{sat}) increases were higher in C_3 plants compared with C_4 plants under e[CO_2] (Ainsworth & Long, 2005). Wand *et al.* (1999) suggest that C_4 plant maintain a higher intercellular [CO_2] (C_i) compare to C_3 plant under current environment whereas some other scientists (von Caemmerer & Furbank, 2003; Leakey, 2009) believe that it is due to the Rubisco carboxylation reaction being saturated at a lower (C_i) in C_4 plants compared with C_3 plants. The ratio between intercellular [CO_2] and atmospheric [CO_2] is 0.65 - 0.8 for C_3 species and 0.4 - 0.6 for C_4 species at ambient conditions (Hetherington & Woodward, 2003). C_4 photosynthesis requires both mesophyll cells and bundle sheath cells to participate, and is much more efficient compared with C_3 plants by pumping CO_2 to Rubisco and therefore increased the CO_2 assimilation (Weber & von Caemmerer, 2010). Along with the photosynthetic capacity being increased in

plants grown under e[CO_2], chloroplast numbers were increased in response to e[CO_2] as well as chloroplast width and profile area, however, the reason for increased chloroplast numbers is still unknown (Teng *et al.*, 2006).

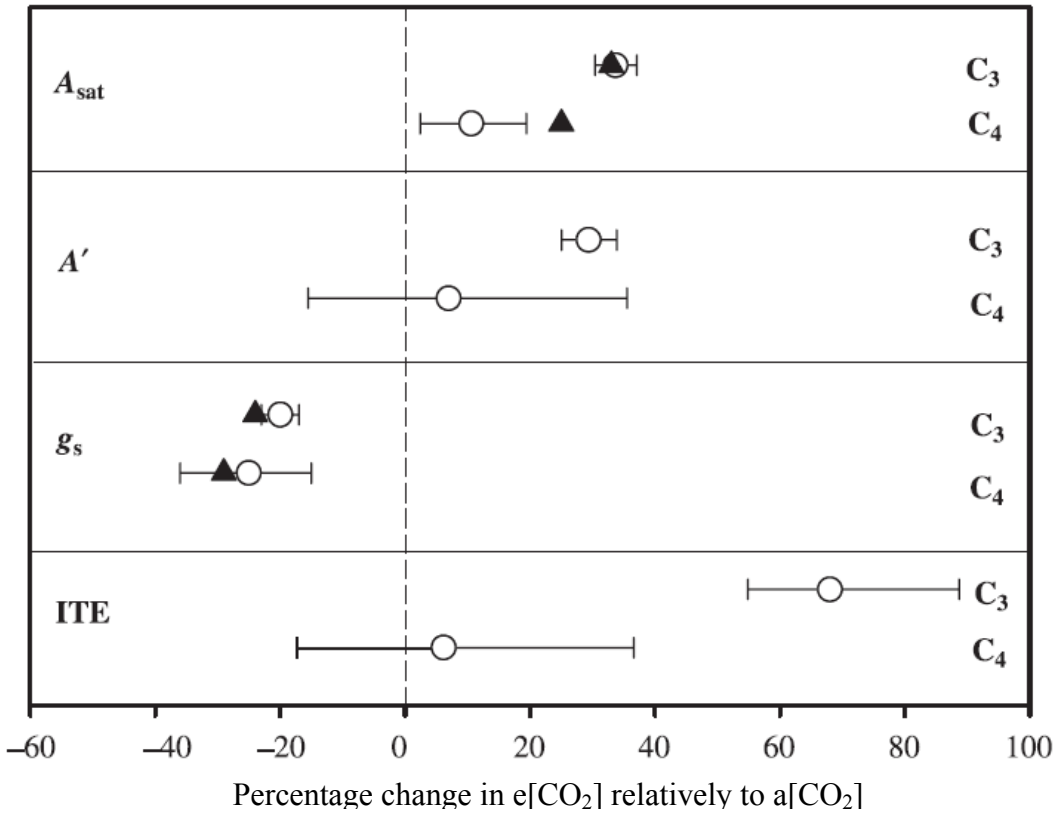


Figure 1.2.1 The comparison of photosynthesis changes in response to e[CO_2] relative to a[CO_2]inC_3 and C_4 species. A_{sat}, light-saturated CO_2 uptake. *A'*, diurnal photosynthetic carbon assimilation. g_s, Stomatal conductance. ITE, instantaneous transpiration efficiency (calculated as A/g_s).*A*, photosynthetic CO_2 uptake. "O" represents data collected by Ainsworth and Long (2005), and "▲" represents data collected by Wand *et al.* (1999).(Figure is taken from Ainsworth and Long (2005))

The direct effect on stomatal aperture of atmospheric [CO_2] is that stomata close when [CO_2] is increased and *vice versa*, regulating leaf gas exchange (Farquhar *et al.*, 1978). An *Arabidopsis* gene *HIGH LEAF TEMPERATURE 1*(*HT1)* has been reported to play a role in diminishing the CO_2 induced stomatal closure effect (Hashimoto *et al.*, 2006) as did AtABCB14 (ABC transporter), which is a guard cell plasma membrane malate (product of Calvin cycle) uptake transporter in guard cells that influences the osmotic stress in the guard cell causing the cell to swell(Lee *et al.*, 2008). Stomatal aperture directly controlled the stomatal

conductance (g_s) to water vapour and CO_2. FACE experiments and controlled environment experiments both identified decreased g_s in response to e[CO_2] irrespective of whether they were C_3 (21% reduction in average) or C_4 species (28% reduction on average) (Wand *et al.*, 1999; Ainsworth & Long, 2005; Ainsworth & Rogers, 2007) (Figure 1.2.2). Recent research (Hu *et al.*, 2010) has identified carbonic anhydrases that are involved in the upstream part of the CO_2 regulated g_s signalling pathway in guard cells and is insensitive to ABA treatment. They also pointed out that this mediation might be through CO_2/HCO_3^-(bicarbonate) acting as messengers to guard cell CO_2 signal transduction.

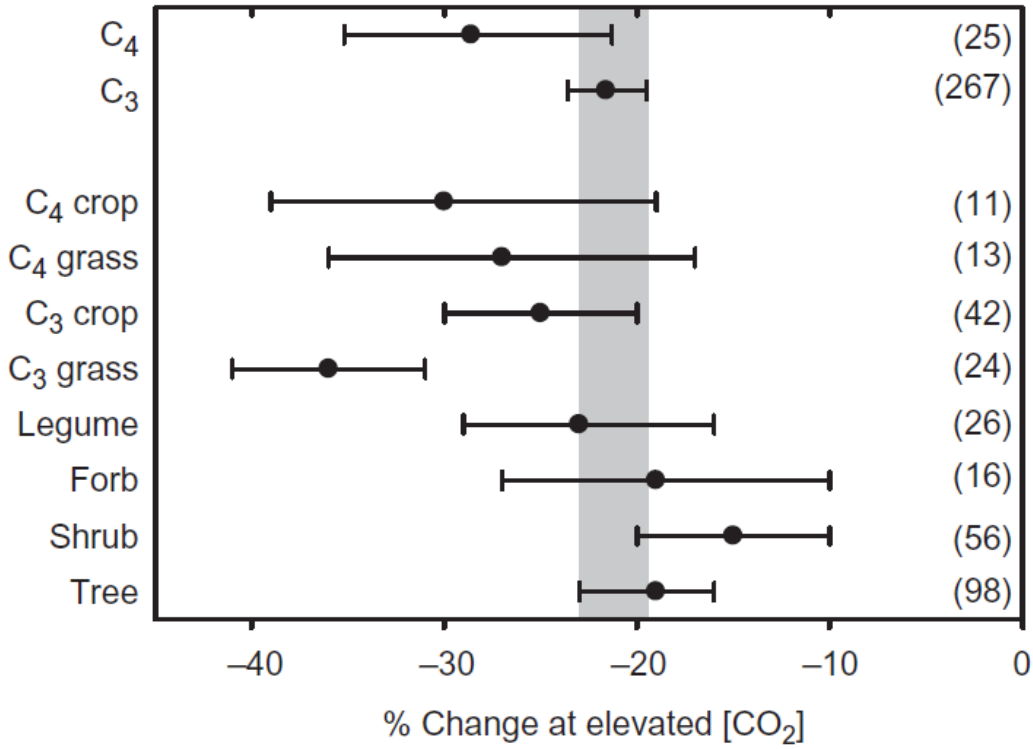


Figure 1.2.2 The stomatal conductance change in response to e[CO_2] in FACE. The grey bar represents the mean of overall results with 95% confidence interval. The numbers in parentheses are the degrees of freedom of each measurement (Figure is taken from Ainsworth and Rogers (2007)).

The general theory is that stomatal densities (SD) increased with falling [CO_2] during the Phanerozoic era (Beerling & Woodward, 1997) and the same trend was observed that SD declined along with increased [CO_2] (Hetherington & Woodward, 2003). However, only three quarters of species showed an average 14.3% SD reduction in response to e[CO_2] in a study of 100 species (Casson &

Gray, 2008) and the same phenomenon was reported by another group, where change in SD was in a range of -10% ~ +10% in response to e[CO_2] (Ainsworth & Rogers, 2007). The compensatory relationship between stomatal size (S) and SD is identified by Hetherington and Woodward (2003) by using fossil records of 64 species which means stomatal size responds negatively to reduced [CO_2]. They suggested that increased densities of small stomata is selected for with falling [CO_2] which maintains higher photosynthetic capacity and higher transpiration rates, Franks and Beerling (2009) concluded from published paper that slightly low SD but much larger S is associated with high atmospheric [CO_2] which restrict transpiration rates therefore improving water use efficiency. The same relationship between SD and S was shown in another experiment, specifically that *Arabidopsis* produced lower SD and larger S under 450 ppm CO_2(future atmospheric [CO_2] in 25-30 years)associated with reduced transpiration, greater biomass, and a higher tolerance to water limitation (Doheny-Adams *et al.*, 2012). They pointed out that the benefit of this phenomenon is that plants favour higher leaf temperature to maintain high CO_2 assimilation rate due to the combination of reduced low transpiration rate and higher [CO_2] environment. In addition, there is less metabolic cost by developing less guard cells as mentioned by Franks *et al.* (2009). Although it is known that stomatal development is influenced by CO_2 during early leaf development, the receptors of [CO_2] are located in the mature leaves which pass down the signal to new leaves for stomatal regulation (Lake *et al.*, 2001). Exposure of young leaves alone to high [CO_2] does not alter the SD. Gray *et al.* (2000) found an *Arabidopsis* gene *HIGH CARBON DIOXIDE* (*HIC)*, which encodes a putative 3-keto acyl coenzyme A synthase (an enzyme involved in very-long-chain fatty acids synthesis), is involved in the CO_2 induced stomatal development pathway. Both *Arabidopsis hic* and *hic/+* (wild type with antisense *HIC* mRNA) mutants showed significantly increased SD and stomatal index (SI) under e[CO_2]. There are also papers suggesting that the mechanisms for CO_2 regulation of stomatal development are overlapped with mechanisms for combatting pathogen attack (Lake *et al.*, 2002; Hetherington & Woodward, 2003).Both jasmonic acid and ethylene signalling pathways synergize to response to pathogen attack and induce the response against the attack (Kunkel & Brooks, 2002; Turner *et al.*, 2002). In addition, the *fad-4* (fatty-acid desaturase-deficient) mutant, which is jasmonic

acid-deficient, and the *ein-2* mutant, which is ethylene insensitive, showed no stomatal number regulation under increased [CO_2] and the *ein-2* mutant also showed susceptibility to pathogens attack.. Despite the CO_2 impact on S, the genome size is also important showing a positive relationship with S in angiosperms. After measuring 101 angiosperm species,Beaulieu *et al.* (2008)found that plants with larger genomes showing larger S and lower SD. However, the regulation system between SD, SI and g_s is still not very clear and further investigation is needed in this area.

A review of FACE experiments concluded that total plant growth and above and below ground biomass production were generally increased in response to e[CO_2] with respect to branching and leaf number, height and stem diameter (Ainsworth & Long, 2005). The enhanced biomass between different functional groups in response to e[CO_2] were a 47% increase for C_3 species, a 21% increase for CAM species and an 11% increase for C_4 species, and crop species showed higher biomass compared with woody species, following by herbaceous plants, indicating that fast growing plants are more sensitive to elevated CO_2 (Poorter *et al.*, 2002). Larger leaf area at e[CO_2] were also generally observed in trees including poplar (Ferris *et al.*, 2002; Walter *et al.*, 2005a) and pine (Tissue *et al.*, 1997). Taylor *et al*. (2003) suggested leaf size enlargement is due to cell expansion at the early leaf growth stage and a highly spatially stimulated cell production by using *Populus x euramericana* in a closed-canopy forest following long-term exposure to e[CO_2]. The same result was shown in SoyFACE (Ainsworth *et al.*, 2006); a higher expression of transcripts involved in cell growth and cell proliferation were shown in leaves growing under e[CO_2] compared to leaves grown under a[CO_2] using microarray data, and this increase is associated with an increase in maximum leaf area index (LAI) and aboveground biomass in response to e[CO_2] (Morgan *et al.*, 2005; Dermody *et al.*, 2006).

It is believed that e[CO_2] influences leaf development and senescence through regulation of the sugar-sensing mechanisms as feedback regulation (Paul & Pellny, 2003). Plants generally produce a higher carbohydrate concentration accompanied with acclimated photosynthesis resulting from growth in e[CO_2] (Drake *et al.*, 1997). Sucrose is a major carbohydrate product from photosynthetic cells, and the

leaf sucrose concentration reflects the balance of supply and demand (the balance between photosynthesis and growth demand) (Farrar *et al.*, 2000; Long *et al.*, 2004). Starch accumulated in leaves under e[CO_2] is a major energy and carbon storage supply (Zou *et al.*, 2007). Research has identified that starch grains in leaf chloroplasts were generally increased in number and size in response to e[CO_2] (Oksanen *et al.*, 2001; Zuo *et al.*, 2002; Wang *et al.*, 2004; Teng *et al.*, 2006) which were suggested to lead to increased chloroplast width in *Arabidopsis*(Teng *et al.*, 2006). Inorganic phosphate (Pi) is required for the photosynthesis reaction, and it also binds to photosynthesis sugar phosphate end-product, therefore the rate of sucrose and starch synthesis determined the rate of Pi recycling for photosynthesis (Quick & Mills, 1988; Paul & Foyer, 2001). Leakey *et al.* (2009a)believe that maintenance of photosynthesis stimulation under e[CO_2] is related to the sink's ability to utilise and relocate extra carbohydrate.

Growing in nutrient-limited habitats usually decreases the effects of CO_2 on plants. This has been confirmed for loblolly pine in a FACE experiment, in which trees which were fed extra nitrogen fertiliser and grown under e[CO_2] showed a three-fold growth increase in woody tissue compared with control trees in a[CO_2] (Oren *et al.*, 2001). Trees also exhibited lower nitrogen concentration in their foliage as well as lower Rubisco concentration after long-term exposure to e[CO_2] (Moore *et al.*, 1999). However, if grown in a low-nitrogen (N) supply habitat, plants usually exhibited higher carbohydrate concentration and greater Rubisco acclimation due to sink limitation (Ainsworth & Long, 2005; Leakey *et al.*, 2009a). The photosynthetic nitrogen use efficiency (PNUE) which is the net amount of CO_2 assimilated per unit of leaf N were generally increased in response to e[CO_2] due to enhanced CO_2 uptake (Leakey *et al.*, 2009a).

Extended growth season was thought to be correlated with global climate change; early bud set was found to be related to global warming (Menzel *et al.*, 2006), whereas the delayed autumnal senescence were more related with higher atmospheric CO_2 concentrations (Taylor *et al.*, 2008). Several studies of the effects of elevated CO_2 on leaf or whole plant senescence have produced variable results, with advances (Stomer & Horvath, 1983; Curtis *et al.*, 1989; Sigurdsson, 2001; Bindi *et al.*, 2002; Korner *et al.*, 2005), delays (Li *et al.*, 2000; Korner *et al.*,

2005; Dermody *et al.*, 2006; Rae *et al.*, 2006; Taylor *et al.*, 2008) or no effect reported (Gunderson *et al.*, 1993; Herrick & Thomas, 2003). Taylor *et al*(2008) conclude that this variation in response to e[CO_2] could be due to plants' growth pattern; determinate growth species often showed early senescence whereas indeterminate growth species showed late senescence in response to e[CO_2]. Also the higher concentration of CO_2 altered the flowering times by influencing the expression of floral-initiation genes (Parmesan, 2006; Springer & Ward, 2007; Springer *et al.*, 2008) as well as delayed soybean reproductive development and final maturation (Castro *et al.*, 2009). However, the fundamental mechanism of delayed senescence under e[CO_2] remained unknown (Section 1.5 for senescence mechanisms).

Elevated CO_2 has been reported to decrease the concentration of antioxidants, both metabolites such as glutathione and ascorbate and enzymes such as secondary metabolism in many species (Wustman *et al.*, 2001; Karnosky, 2003). In contrast, strawberries produced significantly higher amounts of antioxidant while growing under e[CO_2] compared with growth in a[CO_2], potentially protecting cells from free radical and oxidant damage (Wang *et al.*, 2003). However, the effects of elevated CO_2 on antioxidant transcripts are still waiting to be fully elucidated. Another study on *Larrea tridentate* showed that the Abscisic acid (ABA) concentrations in the leaves increased at higher CO_2 concentrations, and a regulatory gene *LtWRKY21*(transcription factor) was found to respond to e[CO_2] which is also induced by ABA and Jasmonic acid (JA), which control plant responses to various environmental stresses (Zou *et al.*, 2007).

1.2.2 Genetic and molecular traits of plant response to elevated CO_2

The plant physiological responses observed following exposure to increasing [CO_2] have been well studied in various species in the past two decades. With the invention of microarray techniques (See section 1.7 for technique introduction), we could further systematically investigate the molecular and/or genetic mechanisms underlying the physiological response.

Although the photosynthesis rate is often increased following increases in [CO_2], the genes involved in the photosynthesis pathway including the chloroplast 30s ribosomal protein, the light reactions and Calvin cycle were all reduced under e[CO_2] relatively to a[CO_2], and this is generally found in all C_3 plants studied to date, including *Arabidopsis*(Li *et al.*, 2008), poplar (Gupta *et al.*, 2005; Taylor *et al.*, 2005; Cseke *et al.*, 2009), soybean (Leakey *et al.*, 2009b) and silver birch (Kontunen-Soppela *et al.*, 2010). Some papers suggested it could be pure biochemical regulation that higher requirement of Rubisco carboxylation reduced the availability of Rubisco oxygenation (Cseke *et al.*, 2009), while others suggested the feedback regulation by larger amounts of carbohydrate induced by e[CO_2], which reduced the oxygenation of RuBP (Paul & Pellny, 2003).

The genes involved in the soluble sugar and starch pathway were mainly increased following the trend of increased sugar and starch content under e[CO_2] relative to a[CO_2] (Cseke *et al.*, 2009; Leakey *et al.*, 2009b). In soybean the genes involved in the starch synthesis and degradation pathway were in abundance implying a higher respiration rate during the night, which was supported by starch metabolism (Leakey *et al.*, 2009b). Highly expressed genes involved in glycolysis as well as in the TCA cycle and mitochondrial electron transport chain were also observed in soybean grown under e[CO_2] compared to a[CO_2] (Ainsworth *et al.*, 2006; Leakey *et al.*, 2009b; Kontunen-Soppela *et al.*, 2010).The genes involved in secondary metabolism did not show consistently up or down-regulation under e[CO_2] from different experiments. An *Arabidopsis* experiment showed genes involved in secondary metabolism were highly expressed under e[CO_2] (Li *et al.*, 2008) and the same response under e[CO_2] was found in soybean (Ainsworth *et al.*, 2006). However, two *Populus* clones 216 and 271 (*Populus tremuloides*), which have been planted under the same conditions since 1997, showed completely opposite gene expression in secondary metabolism pathways in response to e[CO_2] even though they both showed up-regulation in primary metabolism under e[CO_2] (Cseke *et al.*, 2009). It was suggested this could be due to different carbon utilisation strategies and reflect the environments to which the plants were adapted and selected for the experiment.

Thinner cell walls were observed on Aspen clone (clone 271) in AspenFACE (Oksanen *et al.*, 2004),and the corresponding genes involved in cell synthesis, including fasciclin-like arabinogalactan protein genes and pectin methylesterase, also showed reduced expression relative to a[CO_2] (Cseke *et al.*, 2009).Another Aspen clone (clone 216) from same sites showed no leaf cell wall thickness under e[CO_2] compared to a[CO_2] but a higher cell wall percentage in plant(Kaakinen *et al.*, 2004),along with a significant up-regulated transcription factor NAC(Cseke *et al.*, 2009), which could activate the secondary cell wall biosynthetic gene expression results in a extra deposition of secondary walls in cells (Zhong *et al.*, 2006).In *Arabidopsis*, the genes involved in cell wall biosynthesis and the genes encoding cell wall protein and cellulose synthesis enzymes were showed up-regulation under e[CO_2] compared with a[CO_2] (Li *et al.*, 2008), thicker cell walls also have been observed on *Arabidopsis* grown under e[CO_2] compared to a[CO_2] (Teng *et al.*, 2006). It seems that whether e[CO_2] induce thicker or thinner epidermal leaf cell wall is dependent on species.

In poplar, faster epidermal cell growth rate in developing leaves and larger leaf size were observed under e[CO_2] compared to a[CO_2] (Ferris *et al.*, 2001; Taylor *et al.*, 2003). The responsible genes which encodexyloglucan endotransglycosylase (XET), the cell wall-loosening enzyme that enables cell enlargement, as well as cycle-cycle genes were highly expressed under e[CO_2] compared to a[CO_2] (Ferris *et al.*, 2001; Taylor *et al.*, 2003; Gupta *et al.*, 2005). The genes encoding auxin-binding proteins (ABPs) were also found up-regulated under e[CO_2] compare to a[CO_2] corresponding to the physiological change mentioned above(Gupta *et al.*, 2005; de Souza *et al.*, 2008). The ABPs are responsible for cell expansion mediation by stimulating genes involved in cell cycle and cell division(Timpte, 2001; Perrot-Rechenmann, 2010; Tromas *et al.*, 2010).Higher expression of genes involved in cell cycle and cell wall loosening under e[CO_2] compared to a[CO_2] were also found in Soybean (Ainsworth *et al.*, 2006).

The nitrogen fixation genes were also up-regulated in e[CO_2] relative to a[CO_2] suggesting an increased nitrogen uptake from soil(Kontunen-Soppela *et al.*, 2010). It is found that in *Arabidopsis*, the expression change of genes involved in

reactive oxygen species (ROS), secondary metabolism under a N deficient environment is similar to the gene expression change under e[CO_2]when compared to plants grown in an ambient environment (Li *et al.*, 2008). This agrees with the finding of Finzi *et al.* (2007) that improved productivity of forest when grown under e[CO_2] is due to increased nitrogen uptake rather than an increase in the nitrogen use efficiency.

1.3 Plant evolutionary adaptation in response to increased [CO_2]

Increased atmospheric [CO_2] influences plant physiological and biochemical processes, but the question remaining is whether this might lead to gene-level changes, adaptation and microevolution. There are two ways in which plants can cope with such climate changes (Meyers & Bull, 2002). One way is to cope with the environmental variation by behavioural or physiological modification (plastic change), which is what we have seen from FACE experiments or other short-term experiments which are not permanent changes in future generations at the genetic level. Another way is through the regulation from offspring by producing diverse offspring and this diversity between offspring can be temporary (non-genetic) or permanent (genetic) differences (evolutionary adaptation).

The genetic evolutionary adaptation in response to climate change has caught the attention of scientists recently. The United States National Science Foundation (NSF) has spent millions of dollars on collecting 12 million seeds to study the adaptive evolution induced genetic change (Pennisi, 2011). The seeds will be stored in a time capsule for 5,10, or even 50 years for the future evolutionary scientists, covering the study on the effect of global warming, species invasions, new land-use patterns or surroundings change on those plants. Species tend to adapt to climate change to take advantage of favourable conditions, such as higher growth rate, earlier spring phenophase attributed to rising atmospheric temperature and extension of growing season influenced by high [CO_2] (Menzel *et al.*, 2006; Tallis *et al.*, 2010; Hoffmann & Sgro, 2011). Hoffmann and Sgro (2011) mentioned that more studies had successfully justified the genetic

adaptation to growth environment change on a space scale rather than on a time scale, and the only trait which has been identified as evolutionary change in response to climate changes is the timing of activity or reproduction, including the flowering time in plants and migration patterns in birds. This may reflect the limitation in current experimental design rather than the only processes sensitive to climate change.

Evolution changes have been identified in response to falling [CO_2] over the Palaeozoic era. In algae, the *rbcL* gene which encodes the Rubisco large subunit, is positively related to the selection of plant response to falling [CO_2] in ancient times(Young *et al.*, 2012). However, unlike the studies focused on plants' acclimation to increasing [CO_2], there are not many studies which have successfully identified large genetic evolutionary change in response to increasing [CO_2] (Leakey & Lau, 2012). Collins and Bell (2006) collected *Chlamydomonas* from the soil of two CO_2 spring (Bossoleto and Strmec in Italy) and also outside of these spring for comparison and treated them with two different [CO_2] over 1000 generations (430 ppm for ambient and 2000 ppm for e[CO_2] treatment). The results showed that the growth rate is significantly influenced by different CO_2 treatment, whereas the effect of the original environment is ambiguous when comparing spring algae to the ones collected from outside. However, another experiment by Nakamura *et al.* (2011) detected the relative growth rate (biomass increased per day per total fresh mass) and leaf area ratio (plant leaf area per plant weight) of plants were significantly positively correlated with atmospheric [CO_2] by studying *Plantago asiatica* from the Nibu CO_2 spring in Japan. It is generally believed that the high shoot to root ratio (Wieneke *et al.*, 2004) and high productivity (Jablonski *et al.*, 2002) are a favourable response to high [CO_2], and these traits are heritable to the offspring as a result of high [CO_2] selection (Nakamura *et al.*, 2011). The max stomatal conductance of CO_2(g_{cmax}) increased with decreasing atmospheric CO_2 during the Phanerozoic beginning at -400 million years (Myr) until modern times (0Myr) (Franks & Beerling, 2009). Interestingly, C_3 plants (including conifers, angiosperms and fern) further decreased g_{cmax} to adapt to increasing CO_2 by modifying the SD (decreasing) and/or pore size over the past 150 years (Lammertsma *et al.*, 2011). Beerling and Franks (2010) believe this contrasting phenomenon was due to the evolution of

leaf veins (increased) triggered by decreasing CO_2 in ancient times, which improved the transpiration system in plants. The decrease of g_{cmax} as an adaptation to high [CO_2] varied from 17-18% (*Pinus elliottii* and *Quercus laurifolia*) to 55% (*Pinus taeda*) (Lammertsma *et al.*, 2011) and the simulation of this particular trait in adaptation to future increasing CO_2may be further decreased together with transpiration flux until the limits of phenotypic plasticity are reached (de Boer *et al.*, 2011). It is important to notice that the plants adaptation selected by CO_2 occurred during the Phanerozoic era would have important effect on the pattern of plant adaptation to [CO_2] change at modern times.

1.4 Senescence

Senescence is defined as "the age-dependent deterioration process at cellular, tissue, organ, or organismal level, leading to death or the end of the life span" (Nooden & Leopold, 1988). It can be induced by various factors including endogenous signals and exogenous stresses (also known as internal and external factors). Endogenous signals include age, hormones, growth regulators and carbohydrate concentration (Hopkins *et al.*, 2007; Lim *et al.*, 2007; van Doorn, 2008) and exogenous stresses (environment stress) include light, temperature, e[CO_2], O_3 stress, drought, nutrient stress or pathogen infection (Oksanen *et al.*, 2004; Hopkins *et al.*, 2007; Lim *et al.*, 2007; Taylor *et al.*, 2008).

Senescence of non-annual plants has mainly been followed as leaf senescence, as opposed to annual plants where whole plant senescence, which is a form of programmed cell death (PCD), has been studied (van Doorn & Woltering, 2004). A review by Lim *et al.* (2007) on leaf senescence exhibited the complex regulation network induced by various external and internal factors (Figure 1.4.1) that during the senescence process, chlorophyll, membrane and DNA started to degrade or disrupt, while the genes in charge of initiating senescence were up-regulated (Hopkins *et al.*, 2007).

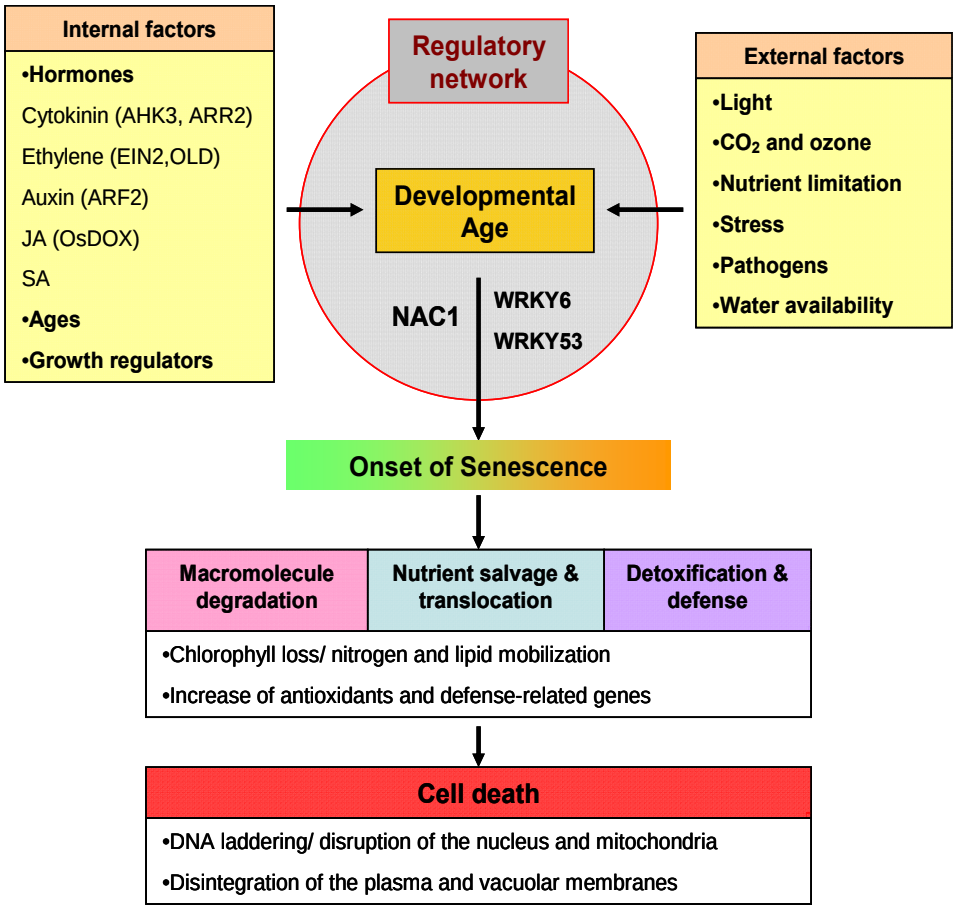


Figure 1.4.1 A model for regulatory pathways in leaf senescence. Figure is from Lim *et al.* (2007).

1.4.1 Pigment and Photosynthesis

One of the visual phenomena during plant senescence is that the leaf pigment changes from green (chlorophyll) to yellow (carotenoids) or red (flavonoids) (Andersson *et al.*, 2004). Andersson and co-workers (2004) studied a free-growing aspen's natural senescence by using their own-created cDNA microarray (custom probes), and revealed the expression trend of transcripts involved in chlorophyll, carotenoid and anthocyanin biosynthesis pathway during the senescence process. Their research showed the transcripts involved in the chlorophyll biosynthesis gradually decreased from the beginning of senescence while the overall trends of carotenoid and anthocyanin were not very clear. Another experiment carried out on the same tree over 3 years on cellular change during senescence showed that the carotenoid concentration decreased at the same

time as chlorophyll degradation but at a much slower rate resulting in the leaf colour shift from green to yellow (Keskitalo *et al.*, 2005). Unlike chlorophyll and carotenoid that exists in the leaves all the time, anthocyanin was only produced during autumn before the leaves fell and gave the leaves a red-purple colour (Archetti *et al.*, 2009). The anthocyanin is a common type of flavonoid that provides colour to flowers in order to attract pollinators and deter herbivores (Hernandez & van Breusegem, 2010). It is also induced by environmental stress including wounding, drought, lack of nutrients and light stress to protect plants from oxidative reactive species (ROS) stress (Hernandez *et al.*, 2009).

Photosynthesis is decreased due to the degradation of leaf chloroplasts during the autumnal senescence process (Huffaker, 1990), and this was also associated with degradation of Rubisco (Wingler *et al.*, 2004). However, Nooden (2004) showed that on the same unit leaf area, under saturating light intensity conditions, the photosynthetic efficiency value remained high at the onset of senescence although it sharply decreased at a later time point, while the photosynthetic capacity was decreased during senescence. Another study on *Arabidopsis* also found that the quantum efficiency of photosystem II electron transport started to decline while the photosynthesis rate remains high at the early stage of senescence (Wingler *et al.*, 2004).

1.4.2 Nitrogen and Sugar

The senescence process also plays a fundamental role in nutrient recycling especially nitrogen remobilisation in plants; for example, more than 80% of the nitrogen was remobilised in *Arabidopsis*(Himelblau & Amasino, 2001), and 90% in aspen (Keskitalo *et al.*, 2005). Nitrogenous compounds, including proteins and nucleic acids, were degraded to a nitrogen form and remobilised into storage tissues such as stem waiting to be used for germination in the next year (Nooden & Leopold, 1988). Around 70-80% of the total nitrogen is stored in chloroplasts in mature leaves (Mae *et al.*, 1993), in which, Rubisco, an important enzyme in the Calvin cycle, is the major abundant protein (Feller *et al.*, 2008). During leaf senescence, Rubisco is degraded until it reaches an undetectable level when the

leaf is completely senesced to become the major internal nitrogen source (Nooden, 2004; Feller *et al.*, 2008).

Higher plants' mature leaves (source tissues) which have photosynthetic activity develop during growth and export carbohydrates to photosynthetically less active or inactive tissues such as root, fruits or tubers (sink tissues) which can not produce carbohydrates (Roitsch, 1999). It is suggested that the accumulation of carbohydrate in leaves induces senescence due to causing an imbalance between sources and sink, and therefore accelerate senescence (Wingler *et al.*, 2006). A higher amount of carbohydrate will inhibit photosynthesis by seizing free Pi which is essential for photophosphorylation and CF1-AFPase activity (Paul & Pellny, 2003). However, research on senescence associated genes (SAGs) showed conflicting results. During the early phase of senescence, some SAGs were found to be sugar-inducible, for example SAG21 (Weaver *et al.*, 1998; Xiao *et al.*, 2000; Paul & Pellny, 2003) whereas, some of the SAGs expressed during the later stages of senescence were sugar-repressible, for example SAG12 (Noh & Amasino, 1999). Therefore, there is still a debate about whether the onset of senescence is induced or delayed by higher carbohydrate concentration in plants (van Doorn, 2008). A study of the *Arabidopsis* mutant *pro3* (contains defective *Suc-transporter* 2 gene) showed a higher concentration of carbohydrate in leaf significantly induced the anthocyanin accumulation through an induction of all genes involved in the anthocyanin biosynthesis pathway as well as three regulators*MYB75/PAP1*, *MYB90/PAP2* and bHLH protein (basic helix loop helix protein) TT8 (Lloyd & Zakhleniuk, 2004; Solfanelli *et al.*, 2006).

1.4.3 Free Radicals and Oxidative Stress

ROS including singlet oxygen ($^1O_2$), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO\cdot$) are the main free radicals produced in mitochondria and chloroplasts in which oxidising metabolites were highly activated or electron flow rate were intensively produced (Mittler *et al.*, 2004). During the senescence process, chlorophyll, protein and RNA were degraded which produced an increased number of free radicals (Zimmermann & Zentgraf,

2005). The free radicals are naturally toxic and could induce oxidative stress (Nooden, 2004) leading to further associated protein degradation and senescence (Zimmermann & Zentgraf, 2005). There is a scavenging system excited by the oxidative stress called ROS-scavenging enzymes including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPx) and Peroxiredoxin (Prx) (Mittler *et al.*, 2004) and non-enzymatic antioxidative molecule like ascorbate, glutathione, α-tocopherol, carotinoids and anthocyanin (Gould, 2004; Zimmermann & Zentgraf, 2005). The superoxide radicals can be divided into three classes depending on the cofactor (1.Cu^{2+} and Zn^{2+}, 2.Mn^{2+} and 3.Fe^{3+}) and catalysed by the SOD to H_2O_2(Ramel *et al.*, 2009); $2O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$(Zimmermann & Zentgraf, 2005). The H_2O_2 will be further detoxified to water (H_2O) via the ascorbate-glutathione cycle (Zimmermann & Zentgraf, 2005).

1.4.4 Hormones

Plant hormones are defined by Davies (2010) as "a group of naturally occurring, organic substances which influence physiological processes at low concentrations". The hormones, including auxins, cytokinins, gibberellins, abscisic acid, jasmonates and ethylene control every single step of the plant lifecycle – growth, development and senescence (Nooden, 2004) in combination with various environmental stresses (Lim *et al.*, 2007).

JA and its methylester (MeJA) induced premature senescence by promoting several SAGs (*SAG14*, *SAG15*, *SEN1*, *SEN4*, *SEN5* and *rVPE*) as well as inhibiting photosynthetic proteins, and it was also found that JA concentration was increased in senescing leaves (He *et al.*, 2002). This function was first discovered in detached Oat (*Avena sativa*) leaves (Ueda & Kato, 1980). Besides the function of inducing senescence, JA also inhibits root growth, seed germination, as well as inducing tuber formation, tendril coiling, nyctinasty, trichome formation, senescence and flower development (Wasternack, 2007). Jasmonates also played important roles on plants stress and defence response especially oxidative stress induced by free radicals (Lamb & Dixon, 1997;

Wasternack, 2007). JA is able to enhance the sucrose-dependent expression of dihydroflavonol 4-reductase (DFR) which is an anthocyanin biosynthesis pathway downstream gene(Chen *et al.*, 2007). The *Arabidopsis* mutant MYC2 (is allelic to the *JASMONATE-INSENSITIVE 1* locus), which is a positive regulator involved in JA-mediated flavonoids biosynthesis, also played roles in regulating ROS scavenging as well as insect pest response (Dombrecht *et al.*, 2007).

Cytokinins have been known for a long time to be associated with the function of delayed leaf senescence (Richmond & Lang, 1957; Buchanan-Wollaston *et al.*, 2003; Lim *et al.*, 2007). Even with senescence specific promoter (*SAG12*), which induces the onset of senescence, tobacco cytokinin over-expression mutants showed delayed senescence (Gan & Amasino, 1995) and the chlorophyll and proteins were degraded at a slower rate compared with wild-type (Wingler *et al.*, 1998). Another study on cytokinin receptors in *Arabidopsis* suggested that cytokinins induce shoot development, chlorophyll retention, increase light sensitive seed germination and regulate root architecture (Riefler *et al.*, 2006). Lara *et al.* (2004) found that extracellular invertases, which supply carbohydrates to sink tissues, were co-induced by cytokinins suggesting that a sink and source balance could be the cue of cytokinin-mediated senescence.

Ethylene is essential for fruit ripening as well as premature leaf senescence in some plants (Buchanan-Wollaston *et al.*, 2003). However, ethylene alone does not induce premature senescence. It also requires age-dependent factors to be involved to function (Lim *et al.*, 2007). This was confirmed in the *onset of leaf death Arabidopsis* mutants(*old*), which exhibit early leaf senescence (Jing *et al.*, 2002). They treated both *old2* mutant, which showed no visible difference with wild type, and wild type *Arabidopsis* with ethylene. The *old2* mutant cotyledon leaves and the first two rosette leaves turned yellow whereas the wild type only showed yellowing on cotyledon leaves, implying that ethylene only induces senescence when plants reach a certain developmental stage. There are also papers indicating that the senescence induced by ethylene could be initiated by free radicals from lipid peroxidation (Arora *et al.*, 2002; Zimmermann & Zentgraf, 2005).

ABA is known to induce seed germination, leaf abscission, stress response, plant growth and senescence (Lim *et al.*, 2007). Hung *et al.*(2004) suggest that ABA-induced senescence is mediated by inducing hydrogen peroxide, which is one of the reactive oxygen species, rather than via a direct effect. However, ABA is also involved in protecting cellular function by inducing genes which could trigger antioxidant enzymes during leaf senescence (Lim *et al.*, 2007).

1.4.5 Transcription factors

Transcription factors (TFs) regulate gene expression by binding to the *cis* element(Guo *et al.*, 2004). Within an *Arabidopsis* leaf senescence cDNA library, 134 transcription factors were identified, including 20 genes in the *NAC* family, 18 genes in *WRKY* family (*WRKY6*, *WRKY18*, *WRKY22*, *WRKY29*, *WRKY53* and *WRKY75*), 27 genes encoding Zinc finger proteins and many others (Guo *et al.*, 2004). WRKY family genes played various important roles in plant defense system (Chen *et al.*, 2012) (Table 1.4.1). *WRKY6* expression is highly induced in senescing leaves and is also involved in early senescence regulation (Robatzek & Somssich, 2001). WRKY53 is involved in inducing early senescence as well as inducing catalase expression, but it is unclear what the role of *WRKY53* is in this oxidative stress regulation (Miao *et al.*, 2004). Another experiment on leaf senescence also identified *WRKY4,6,7* and 11 gene expression were increased at the beginning of senescence that might play a role in senescence regulation, and *WRKY53* is expressed at the very early stage of senescence that could be an early regulator of senescence (Hinderhofer & Zentgraf, 2001). *NAM-B1*, which is a NAC transcription factor in wild wheat, accelerated senescence and resulted in more efficient nitrogen recycling (Uauy *et al.*, 2006).

Table 1.4.1 Summary of major function in abiotic stresses of WRKY family genes. (Table is taken from Chen *et al.* (2012).

Gene	Locus	Induced by abiotic factors	Function in abiotic stress
AtWRKY2	At5g56270	NaCl, mannitol	Negative regulator in ABA signalling
AtWRKY6	At1g62300	H_2O_2, methyl viologen, Pi and B starvation	Negative regulator in low Pi stress and positive regulator in low B stress
AtWRKY18	At4g31800	ABA	ABA signalling, NaCl and mannitao tolerance
AtWRKY22	At4g01250	H_2O_2, dark	Enhanced dark-induced senescence
AtWRKY25	At2g30250	Ethylene, NO, NaCl, mannitol, cold, heat, ABA	Tolerance to heat and NaCl, increased sensitivity to oxidative stress and ABA
AtWRKY26	At5g07100	Heat	Tolerance to heat and NaCl
AtWRKY33	At2g38470	NaCl, mannitol, cold, H_2O_2, ozone oxidative stres, UV	Tolerance to heat and NaCl, increased sensitivity to oxidative stress and ABA
AtWRKY34	At4g26440	Cold	Negative regulator in pollen specific cold response
AtWRKY39	At3g04670	Heat	Tolerance to heat
AtWRKY40	At1g80840	ABA	ABA signalling
AtWRKY60	At2g25000	Wounding	ABA signalling, NaCl and mannitao tolerance
AtWRKY63	At1g66600	ABA	Negative regulator in ABA signalling while positive regulator in drought tolerance
AtWRKY75	At5g13080	Pi deprivation	Positive regulator in Pi starvation
OsWRKY08	05g50610	Drought, salinity, H_2O_2, ABA, NAA	Tolerance to osmotic stress
OsWRKY11	01g43650	Heat, drought	Tolerance to xerothermic stress
OsWRKY23	01g53260	Salinity, ABA, H_2O_2, Osmotic stress, dark	Enhanced dark-induced senescence
OsWRKY45	05g2577	Cold, ABA	Tolerance to salt and drought stress
OsWRKY72	11g29870	Salinity, heat, ABA, NAA, osmotic stress, sugar starvation	Negative regulator in ABA signalling and sugar starvation
OsWRKY89	11g02520	Salinity, ABA, UV-B, wounding	Tolerance to UV-B radiation
GmWRKY13	DQ322694	Salt, drought	Increased sensitivity to salt and mannitol while decreased sensitivity to ABA
GmWRKY21	DQ322691	Salt, drought, cold	Cold tolerance
GmWRKY54	DQ322698	Salt, drought	Salt and drought tolerance
TcWRKY53	EF053036	Salinity, cold, drought	Negative regulator in osmotic stress
HvWRKY34	DQ863118	Sugar	Sugar signalling
HvWRKY41	DQ863124	Sugar	Sugar signalling
HvWRKY46	(SUSIBA2)	Sugar	Sugar signalling
NaWRKY3	AY456271	Wounding	JA signalling
NaWRKY6	AY456272	Wounding	JA signalling

1.5 Important experimental sites and systems for studying plants response to the future [CO_2]

1.5.1 The Free Air Carbon dioxide Enrichment Experimentation

The Free-Air CO_2 Enrichment (FACE) experiments have provided artificial environment sites that allow the study of how plants and ecosystems will respond to a future atmospheric [CO_2] compared with ambient [CO_2] (a[CO_2]) (Hendrey & Miglietta, 2006). According to the POPFACE fumigation system paper (Miglietta *et al.*, 2001)(Figure 1.5.1), the FACE technology was first developed and applied to an experiment in the 1970s by Harper and his co-workers (Harper *et al.*, 1973) and later modified by Mooi (1983)in the 1980s, and at present there are more than thirty FACE experimental sites established around the world with various designs (Hendrey & Miglietta, 2006). The trees or grasses were grown in a fully open field and surrounded by circular or octagonal pipes around or above their top which consistently released either CO_2 or ozone (O_3) or a combination of CO_2 and O_3 at the upwind side, the calculation of [CO_2], wind direction and velocity were measured at plot centre(Long *et al.*, 2006; Nosberger *et al.*, 2006). The FACE technology minimised the artefacts (Woodward, 2002), for example, providing no limitation to root development. Compared with previous experiments where plants were grown in pots, the limitation could potentially restrain plant growth and suppress the plant's response to e[CO_2], especially with respect to biomass (Long *et al.*, 2004). Current FACE experimental rings exist from 1metre (m) to 30m in diameter (Hendrey & Miglietta, 2006), with reduced edge effects due to the large diameter and enabling multiple conditions to be applied to the vegetation including change in temperature, water and nutrient compared with previous controlled environment chambers, and transparent enclosures or open-top chambers (Long *et al.*, 2004). FACE also allows research into ecosystem response to e[CO_2], consistently during long-term exposure that could last for several years (Taylor *et al.*, 2005). The main forest FACE sites are given in Table 1.5.1. Hendrey and Miglietta (2006) conclude that the FACE technology also has disadvantages: 1) The FACE experiment could not simulate the gradient increase of [CO_2] as current natural environmental changes; 2) The pipe around vegetation

releases pure CO_2 from time to time, so there is a high frequency of variability of $[CO_2]$ received by the leaf which might affect the temperate ecosystem response; 3) Although FACE reduced the edge effects, there is still a limitation on plot size and the variation of plants growing in the ring.

Figure 1.5.1 Example of FACE experiment layout.(Picture was obtained from PopFACE website, http://www.unitus.it/dipartimenti/disafri/progetti/popface/Site.htm).

Table 1.5.1 Experimental descriptors of the main global forest FACE sites.ppm: parts per million

Project	Location	Started year	e[CO_2] concentration	Main vegetation
SwissFACE	Eschikon, CHE	1993	600 ppm	Grassland
DukeFACE	Duke forest, North Carolina, USA	1995	Ambient + 200 ppm	Pine, Pine-hardwood, Upland hardwood
AspenFACE	Rhinelander,WI, USA	1998	Ambient + 200 ppm	Aspen, Red maple, Paper birth
POP/EuroFACE	Viterbo, ITA	1999	550 ppm	Poplar
WebFACE	Hofstetten, CHE	2000	600 ppm	Mature temperate forest (*Fagus, Quercus, Carpinus, Prunus, Acer, Tilia*)
SoyFACE	Champaign, IL, USA	2001	550 ppm	Soybean (*Glycine max*)
ChinaFACE	Wuxi, Jiangsu, CHN	2001	Ambient + 200 ppm	Rice, Winter wheat
TasFACE	Pontville, Tasmania, AUS	2002	550 ppm	Native grassland
AGFACE	Horsham, AUS	2007	550 ppm	Rainfed wheat, Field pea
TsukubaFACE	Tsukuba, Ibaraki, JPN	2010	Ambient + 200 ppm	Rice, Paddy field
BangorFACE	Bangor, GBR		Ambient + 200 ppm	Deciduous forest (*Betula pendula, Alnus glutinosa, Fagus sylvatica*)

The information was collected from FACE Data Management System (http://public.ornl.gov/face/global_face.shtml).

1.5.2 Natural high-CO_2 spring

There are several sites which are naturally abundant with high [CO_2] in the atmosphere (Table 1.5.2). There is a variety of vegetation at these sites including trees, shrubs, herbs and grasses, which have been growing under these atmospheric conditions for hundreds of years (known from local records since 19th century) allowing time for adaptation to local environment (Bettarini *et al.*, 1999). Studying the plants from inside spring sites and comparing them with those outside the spring site from the same area will provide us with a straightforward approach to understand the plant and ecological acclimation and genetic adaptation to the future environment, in terms of [CO_2] in the atmosphere.

The Bossoleto spring is located in Tuscany in central Italy (Lat. 43°17', Long. 11°35'), which is shaped as a circular crater measuring 80 m in diameter and 20 m in depth, and is surrounded by ancient walls to prevent human disturbance (van Gardingen *et al.*, 1997; Bettarini *et al.*, 1999). The abundant CO_2 was the main component (96.1%) of the gas released from the bottom of the crater, which also contained 0.02% hydrogen sulphide(H_2S), 0.45% CH_4, 3.40% nitrogen (N_2), and traces of hydrogen(H_2), helium (He) and argon (Ar) (Bettarini *et al.*, 1999). The [CO_2] generally ranged from 500 - 1000 ppm through the day and could reach as high as 5000 ppm during January and February (Bettarini *et al.*, 1999). The soil of this crater did not develop strong acidity due to the contrasting salts, such as calcium carbonate, in the spring (Selvi & Bettarini, 1999). Outside of the Bossoleto spring, there was similar vegetation grown under ambient atmospheric air including species such as *Plantago lanceolata*, *Quercus ilex* and *Agrostis stolonifera*(Selvi & Bettarini, 1999).

Table 1.5.2 Summary of major naturally high CO_2 springs. The table was modified from Grace and van Gardingen (1997).

Country	Region	Source of CO_2 emissions	Maximum CO_2 exposure	Vegetation type	Reference
Italy	Tuscany (Bossoleto)	Mineral spring	1000 ppm	Forbs, shrubs and trees	Bettarini *et al.* (1998)
Italy	Campania (Solfatara)	Mineral spring	600 ppm	Trees and shrubs	Schulte *et al.* (1999)
USA	Yellowstone national park	Mineral spring	n/a	Alga	Rigano *et al.* (1997)
USA	Utah	Burning coal seam	900 ppm	Shrub community	Ehleringer *et al.* (1997)
USA	Florida	Mineral spring	575 ppm	Shrubs and trees	Woodward and Beerling (1997)
Iceland	Snæfellsnes Peninsula	Mineral spring	880 ppm	n/a	Cook *et al.* (1998)
Africa	Cameroon	Degassing of lake	n/a	n/a	Mousseau *et al.* (1997)
Northland	Whangarei (Hakanoa)	Mineral spring	994 ppm	n/a	Ross *et al.* (2000)
New Zealand	Kamo (Hakanoa Spring)	Mineral spring	5200 ppm	Forbs	Newton *et al.* (1996)
Japan	National park (Nibu,Ryuzin-numa and Yuno-kawa)	Mineral spring	>500 ppm	Forbs and shrubs	Onoda *et al.* (2009)

1.6 Plant species used in this study

1.6.1 *Populus*

Although plant structures are complex and there is an enormous diversity across the plant kingdom, it is believed that plants have a common evolutionary origin, therefore "research on one organism can provide useful insight into its relatives" (Ellis *et al.*, 2010). Unlike short-lived plants, trees were fixed to one location and experience varying abiotic and biotic stress continually over time (Tuskan *et al.*, 2006). The genus *Populus* has been established as a genetic model system for trees due to its long life spans and generation times, and woody perennial growth habits (Taylor, 2002; Jansson & Douglas, 2007). Another advantage of using poplar as a model system for woody perennial plants and trees is the rapid and well developed genomic and molecular biology resources (Jansson & Douglas, 2007). The poplar female clone *Nisqually-1* from the black cottonwood tree, *Populus trichocarpa*, was chosen for genome sequencing in 2003 (Tuskan *et al.*, 2006). The final sequencing assembly in early 2004 discovered 2447 scaffolds which covered 429 Mb of captured sequence and mapped these to a physical map, and with help of polymorphic microsatellite genetic markers, 335 Mb of genome sequence were fixed to 19 *Populus* linkage groups (Jansson & Douglas, 2007; Ellis *et al.*, 2010). The *Populus* genome analysis provided evidence that the *Populus* genome was more duplicated (92% of the genome) compared with *Arabidopsis*, from which *Populus* diverged lineages from about 100 to 120 million years ago (Tuskan *et al.*, 2006). The assembled genome sequence is available on the website of the Joint Genome Institute (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html). A second assembly of *Populus* genome was completed in 2009 based on the sub-cloning bacterial artificial chromosomes (BACs) and primer walking, in the meanwhile, the newly available expressed sequence tags (ESTs) sets were re-annotated (Ellis *et al.*, 2010). As a traditional model plant, the *Arabidopsis* genome is relatively small and fully developed, and it also has a much faster life cycle; However, *Populus* has the advantage of enabling the study of important plant processes which are absent or poorly developed in *Arabidopsis*, including wood formation, autumn senescence, sex

determination, and long-term biotic interactions (Taylor, 2002; Jansson & Douglas, 2007). There is also the disadvantage of using *Populus* as a model forest tree as it is more closely related to *Arabidopsis* and annual crop species than coniferous tree species, therefore, not applicable for research on evolutionary or taxonomic relatives of trees (Ellis *et al.*, 2010).

1.6.2 Plantago lanceolata

Plantago lanceolata which is a common herb in Europe, has been studied widely in physiological, ecological, and genetic studies (Bettarini *et al.*, 1998; Klus *et al.*, 2001; Sahin *et al.*, 2007; Onoda *et al.*, 2009; Meudt, 2011; Nakamura *et al.*, 2011). *Plantago lanceolata* is a short-lived perennial rosette herb that requires long-day conditions and with adequate nutrients, producing numerous leaves and spiked inflorescences at the end of fibrous stalks (Sagar & Harper, 1964; Fajer *et al.*, 1992). The flowering spans early June through mid-August and fruit maturation and subsequent seed dispersal occur throughout the summer and autumn (Tonsor *et al.*, 1993). *Plantago* is wind pollinated and has a gametophytic self-incompatibility system (Ross, 1973). It is also known to be capable of undergoing rapid evolutionary changes (Wu & Antonovics, 1976; Wolff & Vandelden, 1989; van Tienderen & van der Toorn, 1991; Klus *et al.*, 2001; Bischoff *et al.*, 2006) particularly on seed yield, biomass, leaf number and length and spike number. However, there is very limited research on *Plantago* genomic resources. According to Joint Genome Institute (JGI) project list, the EST of *Plantago ovate* (the only cultivated species in *Plantago*) and the standard draft of both *Plantago lanceolata* and *Plantago media* mitochondrial genomes have been completed, but the resources are not yet available (http://genome.jgi.doe.gov/genome-projects/). Pommerrenig *et al.* (2006) sequenced the *Plantago major*'s ESTs to study the leaf vascular tissue, in which experiment, they constructed 5,900 vascular EST library that represent 3,247 different mRNAs (each ESTs sequence can be found in the GenBank/EMBL database with the accession number listed in this paper).

1.7 The genomic toolbox available to understand molecular and genetic plant response to elevated atmospheric CO_2

1.7.1 The process from DNA to protein

The process of conversion from DNA to protein was explained in the book of Karp (2001). The DNA strand of a gene is converted to RNA strand by RNA polymerases which incorporate nucleotides one by one. The RNA polymerases require transcription factors bind to the promoter area of gene DNA and initiating transcription and the polymerase moves from 3' end to 5' end of DNA strand. There are three types of transcribing enzymes; RNA polymerase I synthesises the larger ribosomal RNAs, RNA polymerase II synthesises messenger RNAs (mRNAs) and most small nuclear RNAs and RNA polymerase III synthesises low-molecular weight RNAs, including the carious transfer RNAs (tRNA) and the 5S ribosomal RNA(Karp, 2001).The RNA chain directly produced after transcription from DNA is called pre-mRNA which will be converted to mature mRNA with an addition of a 5' cap and 3' poly(A) tail to the ends of the transcript and removal of any intervening introns. The mature mRNA will be transported into the cytoplasm from the nucleus and translated to protein in ribosomes(Clancy & Brown, 2008).

During translation, the small ribosomal subunit attached to mRNA's 5'end (near AUG start codon) with the participation of three initiation factor (IF) proteins (IF1, IF2 and IF3) and a tRNA with their attached amino acids. The large ribosomal subunit then binds to this complex and releases the IFs. The ribosome moves along the mRNA with the participation of elongation factors (EFs), GTPs and tRNAs which carried the corresponds codon. The peptide bonds between amino acids and is accompanied with displacement of deacylated tRNA(Karp, 2001).When the ribosome reaches one of three codons – UAA, UAG or UGA, the elongation will be stopped and release the polypeptide associated with the last tRNA with release factors (RFs) participating(Clancy & Brown, 2008).

1.7.2PICME cDNA microarray

cDNA microarrays have become a reliable profiling method for understanding whole genome gene expression in relation to developmental process, whereas previous studies mostly used mutants for analysing developmental pathways (Schmid *et al.*, 2005; Jansson & Thomas, 2008). Street and Tsai (2010) listed the microarray platforms which have been used before and currently for *Populus*, there were both cDNA arrays and oligonucleotide (oligo) arrays which contain the whole genome (Table 1.7.1). Microarray analyses are usually applied on pathway mapping with identified gene sets (Street & Tsai, 2010) providing a quantifiable and global picture on gene expression level on biological process or response to a specific environmental conditions (Lim *et al.*, 2007), including senescence, drought, flood and e[CO_2] (Andersson *et al.*, 2004; Ainsworth *et al.*, 2006; Street *et al.*, 2006; Cseke *et al.*, 2009; Kreuzwieser *et al.*, 2009)

Table 1.7.1 Commonly used array platforms(Street & Tsai, 2010).

Array	Type	Unique probes	Web link
Agilent	Oligo (60mer)	43,795	www.genomics.agilent.com
Nimblegen	Oligo (60mer)	65,911	www.nimblegen.com
Affymetrix	Oligo (25mer)	61,251	www.affymetrix.com/estore/
UPSC-POP2	cDNA	20,390	www.*Populus*.db.umu.se/
PICME	cDNA	26,915	www.picme.at/
UBC	cDNA	15,496	
MTU	cDNA	6,705	

One of the most commonly used *Populus* microarrays in the past decade is the PICME microarray. The PICME (Platform for Integrated Clone Management) *Populus* microarrays are composed of 26,890 elements (Cseke *et al.*, 2009), including 6528 ESTs of *Populus interamericana* from INRA-Nancy (Rinaldi *et al.*, 2007), 12,202 ESTs of *Populus alba* X *Populus tremula* from INRA-Orleans (Dejardin *et al.*, 2004) and 8,160 cDNA clones of *Populus euphratica* from the University of Helsinki (Brosche *et al.*, 2005). The pathway of cDNA microarray process is demonstrated in Figure 1.7.1.

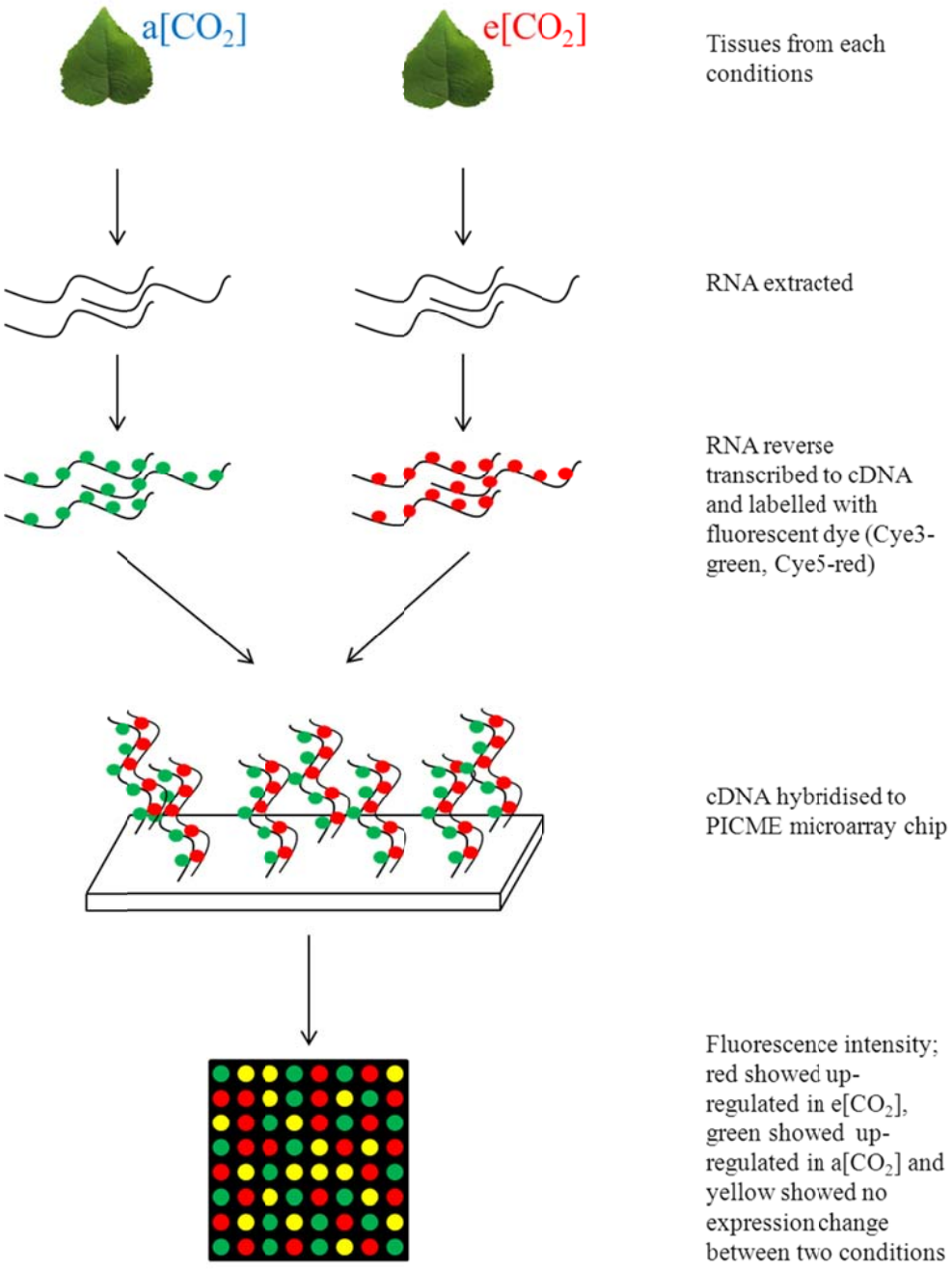


Figure 1.7.1.A brief demonstration of cDNA microarray process. Leaf images were obtained from S. Milner (University of Southampton).

1.7.3Affymetrix microarray

Oligo arrays (Affymetrix, Agilent and Nimblegen) were available for *Populus* after cDNA microarrays were developed with the advantage of containing the whole genome derived from EST sequences (Street & Tsai, 2010). According to

the Affymetrix data sheet available on the Affymetrix website (http://media.affymetrix.com/support/technical/datasheets/poplar_datasheet.pdf), the predicted genes for the Affymetrix microarray set were from the *Populus* genome project (*P.trichocarpa*) and based at the JGI, and the content is designed to detect 7,742 distinct EST/mRNA-based poplar UniGene clusters, and over 23,657 gene predictions. The pathway of Affymetrix microarray process is demonstrated in Figure 1.7.2.

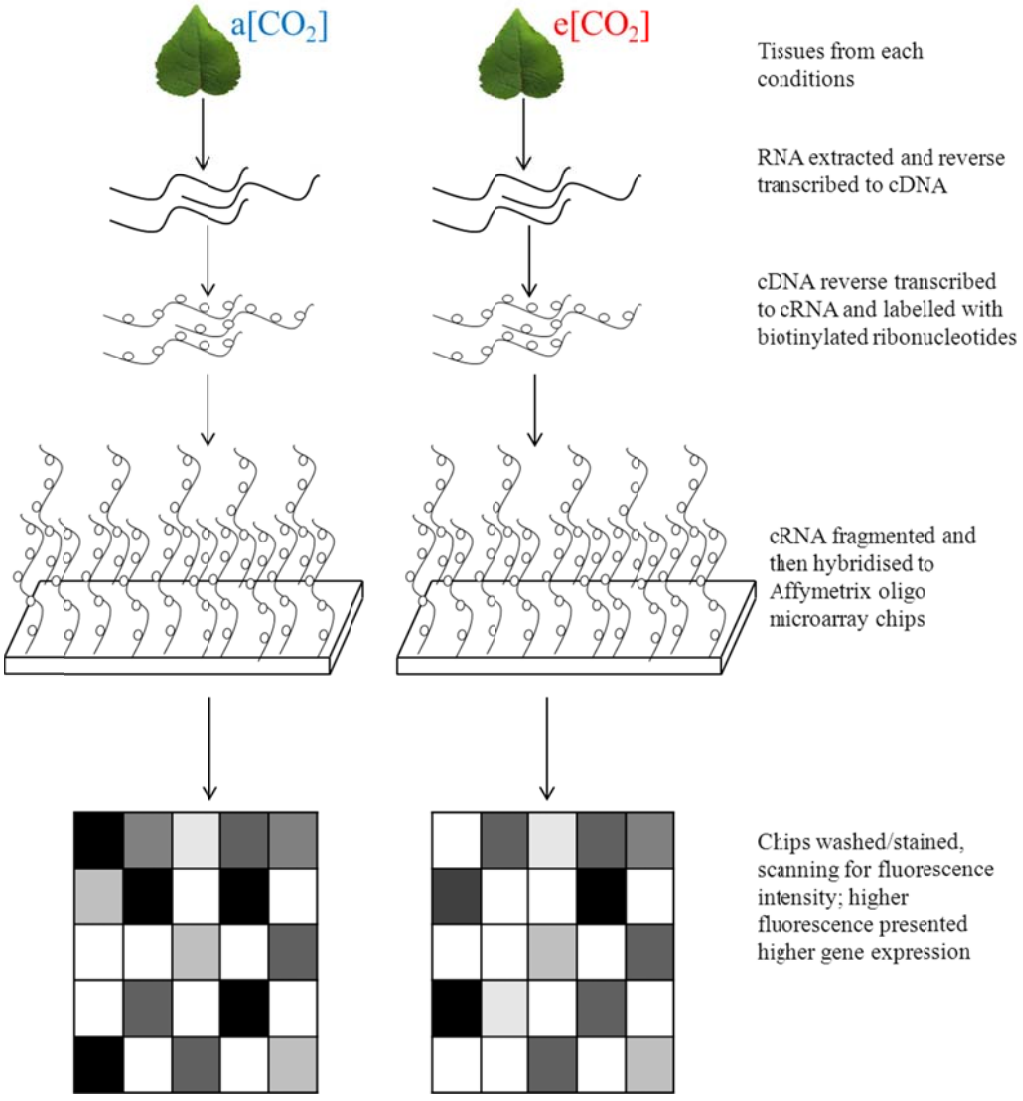


Figure 1.7.2.A brief demonstration of Affymetrix microarray process. Leaf images were obtained from S. Milner (University of Southampton). Figure modified from Affymetrix manual.

The commercial microarray chips can identify transcriptome expression change very easily; however, there is a limitation on transcriptome studies in non-model

species. Shortly after the microarray technique was developed, the cross species microarray (Xspecies GeneChip microarray from Nottingham *Arabidopsis* stock centre (NASC)) were designed around the year 2005 for analysing the transcriptome of un-sequenced or un-annotated species including Banana, Tomato, Potato, Lettuce and several species from *Brassicaceae* family (Hammond *et al.*, 2005). This paper explained the fundamental mechanisms of the Xspecies microarray. The target plant's genomic DNA was hybridised to a selected model-species oligo array (e.g. *Arabidopsis*) which contained multiple oligo probes (each probes represented one gene) to compare the similarity between two species (Hammond *et al.*, 2006). The hybridisation intensity threshold was used to create the probe mask file excluding the probes, which failed to hybridise with target species' sequence. The procedure of RNA hybridisation is very similar to Affymetrix microarrays. The target species' RNA were fluorescently labelled and hybridised to the oligo array. The intensity of signal from each of the probes was captured to quantify the transcripts' expression (Hammond *et al.*, 2006). The target species' transcripts expression were normalised by the probe mask file to correct the expression. Noticeably, this technique does have disadvantages in that it relies on the variety of the probes for each gene to maintain a high level of hybridisation for target species (Davey *et al.*, 2009), and the more different the target species' genome is to the model plant's, the more difficult it is to identify the full transcriptome change will be.

1.7.4Transcriptome sequencing

Transcriptome sequencing (also referred to as RNA-sequencing (RNA-seq)) has been successfully used to identify the expression of transcripts over a long period time (Adams *et al.*, 1991). The transcriptome were translated to cDNA and sequenced based on DNA sequencing technology which has been developed rapidly from the automated Sanger method (referred to as the first-generation technology) to the massive parallel DNA sequencing technologies known as next generation sequencing (NGS) techniques (Metzker, 2009). A review on NGS by Metzker (2009) explained the different approaches of several commercial

sequencing platforms including Roche 454, Illumina, Life/APG, Helicos Biosciences and the Pacific Biosciences in detail (Table 1.7.2).

Compared with traditional microarray techniques, the RNA-seq does not have problems with high background noise due to cross-hybridisation or limited detection due to saturation of signals and background (Wang *et al.*, 2009). Apart from the advantage that RNA-seq can detect the expression of rare transcripts, it can also deliver information on alternative splicing and sequence variation within species, which benefits comparative or evolution studies on large populations (Metzker, 2009). The great advantage of RNA-seq is the ability to identify transcript changes without prior known genomic DNA sequences with much more accurate quantification of expression, even without the availability of a reference genome(Nagalakshmi *et al.*, 2008; Wang *et al.*, 2009).

Table 1.7.2 Comparison between different next generation sequencing platforms. (Table were from Metzker (2009))

Platform	Library/template preparation	NGS chemistry	Read length (Bases)	Run time (days)	Gb per run	Pros	Cons	Biological application
Roche/454's GS FLX Titanium	Fragment, mate-pair/emPCR	Pyrosequencing	330*	0.35	0.45	Longer reads improve mapping in repetitive regions; fast run times	High reagent cost; high error rates in homo-polymer repeats	Bacterial and insect genome; medium scale (<3 Mb) exome capture; 16s in metagenomics
Illumina/ Solexa's GA_{II}	Fragment, mate-pair/solid-phase	Reversible terminators	75 or 100	$4^{\#}$,$9^{\&}$	$18^{\#}$,$35^{\&}$	Currently the most widely used platform in the field	Low multiplexing capability of samples	Variant discovery by whole-genome re-sequencing or whole-exome capture; gene discovery in metagenomics
Life/APG's SOliD3	Fragment, mate-pair/emPCR	Cleavable probe sequencing by ligation	50	$7^{\#}$,$14^{\&}$	$30^{\#}$,$50^{\&}$	Two-base encoding provides inherent error correction	Long run times	Variant discovery by whole-genome re-sequencing or whole-exome capture; gene discovery in metagenomics
Polonator G.007	Mate-pair/emPCR	Non-cleavable probe sequencing by ligation	26	$5^{\&}$	$12^{\&}$	Least expensive platform; open source to adapt alternative NGS chemistries	Users are required to maintain and quality control reagents; shortest NGS read lengths	Bacterial genome resequencing for variant discovery
Helicos BioSciences HeliScope	Fragment, mate-pair/single molecule	Reversible terminators	32*	$8^{\#}$	$37^{\#}$	Non-bias representation of templates for genome and sequence-based application	High error rates compared with other reversible terminator chemistries	Sequence-based methods
Pacific Biosciences	Fragment/single molecule	Real-time	964*	N/A	N/A	Has the greatest potential for reads exceeding 1kb	Highest error rates compared with other NGS chemistries	Full-length transcriptome sequencing; complements other re-sequencing efforts in discovering large structural variants and haplotype blocks

*Average read-lengths. $^{\&}$ Fragment run. $^{\#}$ mate-pair run.Gb, giga base pairs. Mb Mega base pairs. kb, kilo base pairs

1.8 Aims of the project

This project is focused on the morphological, physiological, genomic and genetic changes in plants in response to increased carbon dioxide concentration. Since very few studies on plant response to elevated CO_2 have been undertaken at the molecular level (functional genomics), and even fewer on DNA-level genomic (structural genomics) level, our effort will be focussed here. The first part of this project identifies gene expression changes associated with tree response to elevated $CO_2$by studying the difference of transcript expression of plants exposed to either ambient or elevated [CO_2] in two contrasting FACE experiments. This will allow us to predict plant acclimation to the future atmospheric environment and identify target genes that might be subject to microevolutionary response and adaptive change. The second part of this project studied plants which have been growing in high [CO_2] over hundreds of years and many generations to detect the process of genetic and genomic variation to understand the impact of increasing [CO_2] on plant evolution and to address the question of whether CO_2 may act as a selective agent causing directional microevolution. These two approaches are brought together, synthesised and discussed at the end of the study, with conclusions on whether plants are likely to show adaptations to future climate change, with particular reference to atmospheric CO_2.

Chapter 2: Delayed autumnal senescence in elevated carbon dioxide

2.0 Overview

Increased atmospheric [CO_2] is considered to be the major causative factor contributing to global temperature increase. Along with the increased [CO_2], a delayed autumnal senescence phenomenon has been observed recently. A previous study (Taylor *et al.*, 2008) showed that this delay can be the result of elevated atmospheric [CO_2]. To identify the mechanism underlying delayed senescence in the elevated [CO_2] world, *Populus* leaves from a FACE experiment were used for PICME microarray analysis. Leaves were picked from two different [CO_2] conditions –550 ppm for elevated [CO_2] and 360 ppm for ambient [CO_2]. By analysing the gene expression difference under two treatments, it was confirmed that the secondary metabolism pathways, particularly the anthocyanin biosynthetic pathway, was consistently up-regulated in e[CO_2] which was a consequence of higher sugar content as well as increased [CO_2]. The highly expressed pathways were also confirmed by biochemistry, and anthocyanin, sugar and starch content in leaves were enhanced in e[CO_2]. Therefore, we propose that in a CO_2 rich environment, the increased accumulation of sugar resulted in changes in genes expression involved in secondary carbon metabolism, which potentially reduced the oxidative stress and enabled prolonged leaf longevity and is a positive benefit for carbon balance and stress tolerance.

2.1 Introduction

The atmospheric carbon dioxide concentration has increased from 280 ppm at the beginning of the industrial revolution, early in the 19th century, to 369 ppm at the start of this century due to human activities (Woodward, 2002). It is predicted to continually increase to around 550ppm by the year 2050 (IPCC, 2007). Extended growth season was thought to be correlated with global climate change and early bud set was found to be related to global warming (Menzel *et al.*, 2006). However, delayed autumnal senescence has been shown to be more related to higher CO_2 concentrations (Taylor *et al.*, 2008). Several studies on the effects of elevated CO_2 on leaf or whole plant senescence have produced variable results; with advances (Stomer & Horvath, 1983; Curtis *et al.*, 1989; Sigurdsson, 2001; Bindi *et al.*, 2002; Korner *et al.*, 2005), delays (Li *et al.*, 2000; Korner *et al.*, 2005; Dermody *et al.*, 2006; Rae *et al.*, 2006; Taylor *et al.*, 2008) or no effect (Gunderson *et al.*, 1993; Herrick & Thomas, 2003). Taylor *et al.* (2008) conclude that this variation in response to e[CO_2] could be due to plants' growth pattern; with determinate growth species often showing early senescence whereas indeterminate growth species showing late senescence in response to e[CO_2]. Also the higher concentration of CO_2 altered the flowering times by influencing the expression of floral-initiation genes (Parmesan, 2006; Springer & Ward, 2007; Springer *et al.*, 2008) as well as delayed reproductive development and final maturation in soybean (Castro *et al.*, 2009). However, the fundamental transcript regulation of delayed senescence in response to e[CO_2] is not clear.

Oxidative stress is supposed to have a major effect on senescence and associated degradation events, due to the increased free radicals produced by chlorophyll and membrane degradation during senescence (Zimmermann & Zentgraf, 2005). The plant reacts to this by activating a series of antioxidative enzymes, such as catalases, superoxide dismutases and components of the ascorbate-glutathione cycle, to scavenge these toxic metabolites (Foyer *et al.*, 1994). In addition to the antioxidative enzymes, flavonoid phytochemicals also have the function of inhibiting oxidation, as well as other ecological functions including ultraviolet and visible light screens, pigmentation, or signals (Harborne & Williams, 2000). The

major flavonoid product is anthocyanin which plays important roles in response to stresses such as wounding, pathogen attack or ultraviolet (UV) light stress (Constabel & Lindroth, 2010), and it also scavenges free radicals (Gould, 2004). Elevated CO_2 has been reported to decrease the concentration of antioxidants including both metabolites such as secondary metabolism, and enzymes including glutathione and ascorbate in many species (Wustman *et al.*, 2001; Karnosky *et al.*, 2003). Higher amounts of antioxidants were produced in strawberries grown under e[CO_2] compared with those grown under a[CO_2], potentially as antioxidants were accumulated in order to protect cells from free radical and oxidant damage (Wang *et al.*, 2003). However, the effects of elevated CO_2 on antioxidants at the gene level remain to be identified. *Populus x euramericana*were grown in experimental plots at the POP/EUROFACE site since planting in June 1999. The site was located on a nutrient rich, clay soil in Tuscania, Italy (42°37'04''N, 11°80'87''E, alt.150m) (Lukac *et al.*, 2003). The *Populus* were exposed to a[CO_2] (approximately 360 ppm) and e[CO_2] (approximately 550 ppm) separately, which was supplied from bud burst until leaf fall (March - November), during the day. The *Populus* leaves were collected at four timepoints (21.09.04, 05.10.04, 18.10.04 and 04.11.04) by Dr M. J Tallis (University of Southampton, UK), and the 11th leaf down from the closed apical bud were chosen and 12 leaves from each treatment were collected from the third timepoint (18.10.04). Only the RNA of nine randomly selected samples from each treatment were selected and used for PICME (http://www.picme.at) cDNA microarray analysis to study the molecular and genetic mechanisms underlying the delayed senescence response observed in POP/EUROFACE (Tallis, 2007) (Figure 2.1.1). Tallis (2007) produced 20,243 possible ESTs in which only 2,696 existed across all nine replicate microarrays. In his study, there were 53 genes identified which were significantly up-regulated (at least two-fold) and 31 genes which were significantly down-regulated (at least two-fold) in response to e[CO_2]. There were three ESTs which were significantly differentially expressed between a[CO_2] and e[CO_2], and these were fibre lipid transfer protein, dihydroflavonol reductase and leucoanthocyanidin reductase.

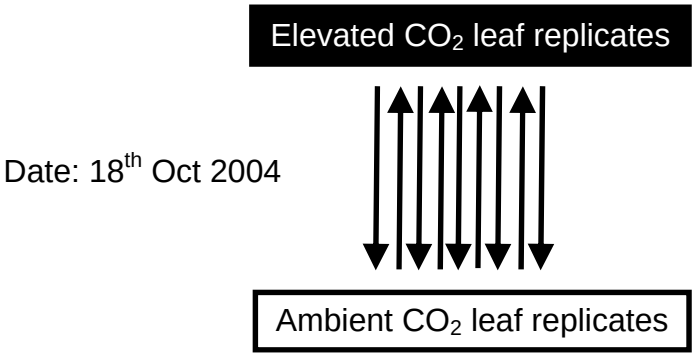


Figure 2.1.1 A schematic of PICME microarray experimental design. The RNA of nine senescence leaves collected from both a[CO2] and e[CO2] in POP/EUROFACE were used for nine randomly paired PICME microarray hybridisation to identify the gene expression change between treatment during senescence(18th Oct 2004). (Figure obtained from Tallis (2007))

The full picture of gene expression differences between e[CO_2] and a[CO_2] at late senescence are presented in this chapter in order to understand the molecular mechanisms underlying delayed senescence in e[CO_2]. The data in this chapter exhibited an overall highly expressed carbon metabolism from photosynthesis through glycolysis and the TCA cycle (tricarboxylic acid cycle) to secondary metabolism under e[CO_2] compared with a[CO_2] in late senescence. The accumulated sugar in e[CO_2] stimulated the anthocyanin regulators, and therefore induced the anthocyanin production to reduce the oxidative stress, enabling prolonged leaf longevity and an improvement to the carbon balance and stress tolerance.

2.2 Material and Methods

2.2.1 Gene expression and pathway analysis

The PICME microarray analysis was followed the MIAME (Minimum information about a microarray experiment) guidelines which can be found at http://picme.at. All microarray data generated from this experiment can be found in the Gene Expression Omnibus (GEO) database under series GSE15874 and platforms GPL8473. All nine Microarray datasets were output from GeneSpring GX and further analysed using Mapman (version 2.1.1, downloaded from http://gabi.rzpd.de/projects/MapMan/) and the overview of the *AraCyc* metabolic map was produced using TAIR (The Arabidopsis Information Resource, http://pmn.plantcyc.org/ARA/expression.html). These two tools were both able to display systematic trends by diagrams of metabolic pathways or other processes based on the large data sets. Both Mapman and TAIR metabolic maps were based on *Arabidopsis* metabolic pathways and required the input of the AGI number which was associated with the EST and the $\log_2$ fold change ratio. EST sequences were searched from the *Populus trichocarpa* database in the National Center of Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) from the output of GeneSpring GX. The sequences were then blasted using tBLAST X algorithm against the *A. thaliana* database via TAIR (http://www.arabidopsis.org/) for the *Arabidopsis* genome initiative (AGI) number and annotation, and regularly checked for updates via TAIR. Log_2 fold change ratios were the average of the overlapping ESTs or the same AGI number. *Populus* gene models were identified by *Populus* EST senescence data and searching TAIR for the *Arabidopsis* orthologue and the annotation.

All genes were input into agriGO v 1.2 (http://bioinfo.cau.edu.cn/agriGO/) for gene ontology (GO) analysis (Du *et al.*, 2010). The analysis mapped the genes into different GO processes and calculated the significance for each category using the parametric analysis of gene set enrichment (PAGE) method. The *P*-value was converted from the *Z*-score which was calculated from the number of genes mapped to the term and the mean of mapped genes' expression,

2.2.2 Leaf Biochemistry

2.2.2.1 Anthocyanin content

Frozen poplar leaves were ground using mortar and pestles, and approximately 50 mg of each sample was used for analysis of anthocyanin content according to the method of Martin *et al.*(2002). Anthocyanins were extracted in 300 microliter (µl) of 7% (volume/volume, v/v) hydrochloric acid in methanol overnight at 4 degree Celsius (°C) under gentle shaking. The following morning 200 µl sterile de-ionized water was added to each sample and mixed. 500 µl chloroform was added and samples centrifuged at 13,000 rpm (revolutions per minute) for 2 min at 4 °C. The supernatant (400 µl) was transferred to a new centrifuge tube, to which 600 µl of 1% (v/v) hydrochloric acid in methanol was added and centrifuged as above to remove remaining particles. The supernatant was used for absorbance measurements at 530 and 657 nanometre (nm). Relative anthocyanin concentrations were calculated as absorbency at 530 nm minus absorbance at 657 nm and then divided by 50 microgram (mg) to get a per mg reading.

2.2.2.2 Soluble carbohydrates and starch

Extraction and measurement of glucose, fructose, sucrose and starch content was carried at by Dr Alistair Rogers (Brookhaven National Laboratory) (Rogers *et al.*, 2006). Glucose, fructose and sucrose were extracted from frozen ground material using sequential incubations in ethanol. Starch was extracted from the residual material and converted to glucose. Glucose, fructose, sucrose and the glucose resulting from the starch degradation were then assayed using a continuous enzymatic substrate assay.

2.2.2.3 Statistical analysis

Anthocyanin, soluble sugar and starch content were statistically analysed by analysis of variance (ANOVA). Anthocyanin contents were transformed by

arcsine normalisation, and soluble sugar contents were normalised using logarithmic transformation. The statistical model for the single timepoint is Plot(CO_2)+ε, and the statistical model for cross time effect is Plot(CO_2)|time. Parenthesis represents nesting meaning Plot is dependent on [CO_2], and "|" represents an interaction between [CO_2] and time, and "ε" represented residual error which is a random variation in the response against the effect tested (Doncaster & Davey, 2007). All statistical analyses were carried out using Minitab 15 (Minitab Inc., 1991, State College, PA, USA).

2.3. Results

2.3.1 Gene expression in senescence under different CO_2 concentration

The microarray produced 20,243 possible data points after intensity normalisation of each EST. The EST sequences indicated that the complete set of 20,243 clones represents 6,513 unique gene models, and 72% of the ESTs were found to be expressed in this experiment. These EST sequences were then blasted using tBLAST X algorithm against the *A. thaliana* database via TAIR (http://www.Arabidopsis.org/) for the AGI number and annotation. 12,491 ESTs exhibited homology with the *Arabidopsis* genome, and unique *Arabidopsis thaliana* orthologues numbered 4,712 (see Appendix IV for full gene list).

Gene Ontology is intended to aid the annotation of homologous genes and protein sequences in multiple organisms using a common vocabulary that results in the ability to query and retrieve genes and proteins based on their shared biology (Ashburner *et al.*, 2000). The hierarchical tree of significant GO terms defining the microarray data was constructed in order to reveal the expression patterns of biological process during senescence influenced by different concentrations of CO_2 (Figure 2.3.1). The average expression of each unique AGI number was used for GO term analysis. There were 21 GO terms which showed a significant up-regulation response to e[CO_2] – the flavonoid biosynthetic process showed the highest significance following by flavonoid metabolic processes, translation, aromatic compound biosynthetic process and response to cytokinin stimulus. Both photosynthesis and the light reaction were significantly up-regulated by e[CO_2], as well as the abiotic response. The genes involved in the significant regulated GO terms were studied in detail in the following pathways analysis to understand the mechanism of e[CO_2] regulated delayed-senescence.

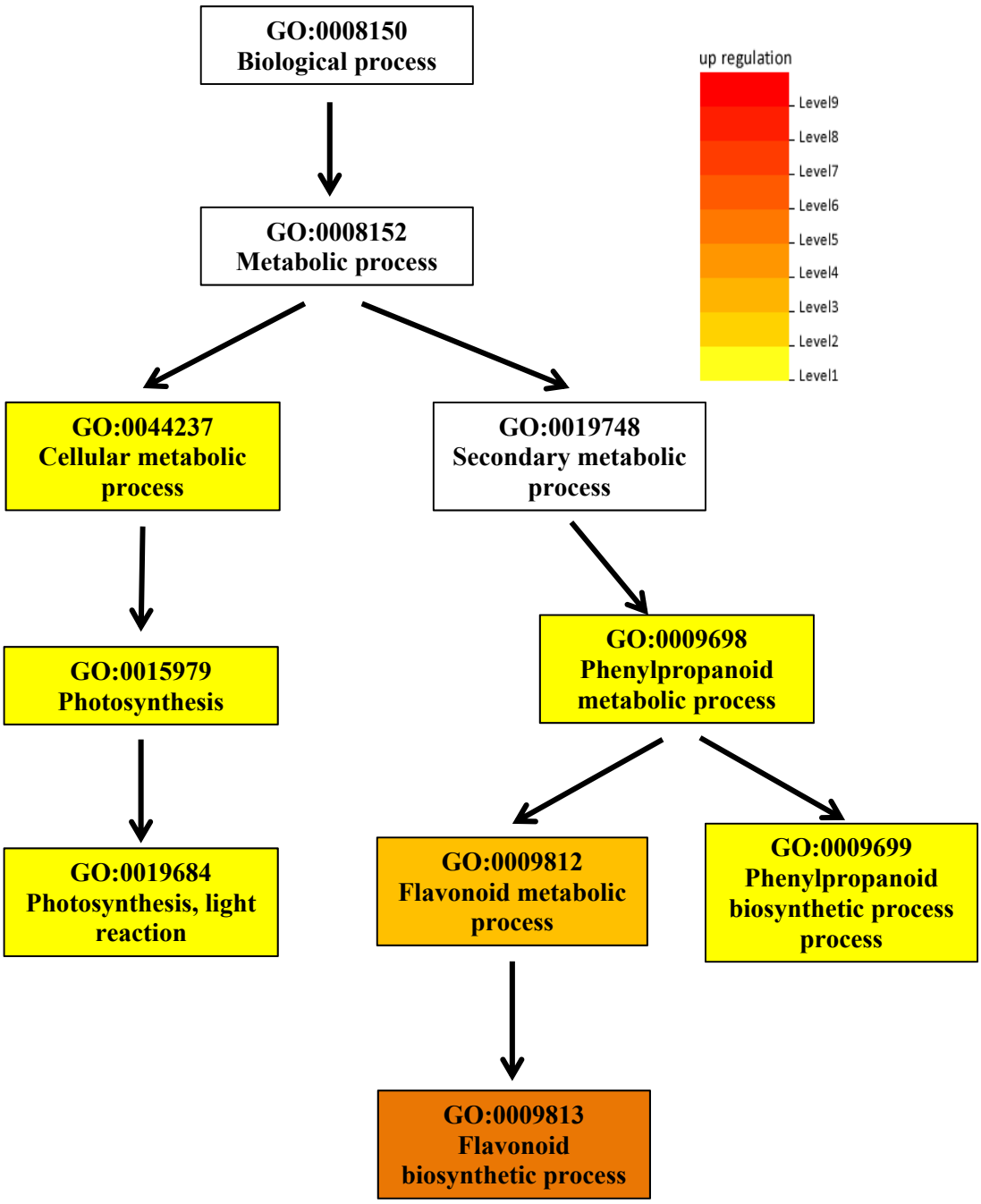


Figure 2.3.1 Hierarchical tree graph of GO terms in biological process category in response to e[CO2] compared with a[CO2] in metabolic process. The coloured boxes represent GO terms which were significantly up-regulated ($p \leq 0.05$) under e[CO_2].

2.3.2 Biosynthesis pathway related to the senescence

2.3.2.1 Carbon metabolism pathway

The full gene list with AGI ontology IDs was also imported into MapMan to determine which pathway showed similar patterns of expression for the genes involved in that functional group. The genes were sorted into different functional group (Bins), and the significance of each Bin was calculated by using the Wilcoxon Rank Sum Test with Benjamini Hochberg correction. Six functional groups and sub-groups showed significant differential expression under e[CO2] compared with a[CO_2] (Table 2.3.1).

Table 2.3.1 Functional groups and sub-groups with significantly different expression under e[CO_2] compared with a[CO_2].

Bin	Functional group	Transcripts number	P-value
29.2	Protein synthesis	212	<0.00001
1	Photosynthesis	94	0.000073
4	Glycolysis	26	0.0129
16.8	Secondary metabolism. Flavonoids	20	0.0197

The overview map of metabolism was abundant with generally highly expressed genes induced by e[CO_2] compared with a[CO_2] (Figure 2.3.2.a). The genes involved in the photosynthesis pathway including the light reaction and Calvin cycle were generally up-regulated by e[CO_2] implying a highly activated carbon assimilation occurring at late senescence stage in plants which were grown under e[CO_2]. Sugars play an important role in the regulation of plant metabolism and development. Starch is the direct carbohydrate product from the Calvin cycle in chloroplasts, which is then converted to a different carbohydrate form, such as maltose, glucose, sucrose or fructose. At late senescence stages, both sucrose and starch synthesis pathways were highly expressed in plants grown under e[CO_2] which suggested higher amounts of sucrose and starch were accumulated. The α-AMYLASE-LIKE 3, STARCH EXCESS 1 and DISPROPORTIONATING

ENZYME which are involved in starch degradation to maltose and glucose were also highly expressed under e[CO_2] compared with a[CO_2] showing an activated starch pathway at late senescence stage when exposed to e[CO_2], which implies an activated growth during the night. The genes involved in glycolysis TCA cycle and mitochondrial electron transport chain were also up-regulated by e[CO_2] at the late senescence stage. The entire metabolism pathway from photosynthesis to carbohydrate synthesis, glycolysis and TCA showed consistent up-regulation at the late senescence stage under e[CO_2] compared with a[CO_2], suggesting that the plants grown in e[CO_2] had a higher rate of carbon assimilation, as well as utilization.

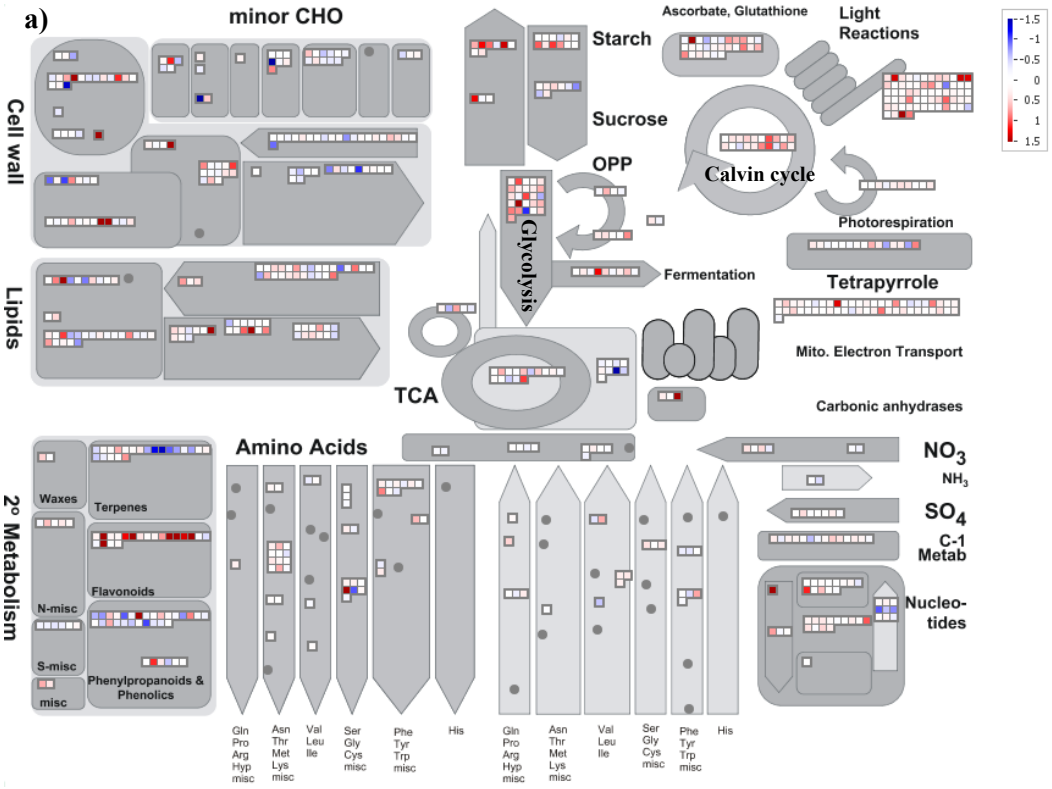


Figure 2.3.2 Transcript expression differences under e[CO_2] relative to a[CO_2] in metabolism and sub-metabolism pathways. Each square represents an *Arabidopsis* orthologue and the colour represents the $\log_2$ average expression under e[CO_2] compared with a[CO_2]. The colour scale bar ranges from dark blue (-1.5 representing ~2.8 fold down-regulated in e[CO_2] relative to a[CO_2]) to dark red (1.5 representing ~2.8 fold up-regulated in e[CO_2] relative to a[CO_2]). (Maps obtained from MapMan).

Secondary metabolism pathways were also abundant with associated genes, particularly the flavonoids, differentially expressed between e[CO_2] and a[CO_2]. The genes involved in anthocyanin synthesis (the final product of the flavonoid pathway) were all highly expressed under e[CO_2] (Figure 2.3.3). *Populus* has more protein-coding genes than *Arabidopsis*, with an average ranging from 1.4 to 1.6 of putative *Populus* homologues for each *Arabidopsis* gene (Tuskan *et al.*, 2006). It is not clear which *Populus* gene model is the main function gene in *Populus* anthocyanin biosynthesis pathway during senescence, therefore, the mean expression of each *Populus* gene model for the same *Arabidopsis* orthologue is used when referring to the expression of such genes as they were all up-regulated under e[CO_2] relatively to a[CO_2].This up-regulated anthocyanin biosynthesis pathway could be the result of highly activated sucrose synthesis pathway. The dihydroflavonol 4-reductase (DFR) enzyme, which is the downstream enzyme in anthocyanin biosynthesis pathway, is regulated by two MYB transcription factors *PRODUCTION OF ANTHOCYANIN PIGMENT1* (*PAP1*) and *PAP2* which positively respond to sucrose concentration (Teng *et al.*, 2005). These two regulators were also identified as putative senescence regulators. However, this experiment did not identify the gene expression of PAP1 and PAP2.

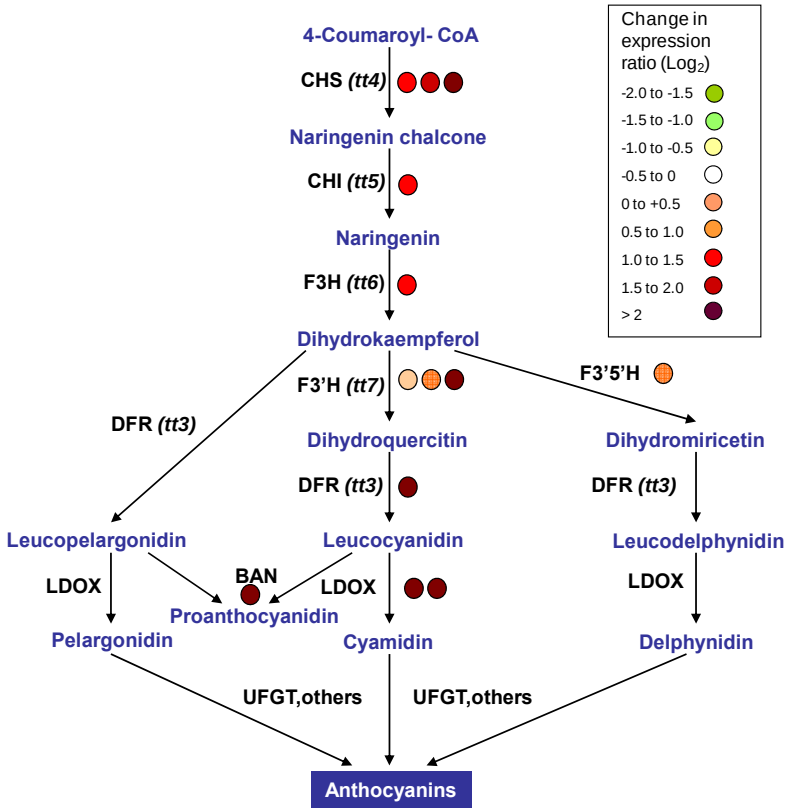


Figure 2.3.3 The effect of higher CO_2 concentration on the anthocyanin biosynthetic pathway. The changes of enzymes involved in the anthocyanin biosynthesis pathway. Genes coding for enzymes in this pathway were represented by the *Populus* gene model. The coloured circles represent the mean Log_2 change expression data for the *Populus* gene model from PICME microarray data. The colour scale corresponds to expression level, with red indicating increased expression in elevated CO_2 and green representing decreased expression.

2.3.2.2 Hormone regulation-Abscisic acid

Hormones are also important regulators in autumnal leaf senescence. *AHK3* (*ARABIDOPSIS HISTIDINE KINASE 3*) which is cytokinin receptor, is up-regulated under e[CO_2] relatively to a[CO_2]and it has been reported to play a major role in cytokinin regulated leaf longevity (Kim *et al.*, 2006).

Although the ABA synthesis genes did not show a consistent up or down expression change under e[CO_2] compared to a [CO_2], the highly expressed ABA signal transduction genes, which are positively related to ABA concentration,

under e[CO_2] relative to a[CO_2], suggesting e[CO_2] induced the ABA concentration during senescence(Table 2.3.2). All the genes except one for GRAM-domain containing protein are up-regulated in e[CO_2] compared to a[CO_2] including three ABA-responsive HVA22 family genes and ABA-responsive element binding protein 3 (Table 2.3.2). ABA has been reported associated with responding to environmental stress and inducing leaf senescence, as well as regulating stomatal closure(Rook *et al.*, 2006). *ABF3* and *ABF4*are involved in ABA-controlled stomatal closure (Kang *et al.*, 2002) and the HVA22 family could inhibit gibberellic acid (GA) induced programme cell death (Guo & Ho, 2008); they all positively response to ABA level change in plant tissues. There is no significant gene expression difference between CO_2 treatments observed in indole-3-acetic acid (IAA), brassinosteroid (BRs), ethylene, JA and GA (Figure 2.3.4).

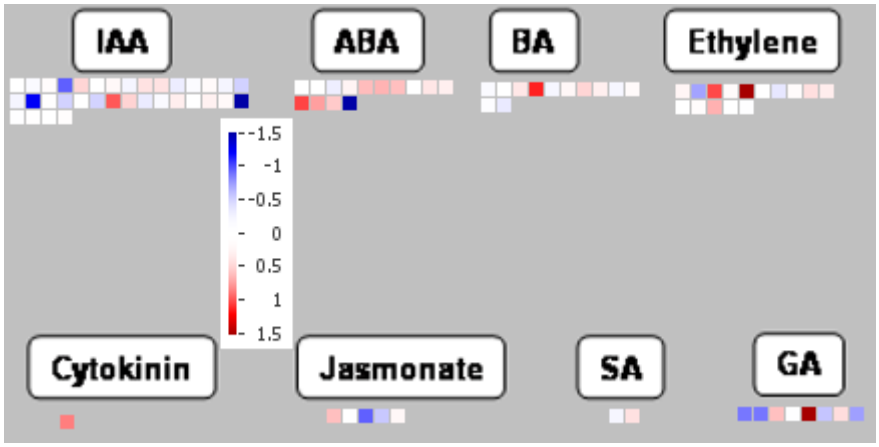


Figure 2.3.4 Transcript expression differences under e[CO_2] relative to a[CO_2] in hormone regulation. Each square represents an *Arabidopsis* orthologue and the colour represents the $\log_2$ average expression under e[CO_2] compared with a[CO_2]. The colour scale bar ranges from dark blue (-1.5 representing ~2.8 fold down-regulation in e[CO_2] relative to a[CO_2]) to dark red (1.5 representing ~2.8 fold up-regulation in e[CO_2] relative to a[CO_2]). (Figure obtained from MapMan).

Table 2.3.2The expression of genes involved in ABA synthesis, degradation, signalling and responsive to ABA under e[CO_2] relatively to a[CO_2]. The gene expression difference between e[CO_2] and a[CO_2]is presented inlog_2transformed form.

Category	AGI number	Gene name	Gene expression
Synthesis-degradation	AT5G67030	ABA DEFICIENT 1 (ABA1). Zeaxanthin epoxidase	-0.03
	AT3G14440	9-CIS-EPOXYCAROTENOID DIOXYGENASE 3 (NCED3)	-0.32
	AT1G52340	ABA DEFICIENT 2 (ABA2); alcohol dehydrogenase	0.25
	AT2G27150	ABSCISIC ALDEHYDE OXIDASE 3 (AAO3)	0.62
Signal transduction	AT3G19290	ABF4 (ABRE BINDING FACTOR 4); Transcription factor	0.66
	AT4G34000	ABF3 (ABA RESPONSIVE ELEMENTS-BINDING FACTOR 3); Transcription factor	0.60
	AT5G20910	zinc finger (C3HC4-type RING finger) family protein	0.03
	AT3G63210	MEDIATOR OF ABA-REGULATED DORMANCY 1 (MARD1)	0.38
Induced-regulated-responsive-activated	AT5G42560	ABA-responsive HVA22 family protein	0.03
	AT1G74520	Arabidopsis ABA responsive protein. ATHVA22A	0.30
	AT5G50720	Arabidopsis ABA responsive protein. ATHVA22E	1.04
	AT3G56850	ABA-RESPONSIVE ELEMENT BINDING PROTEIN 3 (AREB3). Transcription factor	0.74
	AT5G38760	unknown protein	0.55
	AT5G08350	GRAM domain-containing protein / ABA-responsive protein-related	-2.05

2.3.3 Leaf biochemistry – analysis of anthocyanin and sugar content

Anthocyanin and sugar content were measured after microarray data indicated these pathways were important in relation to senescence. Leaves from POP/EUROFACE were collected in August, October and November in 2004. Irrespective of CO_2 treatment, leaf anthocyanin increased over time from late August to mid-November. Moreover, anthocyanin content was significantly increased in e[CO_2] from August to November compared with a[CO2]($F_{4,34}$=3.55, *P*=0.016); this increase was up to 120% greater (Figure 2.3.5)

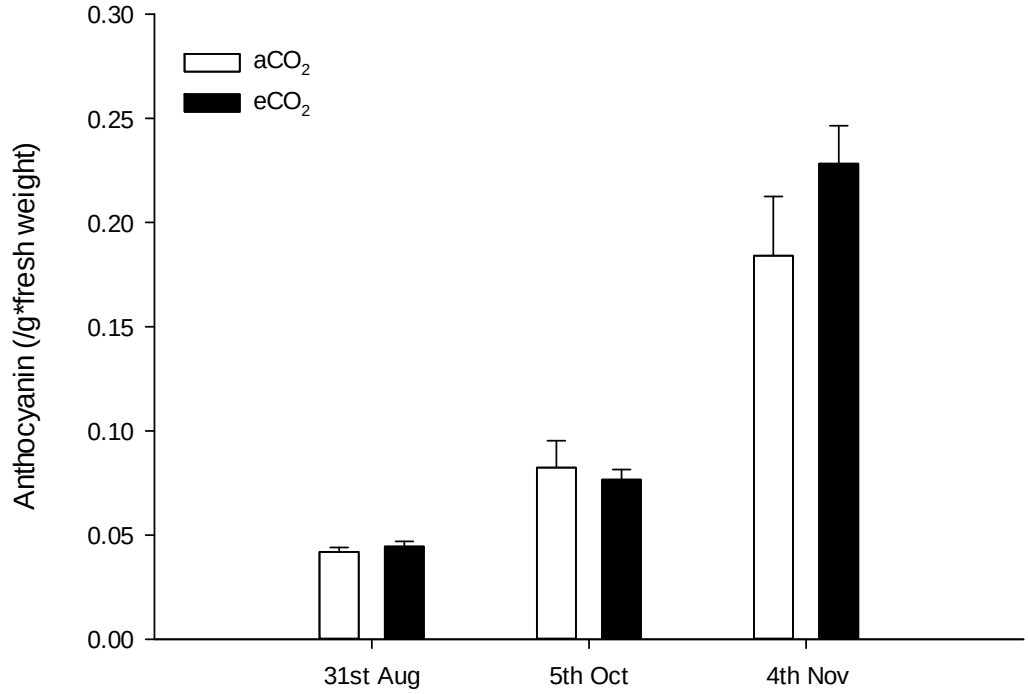


Figure 2.3.5 Anthocyanin content changes during senescence under different CO_2 concentration. Anthocyanin content of leaves sampled from ambient (□) and elevated (■) CO_2 on three occasions during senescence in 2004. Mean data (n = 8 replicates) and standard errors are shown. Four leaves from each plot contributed to the plot average. FW indicates fresh weight.

The measurement of soluble carbohydrates and starch content in senescing leaves also revealed differences between CO_2 treatments (Figure 2.3.6). There was no significant CO_2x time effect detected for carbohydrate content during senescence. However, the sucrose displays a gradual increase which is of greatest significance in August ($F_{1,13}$=9.49,*P*=0.012) and November ($F_{1,15}$=15.91, *P*=0.002) in plants growing in e[CO_2] compared with plants grown in a[CO_2]. Starch content decreased suggesting that metabolism contributed to the energy requirements of the leaf during senescence, and it revealed a sharp degradation in late senescence under a[CO_2]. Glucose and fructose also presented a higher content in plants grown under e[CO_2] at late stage of senescence – November.

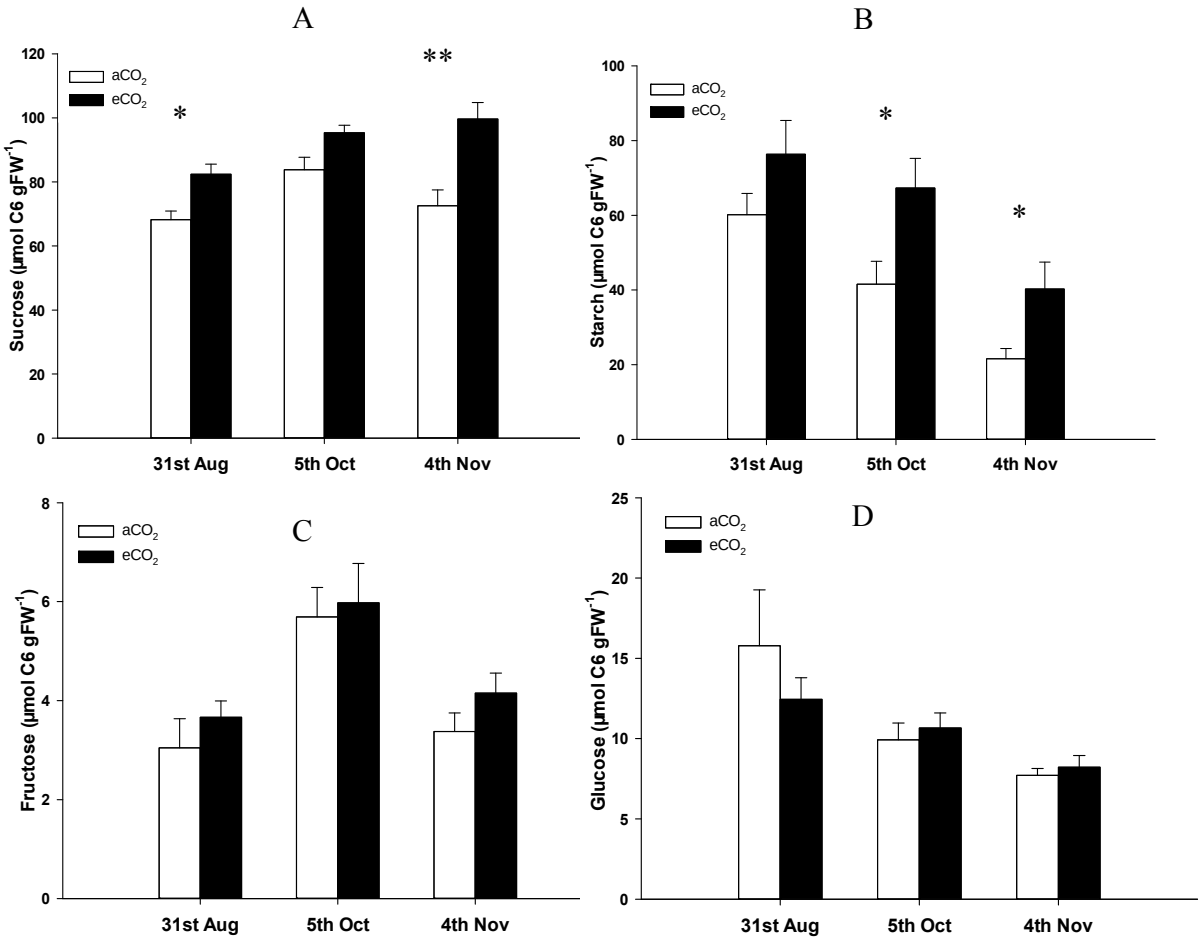


Figure 2.3.6 Carbohydrate contents change during senescence under different CO_2 concentrations. A. Sucrose content of leaves sampled from ambient (□) and elevated (■) [CO_2] on three occasions during senescence in 2004. B. Starch content of the same leaves. C. Fructose content of the same leaves. D. Glucose content of the same leaves. Each point represents four leaves sampled from two plots per treatment and data are shown with standard errors. Significant differences are reported as (* $P \leq 0.05$, ** $P \leq 0.01$). (FW means fresh weight). (Data obtained by Dr A. Rogers).

2.4 Discussion

The PICME microarrays have revealed a global gene picture of natural autumnal senescence in *P. x euramericana* grown in e[CO_2] compared with the same species grown in ambient conditions after many years. The two-fold (or more) differentially expressed genes were clustered majorly in cellular physiological processes and in metabolism. The metabolism was classified as a major functional category of leaf senescence ESTs in *Arabidopsis* (Guo *et al.*, 2004), implying that the changes of genes involved in metabolism and induced by e[CO_2] could be the key factor causing delayed autumnal senescence. The photosynthesis, glycolysis, flavonoid biosynthesis and protein biosynthesis pathways were significantly differentially expressed by e[CO_2] at the late stage of senescence.

The photosynthesis pathway, including the light reaction and Calvin cycle, was significantly highly induced by e[CO_2] in *Populus*, suggested that carbon assimilation was stimulated in comparison with *Populus* grown under a[CO_2]. Thus, assimilation remained higher in ambient compared to elevated conditions during the process of senescence. The highly induced genes expressed in the light reaction under e[CO_2] are surprising since they are the opposite trend of what was observed in other C_3 plants under e[CO_2] (Cseke *et al.*, 2009; Leakey *et al.*, 2009b). However, the down-regulated light reaction genes under e[CO_2] were observed during the active growing season rather than during senescence where it was suggested to be due to feedback regulation of high carbohydrate content or availability of Rubisco. In general, the rate of photosynthesis generally declines during senescence due to the degradation of chlorophyll (Lim *et al.*, 2007). It is therefore likely that in e[CO_2], the higher level of light reaction during senescence might be due to a higher chlorophyll content remaining in the leaves. This has been supported by former lab members who measured the leaves chlorophyll concentration in POPFACE during senescence on 21st Sep and 18th Oct in 2004. They found that the decline of leaf chlorophyll content was significantly reduced in plants grown under e[CO_2] at the senescence stage(Taylor *et al.*, 2008).

Starch was a direct carbohydrate product produced in the Calvin cycle in chloroplasts. The overall increased genes expression in the Calvin cycle under

e[CO_2] induced the higher gene expression in the starch sugar biosynthesis pathway. In addition to the starch synthesis pathway being up-regulated (the genes encoding ADP glucose pyrophosphorylase family protein and starch synthase), the starch degradation pathway (*α-FLUCAN PHOSPHORYLASE 2*, *α-AMYLASE LIKE 3*, *STARCH EXCESS 1* and *DISPROPORTIONATING ENZYMEs*) were also induced by e[CO_2] compared with a[CO_2]. This has been observed in other FACE experiments and it has been suggested that during the night, plants utilise starch until a minimal concentration is reached at dawn, which supports growth metabolism based on source and sink regulation (Smith & Stitt, 2007; Leakey *et al.*, 2009b). Meanwhile, the genes involved in glycolysis and the TCA pathway were also highly activated under e[CO_2] compare with a[CO_2] during senescence, producing ATP energy to support growth and metabolic requirements.

The biochemical analysis also confirmed the up-regulation of genes in the sugar and starch biosynthesis pathway under e[CO_2] at the late senescence stage. Both sucrose and starch were produced significantly more under e[CO_2] and this has generally been seen in many other C_3 plants (Ainsworth & Long, 2005; Cseke *et al.*, 2009; Leakey *et al.*, 2009b). Sucrose content started reducing on 4th November in a[CO_2] whereas in e[CO_2] the sucrose was still increasing at this timepoint. Meanwhile starch reduced much more sharply in a[CO_2] in senescing leaves. There is still an argument uncertainty over sucrose-induced early senescence in plants (van Doorn, 2008). On one hand, the light/dark-induced leaf senescence can be prevented or slowed down by giving additional sugar treatment (Chung *et al.*, 1997; Fujiki *et al.*, 2001). On the other hand, the excess carbohydrate concentration accumulated in plants could induce the leaves yellowing and the expression of SAG12, which is reported induced during *Arabidopsis* leaf senescence (Parrott *et al.*, 2005; Pourtau *et al.*, 2006).

The genes encoding the flavonoid biosynthesis pathway were highly up-regulated during senescence when plants were grown under e[CO_2]. The flavonoid gene family in *Populus* is extensive compared to *Arabidopsis*(Tsai *et al.*, 2006), but the significant difference between multiple copies of genes involved in the flavonoid pathway is still under study (Constabel & Lindroth, 2010). Flavonoids consist of various secondary metabolites such as anthocyanins, which give a deep purple to

red colour to fruits, flowers and leaves, and act as pollination attractants as well as playing important roles in response to biotic and abiotic stress (Stushnoff *et al.*, 2010). The anthocyanin contents were higher in *Populus* growing under e[CO_2], and the increase during senescence were significantly higher in response to e[CO_2] compared with a[CO_2]. This provided strong evidence that anthocyanin plays an important role in protecting plants from oxidative stress, which results in a delayed senescence under e[CO_2].

The increase in gene expression of genes coding different steps in the anthocyanin biosynthesis pathway during senescence also corresponded with another *Arabidopsis* senescence experiment (Buchanan-Wollaston *et al.*, 2005), in which *LDOX* was highly up-regulated in both naturally and dark-induced senescence. Therefore, it is hypothesised that anthocyanin plays dual roles in senescence and both protects senescing tissues from light stress by complementing the chlorophyll role and also reducing oxidative stress by scavenging ROS. The ROS were mainly produced in chloroplasts due to low efficiency of CO_2 fixation, whereby excess radicals from the light reaction were oxidized and became toxic to the plant by injuring cells (Zimmermann & Zentgraf, 2005). This accumulation of ROS could have caused further oxidative stress, resulting in accelerated senescence processes and associated protein degradation. Anthocyanin pigments were reported to play a role in tolerance to stressors, including drought, UV-B, heavy metals, herbivores, pathogens, photo-oxidation, and scavenging free radicals (Gould, 2004), in order to prevent oxidative stress thus preventing the ROS-induced senescence process. Anthocyanin accumulation was reported in sugar maple and it allowed prolonged leaf function and acted to delay leaf senescence (Schaberg *et al.*, 2008). Research on the effects of high [CO_2] on maintaining grape quality also highlighted the concept that anthocyanin preventing the formation of ROS, thus prolonging-low-temperature storage (Romero *et al.*, 2008).

High CO_2 flux leads to a high level of carbohydrate accumulation in leaves, and this excess carbohydrate which is produced under e[CO_2] also leads to enhanced anthocyanin synthesis rather than increased nitrogen metabolism. The *Arabidopsis* mutant *pho3*, which significantly accumulates carbohydrates in the leaves of the plant, induced the expression of genes in the anthocyanin pathway. In particular,

LDOX was up-regulated (190 fold), as well as three transcription factors which regulate anthocyanin biosynthesis (*PAP1*, *PAP2* and *TT8*), suggesting a sink for the excess carbon accumulating in the leaves (Lloyd & Zakhleniuk, 2004). The *MYB75*/*PAP1* is a positive anthocyanin biosynthesis regulator, and was identified as being responsible for QTL, affecting sucrose-induced anthocyanin accumulation (Teng *et al.*, 2005). Tobacco exhibited the same response under 1000 ppm [CO_2] where e[CO_2] caused a shift into secondary metabolite composition and increased pathogen resistance (Matros *et al.*, 2006). Genes involved in the Calvin cycle and glycolysis coincide with increased levels of carbohydrate content and the light reaction produced the necessary energy for prolonged leaf lifespan. This supports the growth-differentiation balance hypothesis, where metabolites are partitioned between growth, storage and defence (Herms & Mattson, 1992). The high carbohydrate concentration in the leaves, induced by e[CO_2], was shifted to anthocyanin production which suggested reducing the ROS damage in leaves. Therefore, the by-product of chlorophyll degradation during senescence process – ROS– is reduced resulting in a prolonged lifespan of leaves.

Hormones have also been reported to interact with sucrose-induced anthocyanin synthesis. GA, ABA and JA were able to enhance the sucrose-dependent expression of *DFR*. In Loreti's experiment (2008),other genes that are involved in the anthocyanin biosynthesis pathway were mainly up-regulated with these hormones present. The *AHK3* which was highly expressed under e[CO_2] is one of three cytokinin receptors in *Arabidopsis*, which have been identified as controlling the cytokinin-regulated-delayed senescence (Kim *et al.*, 2006). The highly expressed ABA signalling genes suggested the ABA concentration were induced by e[CO_2] treatment. This is opposite to what was observed by Teng *et al.* (2006), who found the concentration of ABA is 15.2% less under e[CO_2] when compared to a[CO_2] in *Arabidopsis*. Although the endogenous ABA concentration increase during senescence, and applying ABA treatment could trigger a series of gene expression changes, leading to early leaf senescence(Yang *et al.*, 2003). ABA does not regulate senescence directly but through a group of positive and negative regulators of ABA signalling. The ABA signalling positive regulators, *ABF3* and *ABF4*,regulate the ABA-induced stomatal closure. Overexpression of

mutants of *ABF3* and *ABF4* resulted in lower transpiration rate and enhanced drought tolerance (Kang *et al.*, 2002). The water loss is the key mechanism behind ABA induced early senescence. Zhang *et al.* (2012) found that increase of ABA concentration induces a negative regulator of ABA signalling, *SAG113* (*SENESCENCE ASSOCIATED GENE 113*), which promotes water loss by negatively regulating stomatal movement leading to an early senescence. Under e[CO_2], both *ABF3* and *ABF4* were up-regulated in response to e[CO_2] compared to a[CO_2] in poplar which might diminish the negative effect of increased ABA concentration and function to improve water use efficiency.

From the biochemical analysis, strong evidence was provided that the changes in gene expression here are related to shifts in carbon metabolism; such changes would enable trees of the future to maintain active leaf function for longer during the growing season, with positive effects on carbon balance but potentially negative effects on the development of dormancy.

2.5 Conclusion

The PICME microarray enabled the study of the response of *Populus x euramericana* to elevated [CO_2] (550 ppm) compared with a[CO_2] (~360 ppm) at a late senescence stage. This chapter has identified a consistently up-regulated carbon metabolism pathway under e[CO_2], in which the increased concentrations of sugar, starch and anthocyanin have been verified by biochemical measurement. An activated photosynthesis pathway and TCA cycle under e[CO_2] compared to a[CO_2] indicating that the senescence is delayed under e[CO_2].

The accumulated sucrose concentration under e[CO_2] then triggered anthocyanin regulators such as *PAP1* and *PAP2*, thus inducing the biosynthesis pathway of anthocyanin. The anthocyanin played an important role in scavenging ROS and protecting plants from oxidative stress, therefore delaying senescence in an e[CO_2] environment. Together, with the up-regulated ABA synthesis genes and the positive regulators of ABA signalling, the water use efficiency was improved in poplar grown under e[CO_2] relatively to a[CO_2]. Which might also diminish leaf water loss during senescence therefore delaying the process.

Chapter 3: The transcriptomics of delayed senescence of aspen poplar in a CO_2 - enrichment experiment: AspenFACE

3.0 Overview

The previous chapter hypothesised the mechanism underneath elevated [CO_2]induced delayed autumnal senescence observed in *Populus x euramericana* by studying gene expression differences under e[CO_2] compared to a[CO_2] at a single senescence time point. It illustrated the important relationship between changes in carbohydrate status and anthocyanin content that correlated with delayed autumnal senescence. Here, aspen clone 271(*Populus tremuloides)* which showed the same phenomenon – delayed senescence in e[CO_2] – was studied to understand the regulation by e[CO_2] on senescence from a wider range of time points: mid-growth season (July), onset of senescence (September) and late stage of senescence (October). Affymetrix microarrays were used to identify the key transcripts which affect the trigger of senescence regulated by e[CO_2]. A systematic study presented here on plants grown under different [CO_2] indicates the importance of antioxidant enzymes and antioxidant products induced by e[CO_2], preventing the natural autumnal senescence.

3.1 Introduction

Leaf senescence may be induced by abiotic and biotic environmental factors. Abiotic factors includes drought, nutrient limitation, and oxidative stress, whereas biotic factors includes pathogen infection and shading by other plants (Lim *et al.*, 2007). Plants actively produce ROS as signalling molecules to control processes such as programmed cell death(PCD), abiotic and biotic stress responses and systemic signalling (Mittler, 2002; De Pinto *et al.*, 2012). To protect plants from those highly reactive and toxic enzymes, ROS-scavenging enzymes, including SOD, APX, CAT, GPx and Prx, are produced in cells with highly efficient machinery for detoxifying O_2^- and H_2O_2(Mittler *et al.*, 2004). Flavonoids and anthocyanins were also suggested to play a role of protecting plants against various biotic and abiotic stresses including wounding, pathogen attack, or UV light stress (Pourcel *et al.*, 2007; Constabel & Lindroth, 2010). Together ROS scavenging enzymes and flavonoid products protect plants from oxidative-induced senescence.

Populus is the model tree for understanding unique processes that occur in woody plants compared with *Arabidopsis*, with an advantage of rapid growth and a fully sequenced genome (Taylor, 2002; Jansson & Douglas, 2007). Although there have been some studies on transcriptome level changes of individual tree species' response to e[CO_2] (Gupta *et al.*, 2005; Taylor *et al.*, 2005; Druart *et al.*, 2006; Cseke *et al.*, 2009), the results between species were variable leading to species-dependent reactions to [CO_2] (Cseke *et al.*, 2009). Also, these papers all used the PICME microarray to study the poplar response to e[CO_2]. In this chapter, the technology used was Affymetrix microarrays to identify the key transcriptional responses to different [CO_2]. The Affymetrix microarray contains 61,251 probes (www.affymetrix.com), whereas the PICME cDNA microarray contains only 26,915 probes (www.picme.at) (Street & Tsai, 2010). Thus with more genes identified in plant pathways, a more systemic understanding of transcriptome change in responses to e[CO_2] during delayed autumnal senescence was provided in this study.

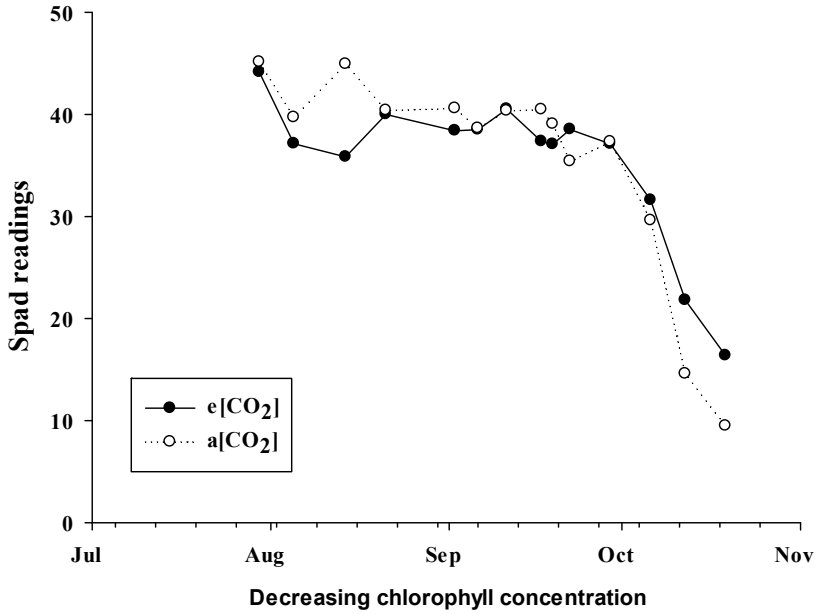
The highly activated carbon metabolism pathway identified in chapter 2 showed a positive regulation of delayed senescence under e[CO_2], in which anthocyanin was suggested to be the key product that reduced oxidative stress with altered carbon metabolism leading to delayed autumnal senescence. However, only one time point was studied at the end of senescence in the last chapter, the full regulation on delayed senescence under e[CO_2] was still unclear. The full transcriptome analysis from the middle of the growing season until late senescence stage will be studied in this chapter using *Populus* which have been grown under different [CO_2] for seven years as part of the AspenFACE experiment.

The AspenFACE facility was located at the United States Department of Agriculture (USDA) Forest Service, Harshaw experimental farm near Rhinelander, WI, USA (89.5°W, 45.7°N) (Gupta *et al.*, 2005) in a continental climate (http://aspenface.mtu.edu/). Four aspen (*P. tremuloides*) clones (clone 8L, 42E, 216, 259 and 271) and birch have been grown exposed to either e[CO_2] (560ppm) or a[CO_2] (~ 360 ppm) since May, 1998 (Oksanen *et al.*, 2004). Many papers have been published on these plants' response to the CO_2, O_3 or a combination of CO_2x O_3 effects from either a morphological or functional aspect (Wustman *et al.*, 2001; Oksanen *et al.*, 2004; Riikonen *et al.*, 2008; Taylor *et al.*, 2008; Cseke *et al.*, 2009). They revealed many leaf physiological changes such as higher photosynthesis, larger leaf area, thicker leaf, higher starch content and even delayed senescence. The plate below was provided by the late Professor David Karnosky as part of the collaboration described here (Figure 3.1.1).

Figure 3.1.1 Example of FACE experiment layout. (Picture was obtained from AspenFACE website, http://aspenface.mtu.edu/index.html).

Aspen clone 271 was chosen out of the other four genotypes due to a suggested sensitive response to [CO_2] with a delayed senescence (Taylor *et al.*, 2008; Cseke *et al.*, 2009). The leaves of aspen clone 271 were collected by our collaborator the late Prof. Dave F. Karnosky's group (Michigan Technological University, MI, US) from AspenFACE on three time points which are 13th July (leaf were mature but not senescing), 21st September (Sept, early senescence stage) and 10th October (Oct)in 2005 (late senescence stage). The leaf senescence stage were determined based on the chlorophyll concentration and photosynthesis data collected in 2004 by our collaborator the late Prof. Dave F. Karnosky's group and part of this data has been published in 2008 (Taylor *et al.*) (Figure 3.1.2).Both chlorophyll concentration and photosynthesis of plants in both e[CO_2] and a[CO_2] started to drop rapidly in late Sept suggesting the beginning of senescence.

a)



b)

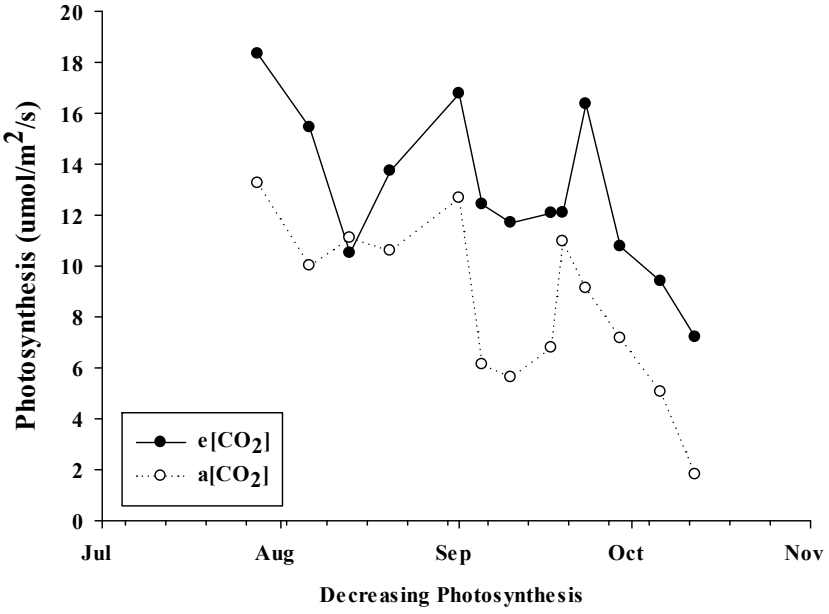


Figure 3.1.2 The chlorophyll concentration and photosynthesis of clone 271 during senescence in 2004. A[CO_2] represented the aspen clone 271 grown under a[CO_2] and e[CO_2]represented the aspen clone 271 grown under e[CO_2] (Data were collected by the late Prof. Dave F. Karnosky's group).

Leaves on one short branch (10-15 leaves) of a randomly chosen tree were sampled together within each a[CO_2] and e[CO_2] ring because all leaves expand at the same time due to short branches having a determinate growth (Cox, 2005). Plant tissue was frozen in liquid nitrogen as soon as they had been harvested and then all samples were delivered to the University of Southampton on dry ice. RNA was extracted by Dr Matthew Tallis from each branch's pooled ground material, and sent to NASC for a quality control test and Affymetrix microarray hybridisation. There were three biological replicate samples from each treatment (2) per time point (3), therefore 18 microarrays in total (Figure 3.1.3).

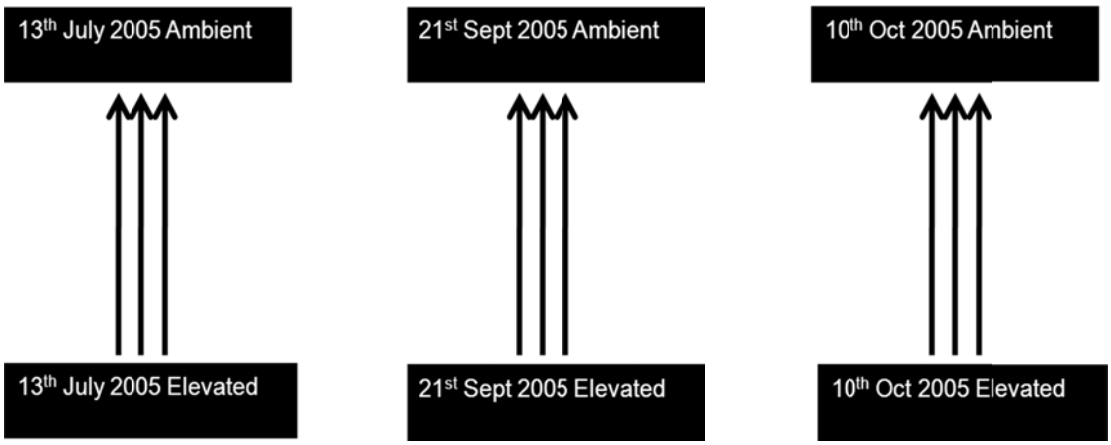


Figure 3.1.3 Affymetrix microarray design model. The arrows between each CO_2 condition represent the direction of normalisation of transcripts expression used in this experiment. There were three replicates in each condition, and each condition at each timepoint contains 10-15 leaves pooled together.

This chapter studied the underlying molecular mechanism of delayed autumnal senescence after exposure to different [CO_2] for seven years; combined with the knowledge of carbon balance between growth, storage and defence from the middle of the growing season until the late stage of senescence.

3.2 Materials and Methods

3.2.1 Analysis and Statistics

The Affymetrix microarray files were analysed using GeneSpring GX (Version 7.3.1, Silicon Genetics, Redwood City, CA). Each file was first normalised using Robust Multi-Array Analysis (RMA) to adjust background, followed by normalisation to median at gene level. The mean gene expression of three biological replicates in e[CO_2] were then normalised by mean gene expression in a[CO_2] for each time point to give the gene fold change under the CO_2 treatment. Filtered gene lists were subjected to a volcano plot with gene specific t-test ($p<0.05$, transcripts expression $Log_2=1$). The volcano plot displayed *loess*-normalized fluorescence intensity ratios from six biological replicates from both ambient and elevated [CO_2] conditions on a two-axis coordinate system. The x-coordinate is the log_2 of the fold change between two [CO_2] and the y-coordinate, corresponding to statistical difference, is the negative log_{10} of the *P* value for the corresponding t-test of differences between two [CO_2](Vermeire *et al.*, 2006).

The Venn diagrams which presented the significantly differentially expressed transcripts in response to e[CO_2] compared with a[CO_2] were formed by GeneSpring GX. All significant transcripts were input into agriGO v 1.2 (http://bioinfo.cau.edu.cn/agriGO/) for GO analysis (Du *et al.*, 2010). The analysis mapped the genes into different GO processes and calculated the significance for each category using the PAGE method. The *P*-value was converted from the *Z*-score, which was calculated from the number of genes mapped to the term and the mean of mapped genes' expression.

The same gene lists were then exported to a text document and the expression data were Log_2 transformed and imported to Mapman (version 3.5.0) for pathway analysis. Candidate gene model name and *Arabidopsis* orthologue gene model was obtained from POParray v1.2 (http://aspendb.uga.edu/poparray) description. The putative function of each gene was obtained from JGI *Populus trichocarpa* v1.1 (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) through the gene

model name. If neither of these sites had annotation information, the putative function was obtained from TAIR (http://www.arabidopsis.org/index.jsp) through *Arabidopsis* homozygous gene names. The hierarchical cluster figures were structured by using Multiexperiment viewer (MeV v 4.8, Dana-Farber cancer institute, USA) (Saeed *et al.*, 2006).

3.2.2 RNA extraction

The ground material of the same samples was used for real time qPCR (RT-qPCR). RNA of the samples used for Affymetrix microarray analysis was extracted using the CTAB protocol modified from Chang *et al.* (1993) and revised by former laboratory member Dr N. Street. 900 µl of pre-warmed (65 ºC) CTAB (2% hexadecyltrimethylammonium bromide (weight (w)/v), 2% polyvinylpyrrolidone (w/v), 100 mM Tris-HCl (hydrochloric acid), 25 mM EDTA Ethylenediaminetetraacetic acid and 2 M NaCl (sodium chloride)) and 50 µl β-mercaptoethanol was added to each Eppendorf tube, and tubes were incubated at 65 ºC for 5 minutes. After incubation, equal volume of (950µl) CHISAM (Chloroform/Isoamyl alcohol) was added into each tube and tubes were centrifuged for 10 minutes at 12x g (gravity). The upper layer (aqueous phase) was transferred to a new tube and 280 µl of 10M lithium chloride was added. After incubation at 4 ºC for 30 minutes, the samples were centrifuged for 15 minutes at 12x g at 4 ºC to form a pellet. After remove the supernatant, he pellet was then dissolved in 700 µl of SSTE (11.7g 1M NaCl (w/v), 0.5% SDS, 10mM Tris-HCl (pH 8.0) and 1mM EDTA), which was prewarmed to 60 ºC, and incubated at 60 ºC for 5minutes. 700 µl CHISAM were added afterward and centrifuged at 12x g for 10 minutes at room temperature. The upper layer was transferred to a fresh Eppendorf and 700 µl of 100% ethanol was added. The tube were incubated at -20ºC for 10 minutes and then centrifuged for 10 minutes at 4ºC. The liquid phase was discarded and the pellet was washed with 1 ml cold 70% ethanol. The samples were centrifuged again and the remaining liquid was discarded. The pellet was air-dried, then resuspended in 20 µl DEPC (diethylpyrocarbonate) treated water. The RNA concentration was measured using

the NanoDrop spectrophotometer (ND100, NanoDrop Technologies, Delaware, USA).

3.2.3 RT-qPCR

The reverse transcription of RNA to cDNA was performed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, USA) following the manufacturer's instructions. 1000ng of RNA was added to 4 µl 5x iScript select reaction mix, 1 µl primer, 2 µl GSP enhancer solution,1 µl iScript reverse transcriptase and nuclease-free water to add up to a 20 µl reaction system. The mixes were then incubated at 42 ºC for 30-60 minutes and the reaction terminated by incubating at 85 ºC for 5mins. The cDNA was stored at -20 ºC.

Genes that were significantly differentially expressed in response to [CO_2] as well as involved in senescence regulation were selected for the RT-qPCR to approve the Affymetrix microarray result. The full genomic sequences of these genes were obtained from JGI website (http://genome.jgi.doe.gov/Poptr1_1/Poptr1_1.info.html). The primers were designed using primer premier 5 (PREMIER Biosoft, CA, USA) with the requirement of product size ranging 150-250 base pairs (bp), more than 50% GC primer content and around 58 ºC annealing temperature (T_m). The reference genes used were *UBIQUITIN-CONJUGATING ENZYME E2*, *CYCLOPHILIN* and *ACTIN* which has been suggested by Cseke *et al.* (2009) for these particular *Populus* genotypes.

Table 3.2.1 The designed primers for each candidate genes.

Gene name	Forward primer (5' to 3')	Reverse primer (5' to 3')
Trx	GTTCTCCGTAACACTCCCGC	TTCAAAGCACAATCCACCCT
Gpx	GGGGTCTTCTCCATCGGTA	GCTTGTCCCAGGCTCTTG
CHS	CGAGAAGCCTTTATTTGAGC	GTTCCAGTCCGAGATGCC
PAL	GCTTCAGGTGATTTAGTCCCATT	TCCATTAACAAGAGCGAGGC
WRKY 6	AGGTAGAACGAATGAAAGTGGAG	CCATACACTGCCTTGGAACTA
WRKY 75	AGCCCAAGTATGCTTTTCAA	CGTCACTACAACACCTTCGTCT

The RT-qPCR was performed using Precision 2x qPCR Mastermix (Primerdesign Ltd, UK). 10 µl Precision 2x qPCR Mastermix, 1 µl primer mix, 5 µl cDNA samples and 4 µl RNAse free water were added to each reaction well on PCR plate. Plates were run on a Chrom4 Real-Time PCR Detection System (Bio-rad Laboratories, Hercules, USA). The reactions were incubated at 95 ºC for 10 minutes followed by 50 cycles of 95 ºC for 15 seconds,60 ºC for 60 seconds and 72 ºCfor 15 seconds. The melting curve was then performed from 60 ºC to 95 ºC with a read every 0.2 ºC and 1 second hold in order to check the RT-qPCR quality. Values were exported from the software Opticon Monitor 3.1 (Bio-Rad Laboratories, Hercules, USA).Amplification efficiency was measured (Livak & Schmittgen, 2001). Ratios were calculated as $2^{-[(e[CO2]Cttarget - e[CO2]Ctreference) - (a[CO2]Cttarget - a[CO2]Ctreference)]}$ in which the Ct is the fluorescence threshold.

3.3 Results

3.3.1 Affymetrix microarray

The transcriptome response during senescence under elevated [CO_2] compared with ambient [CO_2] was analysed with the poplar Affymetrix array (See Appendix V for gene list). The overall expression of all transcripts under the two [CO_2] is shown in (Figure 3.3.1.a). The transcript differences were not dramatically different between the two CO_2 treatments – a finding in agreement with previous microarray studies following long-term exposure to e[CO_2](Gupta *et al.*, 2005; Taylor *et al.*, 2005; Ainsworth *et al.*, 2006). In general, it would appear that few large-scale changes in gene expression occur when plants are exposed to elevated CO_2, rather there are many small changes that are easily observed using microarrays. This was demonstrated by Taylor *et al.* (2005), who revealed that single transcripts such as Rubisco did change in expression, but only by small amounts of approximately 10%. This chapter will focus on these small but important differences induced by different [CO_2] regarding to the delayed senescence phenomenon under e[CO_2].

This experiment detected 1639, 1667 and 2036 transcripts significantly ($p \leq 0.05$) differential expressed in July, Sept and Oct, respectively, out of the 61,419 probe sets in the Affymetrix poplar microarray in elevated versus ambient [CO_2]. Venn diagrams were used to compare the percentage of common transcripts which were significantly differentially expressed under CO_2 treatments between each timepoint (Figure 3.3.1.b). The common transcripts between each timepoint included a thioredoxin family protein, Cinnamoyl CoA reductase (CCR), SAUR (small auxin up RNA) auxin-response protein and a variety of protein kinases involved in plant biological processes.

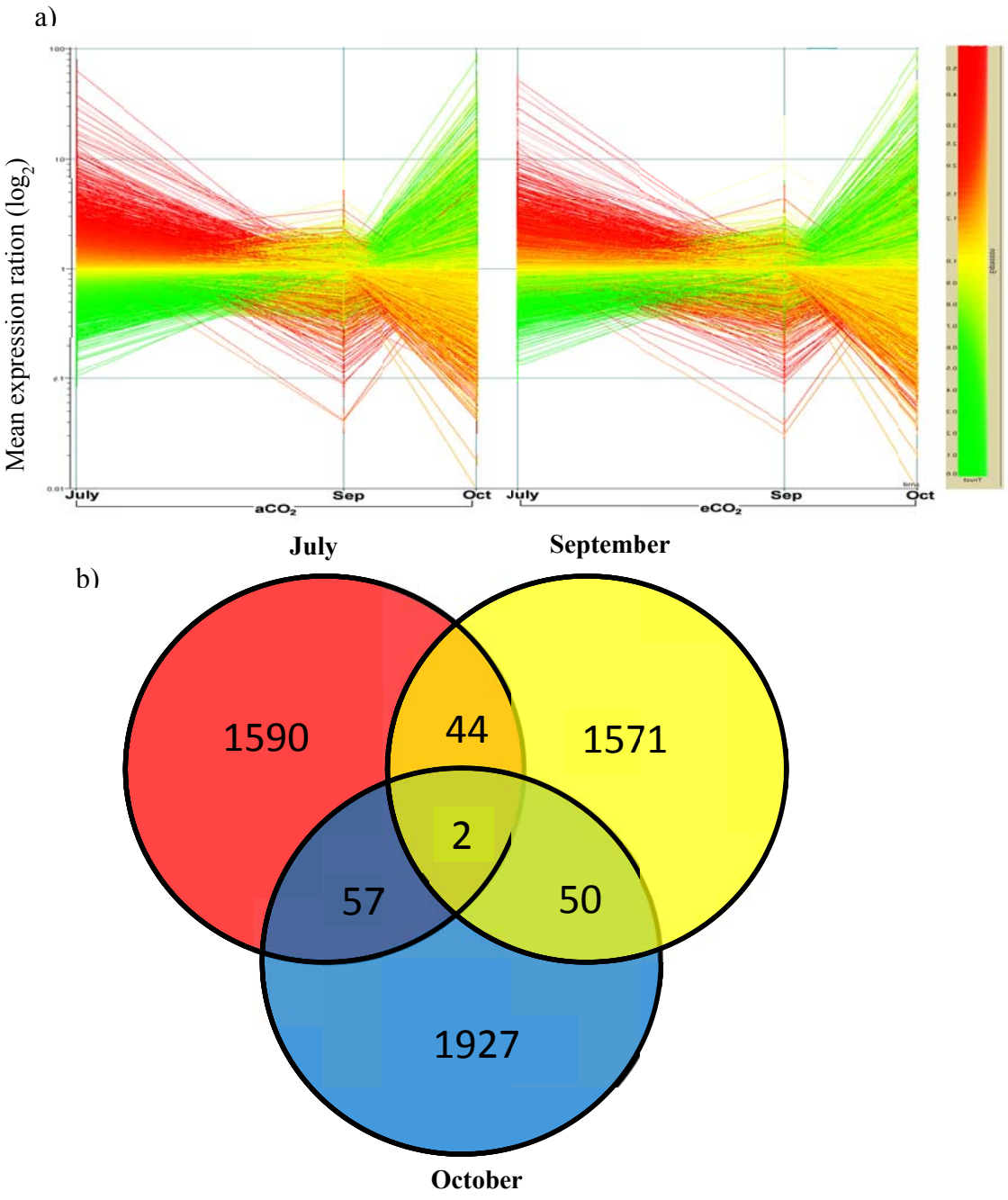


Figure 3.3.1 The transcripts expression change during poplar senescence under a[CO2] (360ppm) and e[CO2] (560ppm) and the Venn diagram. a) The transcripts expression change. The samples for the microarray analysis were taken in July, Sept and Oct, 2005. The expression of e[CO2] normalised by a[CO2] were presented on log scale on y axis, and each line represented one probe on the array. The colour indicated the transcription expression, red represents high expression, whereas the green represents low and yellow represents expression equal to two (Log ratio equals to 1).b) The Venn diagram of transcripts significantly changed in e[CO_2] during growth. The number on the Venn diagram represents the transcripts significantly differentially expressed in response to e[CO2]compared to a[CO2] in July, Sep and Oct.

3.3.2 Transcripts which differently expressed between CO_2 treatment at each time point

The significantly differentially expressed gene lists were also grouped by GO term to identify categories of genes in response to e[CO_2] at each timepoint. The gene list in Sept did not show any clear GO pathway in biological process. In July (middle growth season), cell wall organization, nitrogen compound metabolic process, regulation of metabolic process and regulation of cellular metabolic process were all significantly down-regulated by e[CO_2] compared to a[CO_2] (Figure 3.3.2.a). However, in October (late senescence), the regulation of metabolic process and the following GO term were all up-regulated by e[CO_2] relatively to a[CO_2], while cell wall organization or biogenesis, cellular component biogenesis, secondary metabolism and cellular metabolism were all lower expressed under e[CO_2] compared to a[CO_2] (Figure 3.3.2.b).

a)

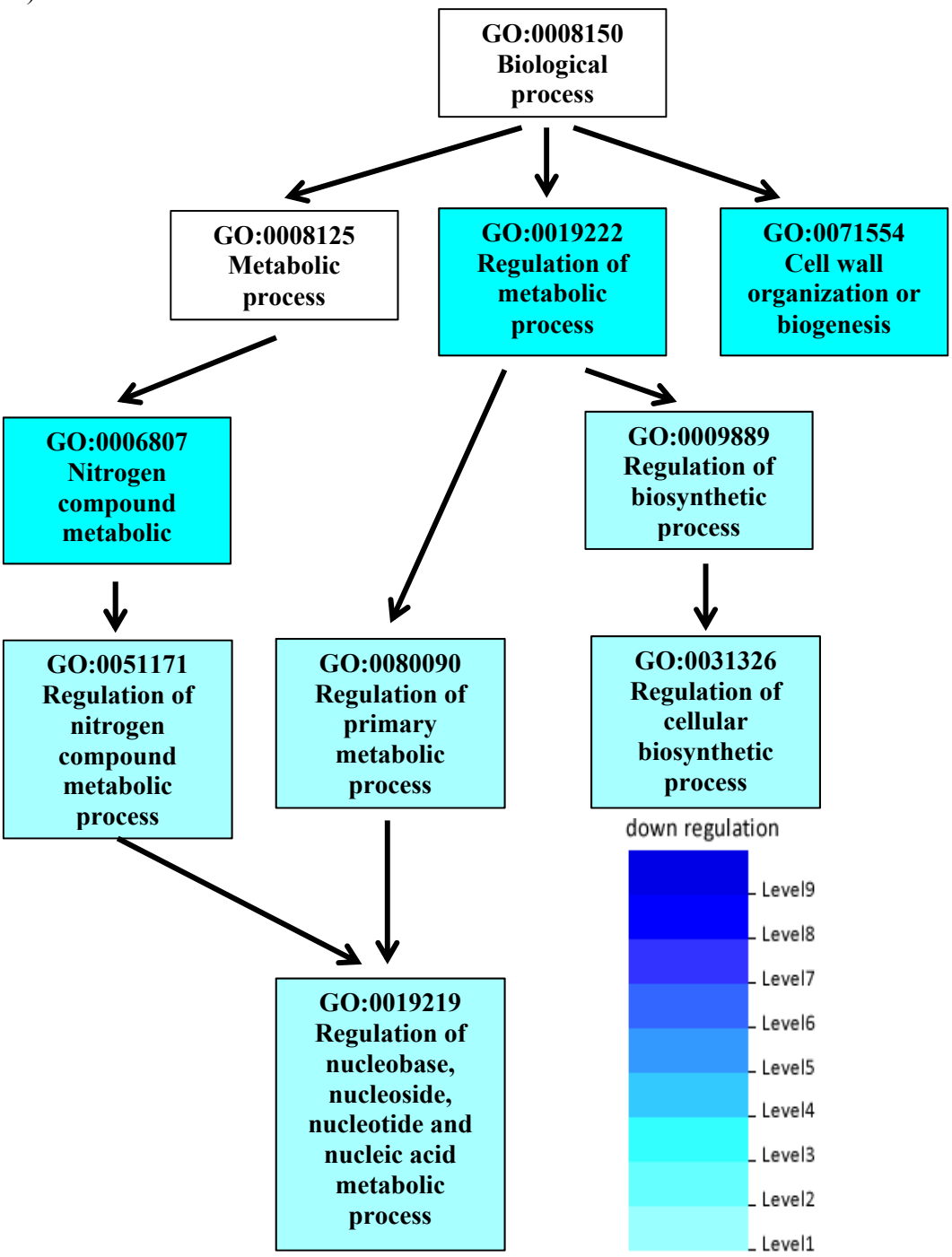


Figure 3.3.2 Hierarchical tree graph of GO terms in biological process category in response to e[CO2] compare with a[CO2]. a) GO tree with significant differentially expressed transcripts in July. b) GO tree with significant differentially expressed transcripts in Oct. The box with colour present GO terms were significantly different ($p \leq 0.05$) in e[CO_2].

b)

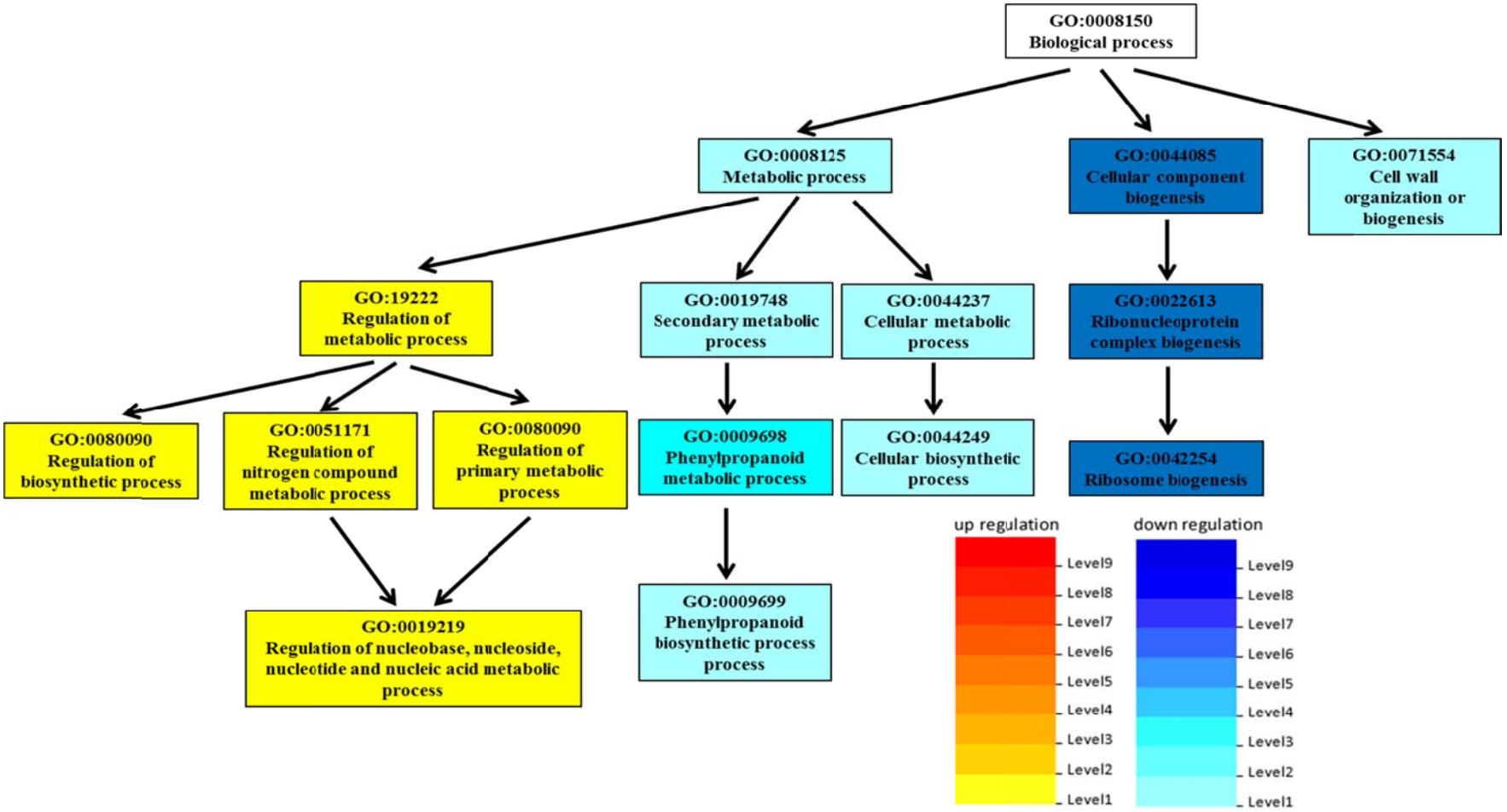


Figure 3.3.2 Continued.

The significantly differentially expressed transcripts were input into MapMan for functional group analysis at each time point. The significant biological pathway identified in response to e[CO_2] were very different between July and Oct (Table 3.3.1). Both the Mapman Wilcoxon rank sum test and hierarchical tree of GO term highlighted the primary metabolism pathway in July and Protein and secondary metabolism in Oct that significantly differentially expressed under e[CO_2] compared to a[CO_2].

Table 3.3.1 Significantly differentially induced by e[CO_2] at three time point.

Time point	Bin	Functional group	Transcripts number	P-value
July	30.1	Signalling. In sugar and nutrient physiology	8	0.012
	20.1	Stress biotic	34	0.0293
Sept	29.2	Protein synthesis	18	0.0254
Oct	29.5	Protein degradation	72	0.0288
	16.2	Secondary metabolism. Phenylpropanoids	8	0.0177
	27.3	RNA. Transcription factors	78	0.0194
	30.5	Signalling. G-proteins	15	0.0382

In July, two functional groups were identified as differentially (down-regulated) regulated by e[CO_2] compared with a[CO_2] which are the ion channel glutamate receptors(GLR) (signalling in sugar and nutrient physiology) and genes involved in response to biotic stress including *CHITINASE CLASS IV*, *PHYTOALEXIN DIFFICIENT 4*, *ROOT HAIR DEFECTIVE 2*, *MILDEW RESISTANCE LOCUS O 12*, and other genes encoding disease resistance proteins, leucine-rich family proteins and pathogenesis-related thaumatin family proteins. The glutamate receptors can be negatively regulated by sucrose concentration and positively affected by nitrogen (N) concentration and induce ABA synthesis which will then trigger the ABA sensing genes, leading to regulation on germination, root growth and stomatal opening (Schroeder *et al.*, 2001; Kang *et al.*, 2002). The down-regulated GLRs could be the result of increased sucrose concentration under e[CO_2] as measured by Cseke *et al.* (2009). The down-regulated stress-related

genes under e[CO_2] compared to a[CO_2] suggest plants which are grown in a[CO_2] showed a better pathogen and stress resistance compared to the plants grown in e[CO_2] in July.

In September, there was only one functional group significantly differentially regulated by e[CO_2] compared to a[CO_2],which is protein synthesis, where, the transcripts encoding ribosomal proteins were down-regulated and the eukaryotic translation initiation factors (EIFs) (except EIF4F), a translation release factor and two genes which encode novel cap-binding protein and elongation factor family protein, respectively, were up-regulated by e[CO_2].The EIFs are essential for initiating the RNA translation to protein process and ribosomes are where the translation occurs(Jackson *et al.*, 2010).It seems that more protein was synthesised and less ribosomes were formed under e[CO_2] compared to a[CO_2].

There were more functional groups that showed significantly different regulation by e[CO_2] compared to a[CO_2]with abundant transcripts in October (late senescence) compared with July and September. Most of the transcripts involved in protein degradation and transcription factors were up-regulated by e[CO_2] relatively to a[CO_2]. However, the assumption of protein degradation and RNA transcription were induced by e[CO_2] during senescence cannot be made. The transcripts involved in phenylpropanoids synthesis including the anthocyanin and lignin biosynthesis pathway were down-regulated under e[CO_2] compared to a[CO_2] at late senescence stage. This is the opposite to the observations made in POPFACE (Chapter 2) where secondary metabolism was up-regulated in response to e[CO_2], during the process of senescence. This might be induced by different climate environment they were growing in or Aspen clone 271 and *P. x euramericana* might have different carbon utilization strategies, particularly with respect to the senescence process in elevated CO_2, despite the fact that senescence was delayed in both FACE experiments. The guanine nucleotide-binding proteins (G proteins), which is the signal transducing molecules in cells, were mainly up-regulated under e[CO_2] compared with a[CO_2]. The highly induced abundant protein, RNA transcripts and activated G-proteins indicated that plants grown under e[CO_2] were at an advanced stage of senescence compared with plants grown under a[CO_2] at late senescence stage. Other functional groups which have

been suggested to be involved in growth regulation induced by CO_2 treatment were also studied in this chapter.

3.3.2.1 The differentially expressed pathways between CO_2 treatments in July

The transcripts involved in light reaction- photosystem centre subunit I (PS I) and PS II were down-regulated during the growing season under e[CO_2], and a similar finding was apparent for photorespiration (Figure 3.3.3.a). However, the transcripts involved in sucrose and starch degradation, as well as glycolysis, were up-regulated by e[CO_2] during this part of the growing season, suggesting a highly activated sugar and starch metabolism under e[CO_2]. The transcripts involved in the TCA cycle were down-regulated by e[CO_2], whereas the transcripts involved in oxidative phosphorylation on the inner mitochondrial membrane were slightly up-regulated compared to a[CO_2]. The electron fluxes in oxidative phosphorylation were positively correlated to the production of energy (ATP), therefore, although the TCA cycle was less activated in plants grown under e[CO_2], there might still be more energy produced during the middle of the growth season. The transcripts involved in phospholipid synthesis were down-regulated in response to e[CO_2]. The *XYLOGLUCAN ENDOTRANSGLYCOSYLASE 6* (*XET 6*) transcripts were down-regulated in e[CO_2] in July but the precursor synthesis was slightly up-regulated compared to the a[CO_2]. In secondary metabolism, CCR involved in flavonoid synthesis and transcripts involved in terpenoides were also increased in e[CO_2] relative to ambient conditions, whereas the transcript ligase 14 involved in the lignin biosynthesis pathway was down-regulated as well as the transcripts involved in the phenylpropanoids biosynthesis pathway compared to the a[CO_2].

Plants grown under e[CO_2] showed less pathogen resistance protein transcripts in July implying aspen clone 271 did not take action in the pathogen defence system compared to the plants grown under a[CO_2] during growth (Figure 3.3.3.b).
The transcripts involved in hormone regulation and response were presented in figure 3.3.3.c. The transcripts encoding auxin-responsive family proteins were

mainly down-regulated except one under e[CO_2] in July. The 1-aminocyclopropane-1-carboxylate (ACC) synthase 1 which is involved in ethylene biosynthesis was down-regulated by e[CO_2] compare with a[CO_2]. However, the ACC oxidases (ACO) were all up-regulated under the same condition. The cytokinin receptor (histidine kinase 3) were also up-regulated in e[CO_2]. The AHK3 was reported to mediate the ethylene-induced leaf longevity by phosphorylation of arr2 which encodes an *Arabidopsis* response regulator protein (Kim *et al.*, 2006). The WRKY transcription factor family, which contains a conserved 60 amino acid DNA-binding domain and a zinc-finger motif, was reported to be involved in the regulation of leaf senescence (Besseau *et al.*, 2012). Under e[CO_2], WRKY 33, 40 and 58 were all found to be down-regulated at the July sampling point.

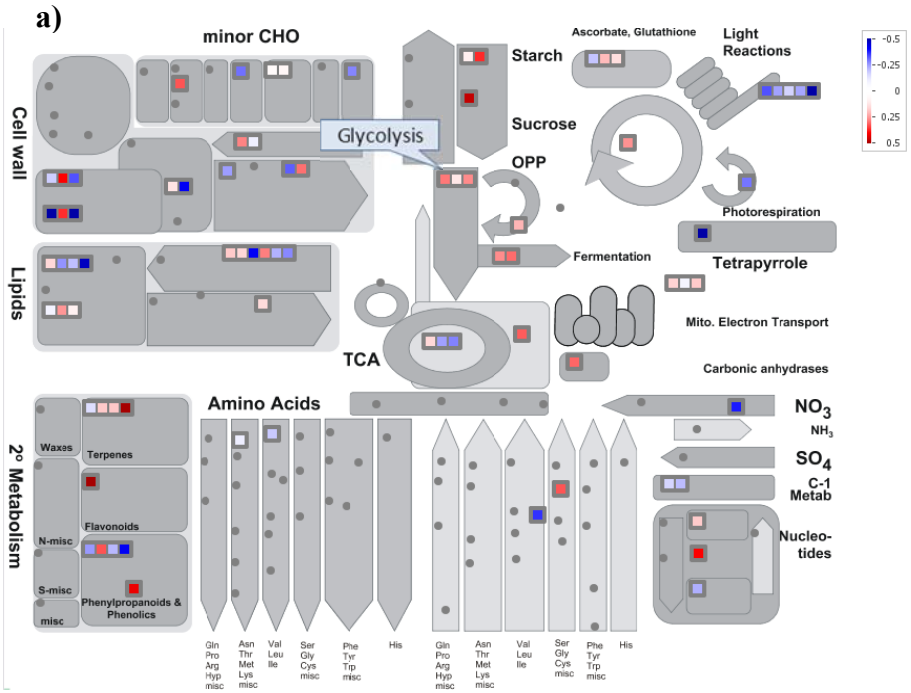
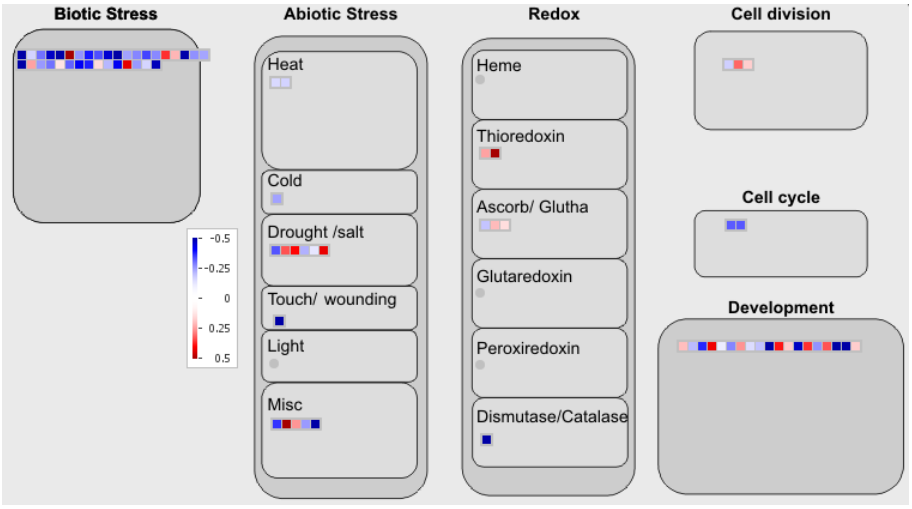


Figure 3.3.3 Mapman map with transcripts significantly influenced by e[CO_2] relative to a[CO_2] at 13th July 2005. a) the metabolism overview map. b) the cellular response overview map. c) the WRKY transcription factors. Each square represents a unique transcript Affymetrix number. The colour of square represents the log fold change of each transcript. Red represents up-regulation by e[CO_2], whereas blue represents down-regulation by e[CO_2]. The scale of gene expression was the same for each figure.
b)



c)

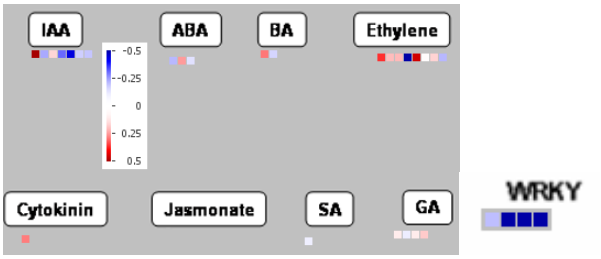


Figure 3.3.3Continued.

3.3.2.2 The differentially expressed pathways between CO_2 treatments in September

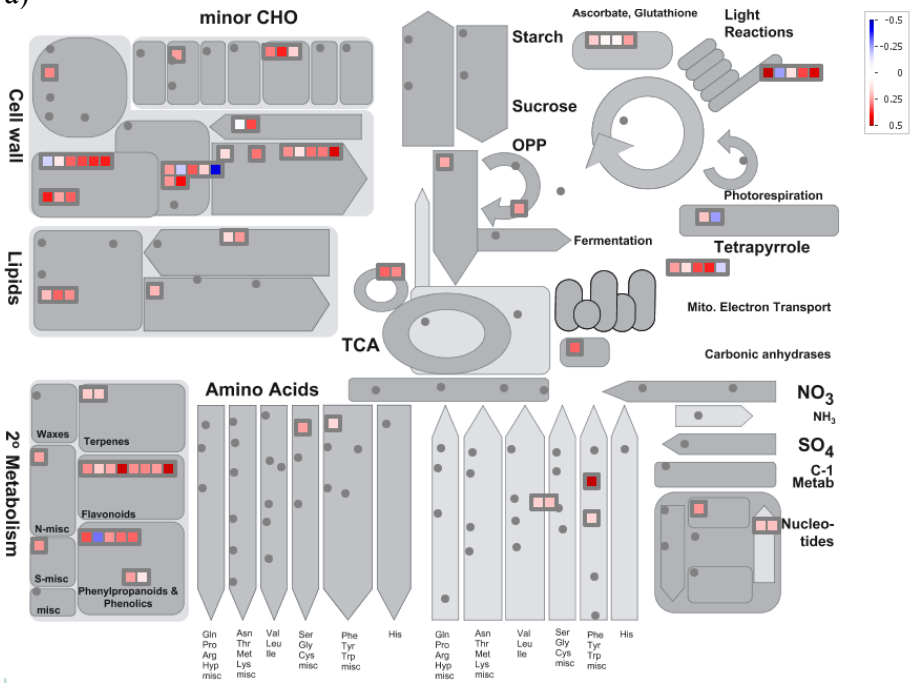
The transcripts identified that were significantly differentially expressed under the two CO_2 treatments at the onset of senescence (September) were mainly up-regulated (1367 out of 1667 transcripts) (Figure 3.3.4), giving support to the notion that senescence is an active process involving re-modelling of the transcriptome. Genes controlling photosynthesis and the TCA cycle were all highly expressed in e[CO_2], compared to ambient conditions, suggesting that autumnal senescence was indeed delayed, as supported by phenotype data of photosynthesis(Taylor *et al.*, 2008). However, there were no transcripts in the sugar and starch synthesis pathways or degradation pathway that showed significant differential expression in response to e[CO_2] in this experiment. Only phosphenolpyruvate carboxylase, which catalyses the reaction of phosphate + oxaloacetate = H_2O + phosphoenolpyruvate + CO_2, was identified in the

glycolysis pathway. The transcripts involved in fatty acid synthesis and elongation, metabolism and degradation all highly expressed under e[CO_2]. Similar responses were also found in the cell wall function group, including pectin methylesterases, glycoside hydrolases, cellulose synthases, and transcripts involved in cell wall degradation. The transcripts involved in secondary metabolism pathways including phenylpropanoids, lignin and flavonoids were up-regulated under e[CO_2] compared to a[CO_2].This pattern of gene expression is similar in many respects to that observed in the POPFACE experiment and suggests that the moderate senescence found in September in northern USA was similar physiologically, to that observed in Italy in late October/November.

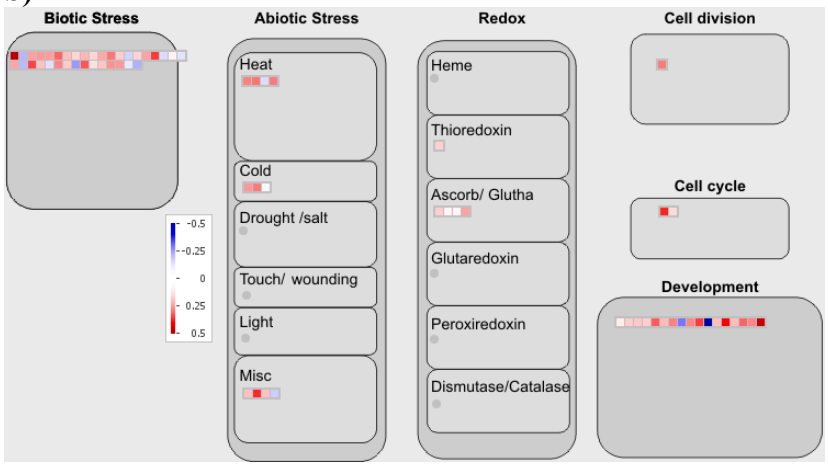
The disease resistance proteins, heat shock proteins and universal stress proteins were all down-regulated under e[CO_2]. The cell division and cell cycle transcripts both showed up-regulation as well as the transcripts involved in development. This up-regulation in response to e[CO_2] suggests that plants grown under e[CO_2] trigger the transcripts involved in growth development and stress resistance system at the onset of the senescence which might be the key regulation timepoint for the high [CO_2]- induced late senescence.

The hormone transcripts were also up-regulated under e[CO_2] including auxin, ABA, ethylene, cytokinin, salicylic acid (SA) and GA. The WRKY transcription factors were induced by e[CO_2] including WRKY 4, 6, 32, 47 and 56, different to those expressed in July.

a)



b)



c)

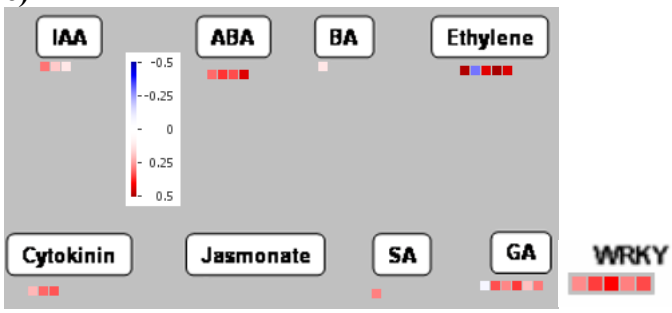


Figure 3.3.4 Mapman map with transcripts significantly influenced by e[CO2] relative to a[CO2] at 21st Sept 2005.a) The metabolism overview map. b) The cellular response overview map. c) The hormone regulation and WRKY transcription factors. Each square represents a unique transcript Affymetrix number. The colour of square represents the log fold change of each transcript. Red represents up-regulation by e[CO_2], whereas blue represents down-regulation by e[CO_2]. The scale of gene expression was the same for each figure.

3.3.2.3 The differentially expressed pathways between CO_2 treatments in October

The transcripts which were significantly differentially expressed in response to e[CO_2] compared with a[CO_2] at the late stage of senescence which showed a completely different trend to that identified in September (Figure 3.3.5), and to that observed in November in the POPFACE experiment in Italy (Chapter 2) on aspect of sucrose and starch synthesis, glycolysis, phenylpropanoids synthesis and flavonoid synthesis. The transcripts to synthesise the light harvesting complex of PSII were down-regulated under e[CO_2], whereas the two transcripts encoding PS II reaction centre protein B and one transcript responsive to electron carrier activity were up-regulated under e[CO_2], suggesting that less light photons were required during photosynthesis but higher NADPH was produced under e[CO_2] compared to a[CO_2]. The phosphatase/fructose-1,6-bisphosphatase in the Calvin cycle were also induced by e[CO_2] at the late senescence stage. Sucrose phosphatase 1, involved in sucrose synthesis, was decreased when exposed to e[CO_2] as well as the mitochondrial ATP/ADP transporter 2 involved in starch biosynthesis. However, the transcripts involved in degradation of sucrose and starch for the glycolysis pathway were up-regulated under e[CO_2] at the late senescence stage, and the sucrose were degraded to fructose rather than UDP-Glucose (uridine diphosphate glucose)according to the two up-regulated transcripts encoding β-fructofuranosidase and one down-regulated transcript encoding the UDP-glycosyltransferase regulated by e[CO_2]. Phospholipid synthesis and lipid metabolism were still up-regulated under e[CO_2] at late senescence, whereas the fatty acid synthesis and elongation were down-regulated as well as lipid degradation. The cell wall showed an interesting response to e[CO_2] at this very late phase of senescence. The cell wall precursor synthesis was up-regulated whereas other modification including pectin methylesterases, expansin, cellulose synthase and transcripts involved in cell wall degradation were down-regulated in response to e[CO_2] compared to a[CO_2]. The transcripts involved in secondary metabolism were also down-regulated in response to e[CO_2] especially in the phenylpropanoid and flavonoid biosynthesis pathways.

The transcripts involved in biotic and abiotic stress response were also mainly down-regulated in response to e[CO_2], except a few disease-resistance proteins and a few heat shock proteins.

The ABA synthesis and ABA-responsive proteins were significantly down-regulated by e[CO_2]. The transcripts involved in ethylene synthesis, signal transduction and responsive proteins, as well as GA signal transduction were also down-regulated in response to e[CO_2] at late senescence stage. The *DEFECTIVE ANTHER DEHISCENCE 1* which regulated the JA synthesis is up-regulated by e[CO_2] at late senescence stage. There were only two WRKY transcription factors showing a significant response to e[CO_2], which were *WRKY50* and *WRKY75*.

a)

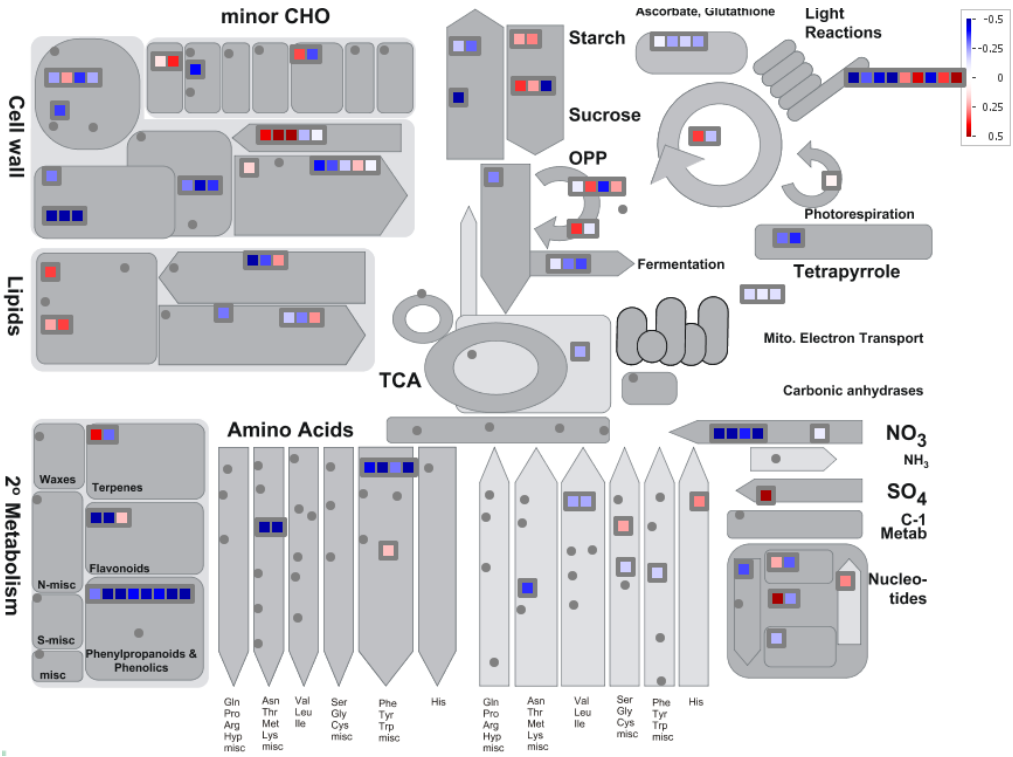
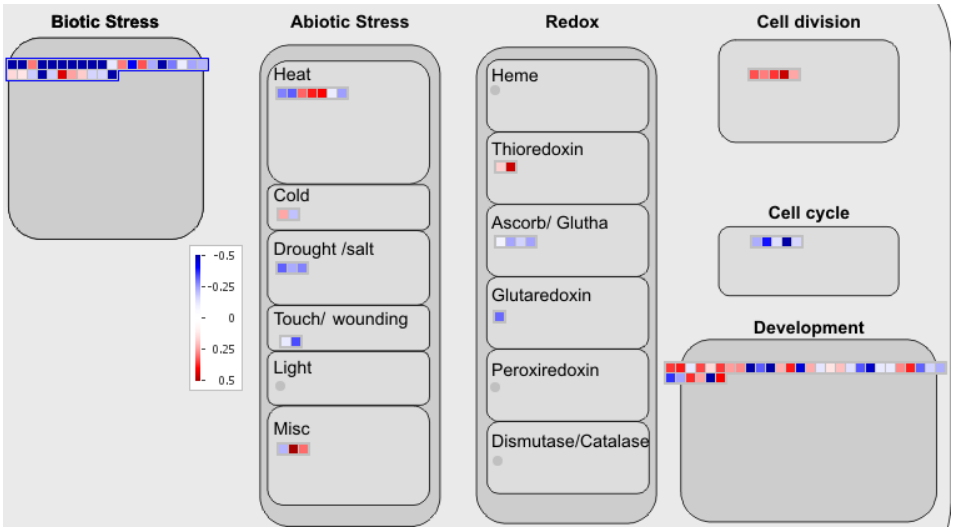


Figure 3.3.5 Mapman map with transcripts significantly influenced by e[CO_2] relative to a[CO_2] at 10th Oct 2005.a) The metabolism overview map. b) The cellular response overview map. c) The hormone regulation and WRKY transcription factors. Each square represents a unique transcript Affymetrix number. The colour of square represents the log fold change of each transcript. Red represents up-regulation by e[CO_2], whereas blue represents down-regulation by e[CO_2]. The scale of gene expression is presented on each figure.

b)



c)

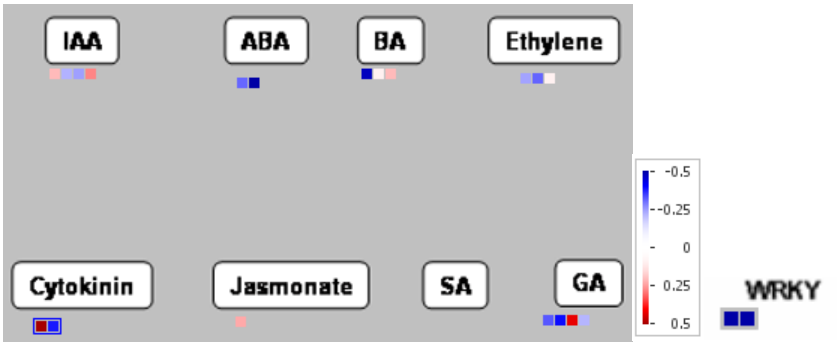


Figure 3.3.5 Continued.

3.3.3 The change of differentially expressed transcripts between CO_2 treatments in July, Sept and Oct

The overall expression change of transcripts that were two-fold difference under e[CO_2] relatively to a[CO_2] at any single timepoint were studied in this section to compare the different growth stage or strategy between plants grown under e[CO_2] and a[CO_2].The transcript hierarchical cluster presented 603 different transcripts expression pattern from the middle growth season until the late senescence stage (Figure 3.3.6). Most of the transcripts expression changes from July until October of plants grown under e[CO_2] were different to the transcripts in plants grown under a[CO_2]. This might be a consequence of delayed autumnal senescence

observed in plants grown under e[CO_2]. The transcripts involved in photosynthesis, sucrose and starch metabolism, the flavonoid biosynthesis pathway, cell wall formation and degradation and WRKY family were studied in the following section allowing the study of potential different carbon allocation in clone 271 when grown under e[CO_2] and a[CO_2] and the mechanism of delayed senescence.

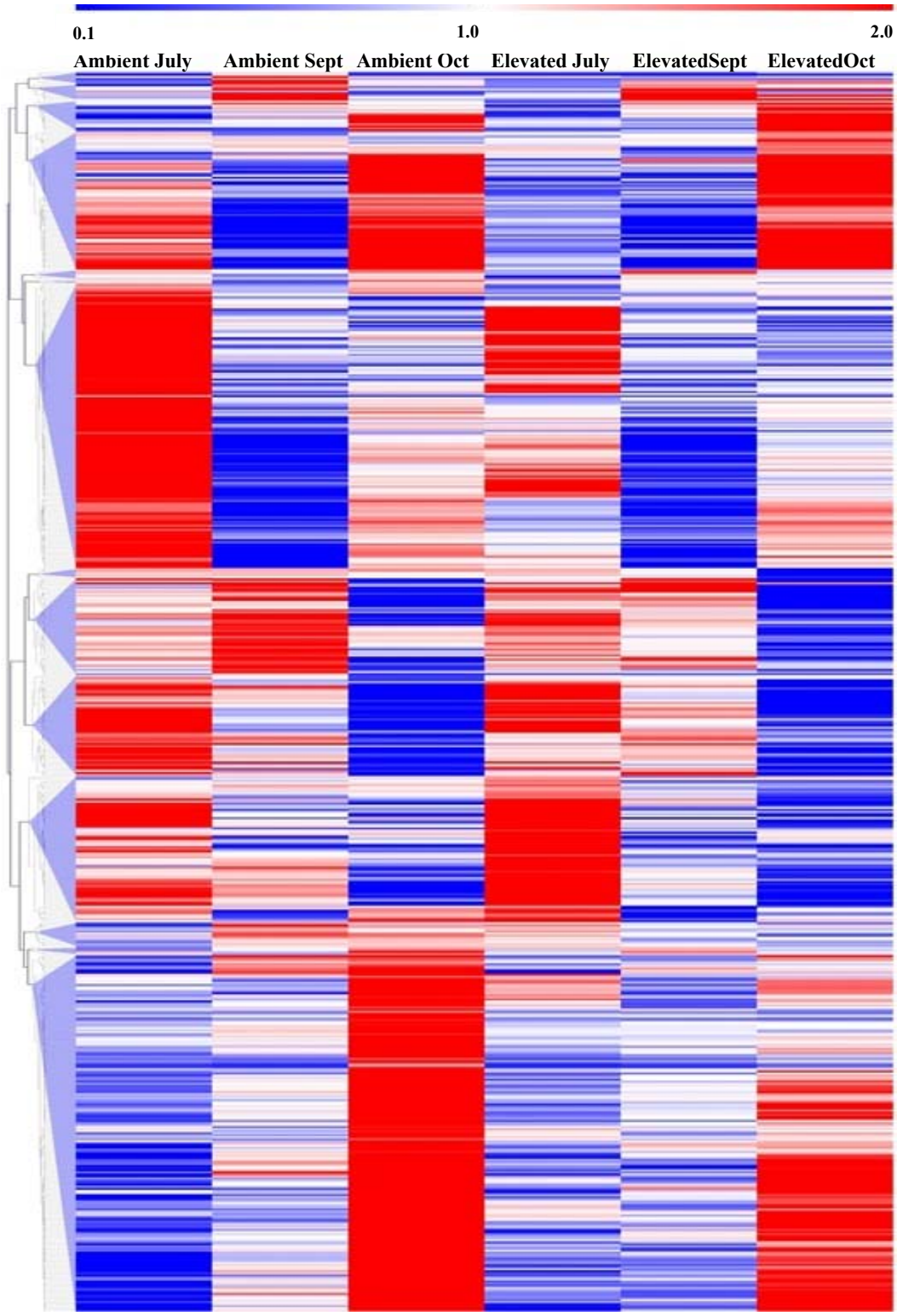
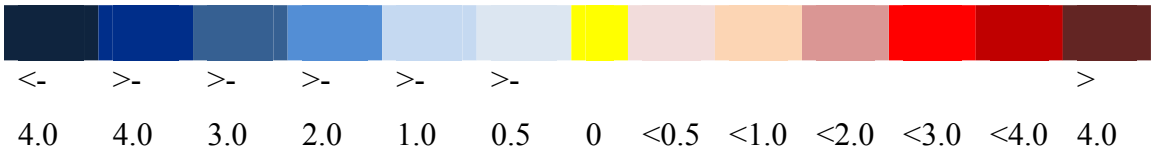


Figure 3.3.6 The Hierarchical cluster of transcript expression in July, September and October under a[CO_2] and e[CO_2]. The transcript expressions of each array were the mean expression of three biological replicates.

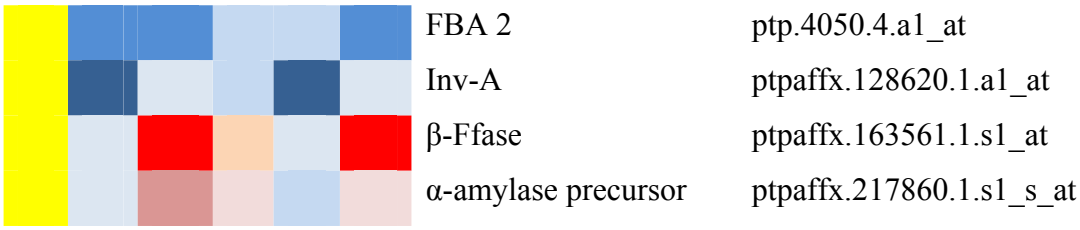
The average of three biological replicate microarrays were used as the expression of each condition: July in a[CO_2], September in a[CO_2], October in a[CO_2], July in e[CO_2], September in e[CO_2] and October in e[CO_2]. All the transcripts expression were divided by the same transcript's expression in July under a[CO_2] and then log_2 transformed to compare the transcripts expression change through time and also under different CO_2 treatments. The hierarchical cluster trees were used to visualise the difference (Figure 3.3.7.a).

The *FRUCTOSE-BISPHOSPHATE ALDOLASE 2*(*FBA 2*) which participates in the Calvin cycle decreased over time under both [CO_2] treatments during senescing. *FBA*s were less expressed in July and Sept under e[CO_2] compared to a[CO_2]suggesting that higher carbohydrate concentration would feedback-regulate the photosynthesis and therefore mediate the Calvin cycle. This lower expression during the growth season could be the reason of rich carbohydrate concentration. The sucrose hydrolysis transcripts Alkaline/ neutral invertase A (Inv-A), which was much less expressed in July under e[CO_2], and beta-fructofuranosidase (β-Ffase), which was slightly higher expressed under e[CO_2], showed relatively similar expression during senescence. The starch degradation transcript- α-amylase precursor, was highly expressed in e[CO_2] in July but decreased dramatically at the onset of senescence while it was highly up-regulated in a[CO_2] condition.

The transcripts involved in secondary metabolism were mainly highly expressed during the growth season under e[CO_2], and some of them were still highly induced at the onset of the senescence. However, at the late senescence stage, most of the transcripts showed much less expression compared with the plants grown under a[CO_2] (Figure 3.3.7.b).



a)



b)

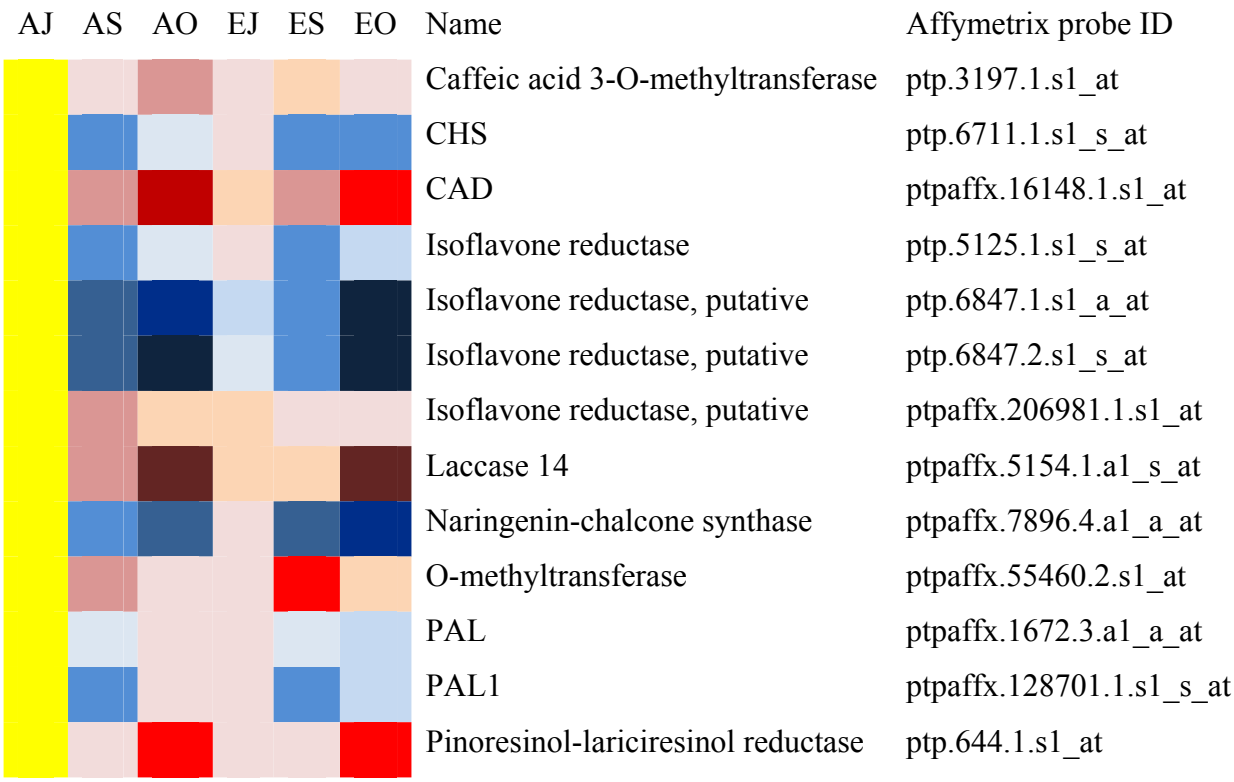
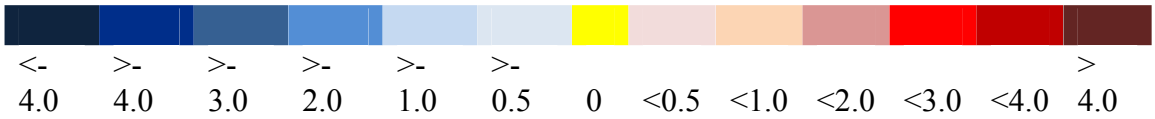


Figure 3.3.7 The Hierarchical cluster of each functional group with the two-fold differentially expressed transcripts over time under two CO_2 treatments. a) The photosynthesis, glycolysis pathway. b) The secondary metabolism. c) The cell wall synthesis, modification and degradation pathway. d) The WRKY families. AJ is July in a[CO_2]. AS means in September under a[CO_2]. AO means in October under a[CO_2]. EJ means in July under e[CO_2]. ES means in September under e[CO2]. EO means in October under e[CO_2]. The colour scale is shown on the top each page.



c)

AJ	AS	AO	EJ	ES	EO	Name	Affymetrix ID
						Cellulose synthase-like 2	ptpaffx.107240.1.s1_s_at
						Expansin	ptpaffx.17914.2.a1_a_at
						Expansin	ptpaffx.17914.3.a1_a_at
						Expansin-like A1	ptpaffx.132592.1.s1_at
						Expansin-like B2 precursor	ptp.5106.1.s1_s_at
						Expansin-like B3 precursor	ptp.5980.3.a1_a_at
						Expansin-like B3 precursor	ptpaffx.95130.1.a1_s_at
						Fasciclin-like arabinogalactan protein	ptp.371.1.s1_x_at
						Pectate lyase family protein	ptpaffx.203635.1.s1_at
						Pectin acetylesterase	ptp.2483.1.a1_at
						Pectin acetylesterase, putative	ptpaffx.117004.1.s1_s_at
						Pectin methylesterase 3	ptpaffx.9932.1.s1_at
						Pectin methylesterase 3	ptpaffx.9932.3.s1_a_at
						Pectinesterase	ptpaffx.11747.2.a1_a_at
						Pectinesterase	ptpaffx.209486.1.s1_at
						XET 6	ptpaffx.102427.1.a1_s_at
						XET 6	ptpaffx.112384.1.s1_s_at
						XET 6	ptpaffx.214395.1.s1_at
						XET 6	ptpaffx.84718.1.s1_s_at
						XTH 9	ptp.2467.1.a1_at
						XTH 9	ptp.2467.1.a1_x_at
						XTH 9	ptpaffx.1041.1.s1_s_at

d)

AJ	AS	AO	EJ	ES	EO	Name	Affymetrix ID
						WRKY 18	ptpaffx.5224.1.a1_at
						WRKY 33	ptp.5112.2.s1_a_at
						WRKY 33	ptpaffx.107379.1.s1_s_at
						WRKY 33	ptpaffx.206337.1.s1_at
						WRKY 33	ptpaffx.213689.1.s1_at
						WRKY 40	ptp.2164.1.s1_s_at
						WRKY 40	ptpaffx.10586.2.s1_at
						WRKY 40	ptpaffx.152367.1.s1_s_at
						WRKY 41	ptpaffx.200375.1.s1_at
						WRKY 53	ptp.2645.1.s1_s_at
						WRKY 53	ptpaffx.132628.1.s1_s_at
						WRKY 6	ptpaffx.217349.1.s1_x_at
						WRKY 69	ptpaffx.11077.4.a1_s_at
						WRKY 70	ptpaffx.211278.1.s1_at
						WRKY 75	ptpaffx.223450.1.s1_at

Figure 3.3.7Continued.

The transcripts that constitute to the expansin gene family were up-regulated during the growth season under e[CO_2], suggesting enhanced cell expansion, but showed a lower expression at the late senescence stage compared with a[CO_2] (Figure 3.3.7.c). The opposite trend was shown in *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9* (*XTH 9*), which was involved in cell wall expansion for cell growth(Maris *et al.*, 2009), with a down regulation under e[CO_2] at the growth season but a lower reduction during senescence compared to the plants grown under a[CO_2]. The *XET* 6, which is part of XTH family, showed the same trends. Both expansin and XTH were involved in cell wall modification, whereas the XTH also enhances the cell wall strength, as well as stimulating loosening whilst expansin is involved in wall loosening only (Cosgrove, 2005). Pectin methylesterase expression was not very different at growth season between e[CO_2] and a[CO_2], but increased much less under the e[CO_2] during senescence. There was no clear trend on pectin acetylesterase and pectin esterase. The pectatelyase family of proteins, which is involved in cell wall degradation, were down-regulated under e[CO_2] during the growth season and the onset of senescence.

The TFs of WRKY families have been reported to play a role in senescence regulation (Figure 3.3.7.d). *WRKY6* and *WRKY53* which were two TFs that were induced at the onset of senescence in e[CO_2], suggesting that they have some important regulatory role in this delayed process in elevated CO_2. *WRKY6* had a lower expression at the onset of senescence compared to the plants under a[CO_2]. *WRKY70,* which works with *WRKY54*together to negatively regulate the leaf senescence through interaction with *WRKY30*(Besseau *et al.*, 2012), and *WRKY40* were both less expressed at the middle growth season under e[CO_2] and showed similar expression during senescence. It is highly possible that these two TFs regulate senescence at an early stage and are less active in elevated CO_2. *WRKY33* was less expressed in the growing season but was increased during senescence in e[CO_2] compared to a[CO_2].

3.3.4 Real time qPCR to confirm the microarray analysis.

Six transcripts were chosen from the Affymetrix microarray analysis, which showed a significantly differential expression in e[CO_2] relative to a[CO_2] at a certain timepoint and were also involved in antioxidative or senescence regulation. The *THIOREDOXIN* (*Trx*), which was consistently up-regulated by e[CO_2] compared with the a[CO_2] in the microarray analysis, and *GPx4*, which was down-regulated by e[CO_2] at the late senescence stage in the microarray analysis, are both involved in free radical scavenging systems, and therefore in the reduction of the oxidative stress. The *CHALCONE SYNTHASE*(*CHS*), which was down-regulated by e[CO_2] from July until October in the microarray analysis, and *PHENYLALANINE AMMONIA LYASE*(*PAL*), which was up-regulated in July following a gradual down-regulation in September and October under e[CO_2] in the microarray analysis, were both involved in the anthocyanin biosynthesis pathway, and also involved in the oxidative stress response system. The *WRKY6*, which was slightly up-regulated under e[CO_2] during July and September, followed by a down-regulation by e[CO_2] in the microarray analysis and the *WRKY75*, which showed the same response as *WRKY6* in the microarray analysis, were suggested to play a role in inducing senescence at the early senescence stage.

Table 3.3.2 Information on the candidate transcripts related to the oxidative stress and senescence. Information obtained from JGI (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.info.html) and TAIR (http://arabidopsis.org/index.jsp).

Gene name	Affymetrix probe ID	Gene Model	*Arabidopsis* orthologue	*Arabidopsis* orthologue annotation	LG position
Trx	PtpAffx.208044.1.S1_x_at	estExt_fgenesh4_pm.C_LG_VIII0540	AT2G01270.1	Encodes disulfide isomerase-like protein, a member of a multigene family within the Trx superfamily. This protein also belongs to the quiescin-sulfhydryl oxidase family, which possess an Erv1-like domain at the COOH terminus in addition to a Trx domain.	LG_VIII:8224040-8231645
GPx	PtpAffx.160545.1.A1_at	estExt_fgenesh4_kg.C_LG_XIV0026	AT2G48150.1	Encodes glutathione peroxidase.	LG_XIV:6688981-6692615
CHS	PtpAffx.200126.1.S1_at	eugene3.00031462	AT5G13930.1	Encodes chalcone synthase, a key enzyme involved in the biosynthesis of flavonoids. Required for the accumulation of purple anthocyanins in leaves and stems. Also involved in the regulation of auxin transport and the modulation of root gravitropism.	LG_XIV:7151030-7153182

Gene name	Affymetrix probe ID	Gene Model	*Arabidopsis* orthologue	*Arabidopsis* orthologue annotation	LG position
PAL	Ptp.3185.1.S1_at	estExt_Genewise1_v1.C_280658	AT2G37040.1	Encodes PAL1, a phenylalanine ammonia lyase. *Arabidopsis* has four *PAL*s: AT2G37040 (*PAL1*), AT3G53260 (*PAL2*), AT5G04230 (*PAL3*) and AT3G10340 (*PAL4*).	scaffold_28:2031644-2035061
WRKY 6	PtpAffx.202497.1.S1_at	eugene3.00002092	AT1G62300.1	Encodes a transcription factor WRKY6. Regulates *PHOSPHATE1*expression in response to low Pi stress.	LG_II:19420400-19422816
WRKY 75	PtpAffx.223450.1.S1_at	gw1.I.3150.1	AT5G13080.1	WRKY75 is one of several transcription factors induced during Pi deprivation. It is nuclear localized and regulated differentially during Pi starvation. RNAi mediated suppression of WRKY75 made the plants more susceptible to Pi stress as indicated by the higher accumulation of anthocyanin during Pi starvation.	LG_I:24197597-24199253

The real-time qPCR (RT-qPCR) results of the six transcripts were compared with their expression from microarray results (Figure 3.3.8). The RT-qPCR result of *PAL* failed to correlate in this experiment, as the *PAL* primer melting curve showed two peaks in the RT-qPCR results. A primer of *PAL* was designed to amplify one gene, the common sequences between *PAL* homologues were avoided. The PCR result with plant DNA which were used to confirm the specificity of primer, proved only one fragment was amplified and detected(Appendix I). The RT-qPCR and microarray results correlated well for transcripts *Trx*, *GPx*, and *WRKY75*. The *CHS* RT-qPCR expression was close to the microarray results. However, a high *WRKY 6* expression was detected by RT-qPCR in October under e[CO_2] compared with [CO_2] with a large error bar. The reason for this difference between RT-qPCR and microarray expressions is not very clear.

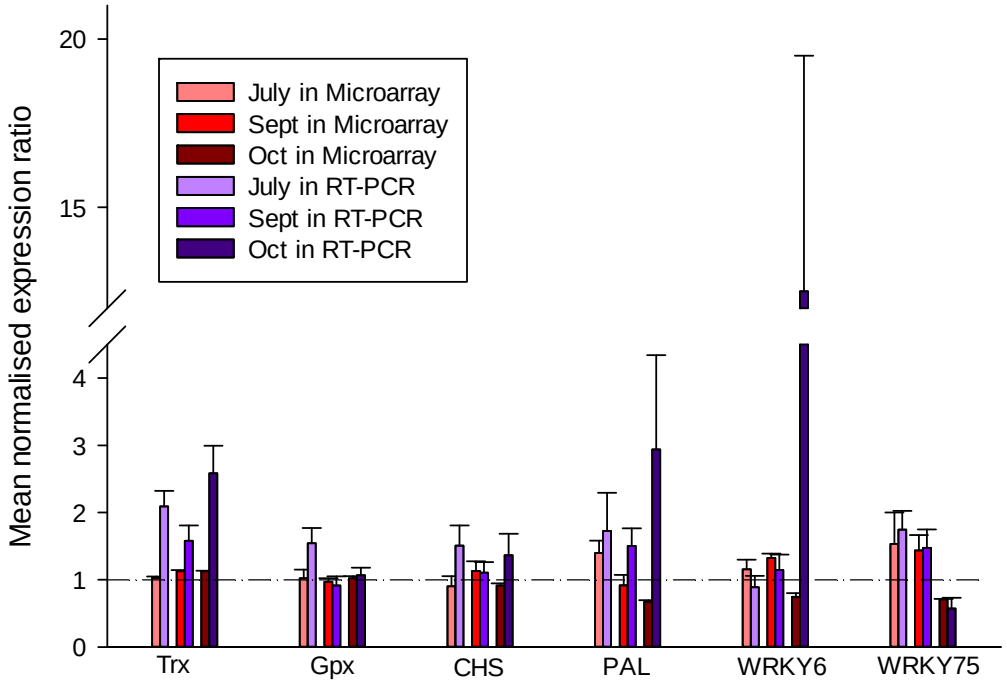


Figure 3.3.8 The comparison of candidate transcripts expression between RT-qPCR and Microarray experiment. The expression of interesting transcripts was calculated using expression under e[CO_2] divided by the expression under a[CO_2]. Expression above one means the transcripts were highly expressed under e[CO_2] and *vice versa*. (±SE)

3.4 Discussion

The aim of this chapter was to investigate the gene expression changes associated with delayed senescence observed in *Populus* grown in the AspenFACE experiment. It is interesting that the same developmental phenomenon was observed in two very contrasting environments (Taylor *et al.*, 2008), and here we wished to investigate the transcript profiling in more detail using Affymetrix microarrays which allow more transcripts to be detected due to higher probes compared to PICME microarrays. The e[CO_2] induced transcriptome regulation of aspen clone 271 was compared with transcriptome change under a[CO_2] at three timepoints (i) during the growing season (July) (ii) during senescence (September) and finally at (iii) late senescence (October). There was a dramatic up-regulation (86% of significantly differentially expressed transcripts) under e[CO_2] in September compared with July and October and it is hypothesised that this was the most active phase of senescence, perhaps similar to the sampling phenotype at the POPFACE site in Italy. Interestingly, many of the similar transcripts displayed in this microarray in response to e[CO_2] compared with a[CO_2] showed opposite and contrasting expression responses between time points.

Net photosynthesis increased by 52% in e[CO_2] consistently in aspen clone 271 over the growing season (Riikonen *et al.*, 2008), and in 2004 the increase even accumulated up to 86% in October under e[CO_2] compared with the control aspen (Taylor *et al.*, 2008). Genes involved in the light-harvesting systems of PSII were less expressed in e[CO_2] compared to a[CO_2] at late senescence stage, the transcripts involved in electrons flow during light reaction were highly expressed under e[CO_2] compared to a[CO_2] during senescence even though they were down-regulated at the middle growth season. The reduction of transcript expression in the photosynthesis pathway during growth season has been observed in other young and mature leaves such as *P. x euramericana*, aspen clone 261, 271, and soybean along with increased net photosynthesis (Taylor *et al.*, 2005; Cseke *et al.*, 2009; Leakey *et al.*, 2009b). It is believed that e[CO_2] increases the efficiency of light use in net CO_2 uptake, as well as water use efficiency (Long *et al.*, 2004). However, when the plants enter the senescence stage, the day length

getting shorter and light becomes limited, the highly expressed electron transfer reaction could maintain the production of reducing power (reduced ferredoxin, NAD(P)H) and energy (ATP) during light reaction for the following CO_2 assimilation by the Calvin cycle (Foyer & Noctor, 2009; Rochaix, 2011).

The chloroplast is the main site of ROS production in plant cells because of the photosynthetic activity (Dos Santos & Rey, 2006). The water-splitting system in PS II, where four electrons are separated from water (from H_2O to $^3O_2$),does not release ROS, but PS I is the major site of superoxide generation (Foyer & Noctor, 2009). The ROS is "toxic" to the plant as they oxidize molecules, including DNA, proteins and lipids (Zimmermann & Zentgraf, 2005). The review by Mittler *et al.* (2004) mentioned that plants detoxifying the O_2^-, and $H_2O_2$by activating the ROS-scavenging system – the antioxidative enzymes, including the SOD, APX, CAT, GPx, Prx, ascorbic acid and glutathione. They also point out that the $^1O_2$ scavenging is mediated by the carotenoids and the $HO\cdot$ formation is prevented by those antioxidative enzymes as well as metal-binding proteins including ferritin.

This influences ROS scavenging enzymes to act as redox buffers and regulate gene expression under biotic and abiotic stress (Foyer & Noctor, 2005; Miller *et al.*, 2008). Thioredoxin (Trx) family proteins are suggested to participate in defence mechanisms linked to the oxidative damage as well as regulate transcription under oxidative stress (Dos Santos & Rey, 2006) and GPx plays the role of protecting membranes from oxidative stress (Mittler *et al.*, 2004). Oksanen *et al.*(2004) suggested that CO_2 enrichment increased scavenging capacity by releasing the resources for peroxisomal antioxidant defence against overproduction of H_2O_2 during oxidative stress. In this experiment, two ROS-scavenging enzyme groups were both up-regulated consistently from growth season until late stage of senescence under e[CO_2] suggesting a possible pathway of delayed senescence through radical-scavenging under e[CO_2] which reinforces other research finding (Buchanan-Wollaston *et al.*, 2003; Tallis *et al.*, 2010).

In the Calvin cycle, FBA (1.20 fold change) and fructose-1,6-bisphosphatase (1.28 fold change) which are the two precursors of starch formation were up-regulated in July and Oct respectively under e[CO_2] compared to a[CO_2]. Starch is

the major carbohydrate product generated during photosynthesis, the increase implying that extra starch was produced during this period under e[CO_2]. Starch accumulated in the day supported by a higher nocturnal metabolism for leaf growth and biomass. Meanwhile, transcripts involved in sugar metabolism were also up-regulated in response to e[CO_2], associated with extra glucose, maltose and sucrose production. There were not any carbohydrate regulating transcripts detected as significantly differentially expressed in response to e[CO_2] in September. This could be due to the restriction of microarray statistic ($p \leq 0.05$) which eliminates some transcripts. The Inv-A and β-Ffase are both invertases, which irreversibly hydrolyses the sucrose to glucose and fructose. The invertases is one of two sucrose catabolism enzymes and have been suggested as the key enzyme for plant growth rather than the sucrose synthase (SUS), the other sucrose catabolism enzymes that catalyse the reversible conversion of sucrose to fructose and UDP glucose (Barratt *et al.*, 2009). The Inv-A isa neutral/alkaline invertase which isoforms in the cytosol, mitochondria and plastids whereas the β-Ffase is an acid invertase that isoforms in the cell wall and vacuole (Barratt *et al.*, 2009). The cell wall invertase has been suggested to be involved in preventing the ABA-induced senescence and increases the seed weight and fruit hexose level in Tomato (Jin *et al.*, 2009). In this study, the dramatic up-regulation of β-Ffase under e[CO_2] compared to a[CO_2] at the middle growth season might also be involved in the delayed-senescence regulation under e[CO_2]. The enhanced carbon uptake accompanied by an increase of photosynthesis under e[CO_2] converted to a greater level of soluble sugars and starch pool in leaves (Leakey *et al.*, 2009b). This is particularly demonstrated on aspen clone 271, which produced a significantly greater carbohydrate pool measured by Cseke *et al.* (2009)compared with aspen clone 216 which did not display an e[CO_2]-induced-late senescence. Research on soybean by Kaschuk *et al.*(2010) suggested that higher carbon sink strength with efficient N_2 fixation could increase the photosynthetic rate and delay leaf senescence.

Increased expression of transcripts in the glycolysis pathway, especially the starch and sucrose degradation pathway, were observed in this microarray experiment from the growing season sample time until the late senescence stage under e[CO_2] compared with a[CO_2], and this could be driven by carbohydrate stimulation

induced by e[CO_2] (Fernie *et al.*, 2004). The increase in transcripts associated with glycolysis was also observed in both developing and mature soybean (Ainsworth *et al.*, 2006; Leakey *et al.*, 2009b) as well as *P. x euramericana* (Tallis *et al.*, 2010). The TCA cycle was up-regulated under e[CO_2] during July and September but down-regulated in October. The expression of transcripts in the mitochondrial electron transport chain was also increased under e[CO_2] compared with a[CO_2] in September then switched to down-regulation in October. The up-regulation of glycolysis and mitochondrial electron transport chain implied a higher respiration metabolism of aspen grown under e[CO_2] from the growing season sampling until the early stage of senescence. This increase provided necessary energy for extended leaf longevity which was induced by exposure to e[CO_2]. The reason transcripts related to respiration metabolism were less expressed under e[CO_2] compared with a[CO_2] at late stage of senescence is not clear. A study on soybean mutant *pan* (chilling sensitive) exhibited an inhibition of CO_2 assimilation & respiration metabolism on nodules under dark chilling (low night temperature) stress (van Heerden *et al.*, 2008). This low respiration metabolism expression in aspen grown in e[CO_2] compared with aspen grown in a[CO_2] could be due to high efficiency of respiration with enough carbohydrate substrate under chilling stress at late stage of senescence.

Transcripts in secondary metabolism followed the same trend of glycolysis; up-regulation in July and September but down-regulation in October. During the growing season sampling and the early senescence sampling, the excess carbohydrate induced the transcripts involved in secondary metabolism, especially the anthocyanin biosynthesis pathway which is the major flavonoid product. A higher amount of anthocyanin protects plants from specific environmental conditions or stresses such as wounding, pathogen attack, or UV light stress (Constabel & Lindroth, 2010), and the increased level of anthocyanin product was suggested to provide a protective role in senescing leaves to extend leaf longevity (Tallis *et al.*, 2010). Trees allocate assimilated carbon between growth, respiration, storage and chemical defence (Cseke *et al.*, 2009) following the growth-differentiation balance hypothesis (Herms & Mattson, 1992). It is also suggested that flavonoid biosynthesis consumes more energy and photoassimilates than phenylpropanoids (Hernandez & van Breusegem, 2010), thus clone 271 seems to

have allocated the extra carbohydrate to starch-sucrose biosynthesis for growth and storage rather than defence at the late stage of senescence. This conclusion agrees with the theory of King *et al.* (2005),that growth-dominated species tend to allocate 'extra' carbon to growth or energy, whereas differentiation-dominated species tend to allocate it to the production of carbon-based secondary compounds. This could explain the different e[CO_2] response within different species of *Populus*.

Pectins are important determinates of cell wall thickness, which are synthesised in the *cis*-Golgi, methylesterified in the medial-Golgi and substituted in *trans*-Golgi cisternae then secreted into the wall (Micheli, 2001). Generally lower expression of PME and pectin acetylesterase was found in this experiment during senescence under e[CO_2]. *Arabidopsis* treated with 10% Methanol (MeOH), which comes from the demethylation of pectin by PME induced transcript expression in the flavonoid pathway, as well as the anthocyanin and flavonoids product (Downie *et al.*, 2004). Another study on detached rice leaves suggested that MeOH is induced during senescence, which is closely associated with the expression of PME1, which may trigger the tryptophan and tryptophan-derived secondary metabolism and lead to delayed senescence (Kang *et al.*, 2011). In our experiment, the PMEs were less increased during senescence reinforcing the down-regulated secondary metabolism under e[CO_2] at the late senescence stage. The cell wall XTH families, which strengthen the cell wall and control cell wall loosening during growth (Cosgrove, 2005), were less-expressed during the growing season sampling under e[CO_2], suggesting smaller cell or weaker cell wall in plants grown under e[CO_2]. However, the decrease of XTH was much slower and less during senescence in e[CO2] compared to the a[CO_2], which might be due to plants grown under e[CO_2] being at a "pre-senescence" stage compared to the plants grown under a[CO_2]; therefore, the cell wall breakdown during senescence delayed under e[CO_2]. The expansins are also the primary wall loosening agents that could extend the cell wall and stimulate larger cells (Cosgrove, 2005). The expansins showed higher expression during the growing season sampling under e[CO_2], which confirms the general phenomenon that plants grown under e[CO_2] produce larger leaf cells by loosening the cell wall (Ferris *et al.*, 2001; Taylor *et al.*, 2003).

Transcription factors regulate a variety of stress-responses in plants. A review from Miller *et al.* (2008)demonstrated the key role of the WRKY family of transcription factors on oxidase stresses. Under e[CO_2], *WRKY18*, *33*, *40* and *70* showed a lower expression at growing season sampling, and during senescence both *WRKY33* and *40*were more highly expressed while *WRKY18* and *70* did not change much compared to a[CO_2]. These four WRKY transcription factors all showed higher expression in a knockout *apx1* mutant, which is a ROS-scavenging enzyme, involved in ROS signalling, and enhanced the pathogenesis-related proteins expression (Davletova *et al.*, 2005). *WRKY75*, can be induced by oxidative stress, showed higher expression in July under e[CO_2] (Gadjev *et al.*, 2006). This is reinforced with ROS-scavenging enzyme expression, and confirmed that e[CO_2] reduces the oxidative enzyme activity through antioxidative enzymes. *WRKY6* and *WRKY53* were two TFs which have been systematically studied for their roles in influencing senescence (Robatzek & Somssich, 2002; Miao *et al.*, 2004). The *WRKY6* is the first reported senescence regulator and positively influenced the senescence- and pathogen defence-associated promoter PR1 (promoter regulation 1) activity (Robatzek & Somssich, 2002). It was less expressed in July and slightly higher in September in e[CO_2] compared to a[CO_2]. *WRKY53*, which induces senescence, showed much less expression under e[CO_2] compared to a[CO_2] which could be another e[CO_2] induced delayed-senescence regulator. Most of the WRKY families showed much less expression under e[CO_2] compared with a[CO_2] during the growing season sampling indicating these TF's regulation of senescence might even start from the growing season, meaning that they are early actors in determining the altered functioning associated with the control of senescence programming.

The transcript changes of aspen clone 271 in response to e[CO_2] compared with a[CO_2] were small compared with other climate change response, such as drought or O_3, and this has been seen in other research studies on clone 271 (Cseke *et al.*, 2009). The transcripts expression was also validated by RT-qPCR. There are several techniques for validating the microarray results including Northern blotting, ribonuclease protection, *in situ* hybridization and RT-qPCR (Dallas *et al.*, 2005). Several candidate transcripts showed a good correlation with the microarray expression except the *CHS* and *WRKY6* (despite the result of *PAL* due to a technical problem). It has been reported that although RT-qPCR and

microarray expression data is strongly correlated, there is still 13 – 16% of genes that do not show the same trend (Dallas *et al.*, 2005).

3.5 Conclusion

The Affymetrix microarray analysis provided a full picture of transcript expression changes in response to e[CO_2] from the growing season until the late stage of autumnal senescence. Thus, the analysis extends that presented for the POPFACE trees in Chapter 2, but the results here were sometimes in contrast to those observed in this previous experiment. It is possible that the aspen and *P. x euramericana* allocate and utilise excess carbon in different ways.

The light harvesting system transcripts were generally down-regulated under e[CO_2] during senescence but the electron carriers were still highly activated providing sufficient energy and the reducing power for the glycolysis and TCA cycle. The highly expressed sucrose and starch degradation transcripts and less expressed secondary metabolism transcripts under e[CO_2] indicated that the excess carbohydrate induced by e[CO_2] was used for energizing the processes of late senescence, not for defence following the growth-differentiation balance theory, in contrast to the finding for POPFACE. The highly activated electron carriers in chloroplast and mitochondria induced a series of ROS which trigger the ROS-scavenging enzymes under e[CO_2]. The ROS-scavenging enzymes suppressed the oxidative stress under e[CO_2], and therefore prevented the ROS-induced autumn senescence.

Both *P. x euramericana* and aspen clone 271 showed delayed autumnal senescence phenomenon, but the carbon utilization were completely different during growth and senescence. The different biological strategies of different poplar species in response to e[CO_2] should be taken into consideration when selecting plantation for future environment.

Chapter 4: Multi- generational response of *Plantago* to a CO_2-enriched environment – elucidating acclimation and adaptation mechanisms

4.0 Overview

There have been few studies on plant evolution and adaptation to CO_2 concentration, with most research mainly focused on morphological and physiological responses to increased atmospheric CO_2. Not many studies have investigated the genetics and evolution of adaptation to CO_2. In this chapter, a common species *Plantago lanceolata* L. (Plantaginaceae)from a naturally abundant CO_2spring in Italy was used to investigate the effect of atmospheric CO_2 concentration as a selective agent for plant microevolution which is a type of evolution in which small scale changes were observed by monitoring natural populations (Freeman & Herron, 2004).

Seeds of plants from the spring site (naturally elevated [CO_2] site) and the outside spring site (control site with ambient [CO_2]) were germinated and grown under two different [CO_2]. Morphological data including leaf and cell traits were collected to compare the difference between plants from the two sites to identify the possibility of genetic evolutionary mechanism in response to e[CO_2]. We tested the hypothesis that plants subjected to elevated CO_2 for many generations are more likely to be adapted to this condition and to show limited response to elevatedCO_2 when exposed in the chamber system at Southampton.

4.1 Introduction

The atmospheric CO_2 concentrations were at a high level (1500-3000 ppm) when the earliest land plants became established in Palaeozoic era, then the concentration of CO_2 dropped to ~1000 ppm between ~250 – 100 Mya (million years ago) before further decreasing to ~280 ppm (Leakey & Lau, 2012). However, since the industrial revolution, atmospheric [CO_2] has increased rapidly due to human activities to 395 ppm nowadays(Rhodes, 2012). The drop in atmospheric [CO_2] was shown to be a strong selective force in both plants and ecosystem on evolution adaptation to the environment change, which have not been detected with increasing atmospheric [CO_2] (Beerling, 2012). Previous chapters have studied plant transcriptomic responses to an elevated [CO_2] growth environment by using the model species – *Populus*–and identified several exciting pathways and key genes, especially the secondary metabolism pathways, which have important roles in plant acclimation to increased [CO_2] as shown in the PopFACE and AspenFACE experiments. These key responses and transcripts associated with them may be the starting point for adaptation and evolutionary change but to study this further, it is necessary to move to multi-generational experiments. Here we chose to use a naturally rich-in-CO_2 spring to help to extend our knowledge further, implicating the genetic modification with respect to both morphology and physiology on a micro-evolutionary level (Woodward, 1999).

There are several naturally rich-in-CO_2 springs around the world that provided a naturally elevated [CO_2] environment for the vegetation grown over many generations (Bettarini *et al.*, 1999; Onoda *et al.*, 2009) (for details see chapter 1.2.2). The CO_2 spring used for this study is Bossoleto CO_2 spring, which is located in Rapolano Terme, near Siena in Central Italy (Lat. 43°17', Long. 11°35') (Bettarini *et al.*, 1998) (Figure 4.1.1). At this natural CO_2 spring, the surrounding air has been at high [CO_2] ranging from 600 to 1200ppm, which is emitted from gas vents (Bettarini *et al.*, 1999; Collins & Bell, 2006). A microevolutionary adaptation of plants to elevated CO_2 (MAPLE) project was carried in the late 1990s trying to identify and characterise the occurrence of microevolutionary traits in native plants from a number of CO_2 springs in Italy including Bossoleto

CO_2 spring (Raschi *et al.*, 1999). This project detected several important changes in morphology, but did not consider underlying genetics. For example Woodward (1999) grew *Plantago lanceolata L.* collected from CO_2 spring site in two different CO_2 treatments (350 ppm and 700 ppm), and a significant reduction in total leaf area and shoot to root ratio with no stomatal index change were detected under e[CO_2] compared to a[CO_2]. This response is in contrast to the CO_2 treatment response observed in *Plantago* which was collected at the spring site and compared to plants grown outside of the spring site. It suggests that response to e[CO_2] may differ depending on the site of original for any given population of plants.Woodward (1999)also grew mixed *Tussilago farfara* collected both from inside and outside of spring, and measured the shoot weight and shoot to root ratio leading to the conclusion that plants from the spring site showed a superior competitive ability during the growing season. However, for the plants from outside the spring (here termed 'control'), Andalo *et al.* (1999) discovered that although there were more seeds produced (*Arabidopsis thaliana*) from plants grown in the CO_2 spring, the germination rate of seeds were much lower compared to the seeds from *Arabidopsis* collected from outside of spring. Plants from both Japanese and Italian CO_2 springs showed an increased light-saturated rate of photosynthesis at mean growth CO_2 concentration accompanied with decreased photosynthesis rate (maximum rate of RuBP carboxylation and maximum rate of electron transport driving RuBP regeneration) (Stylinski *et al.*, 2000; Blaschke *et al.*, 2001; Onoda *et al.*, 2007). This finding is very similar to what was found in the FACE experiments(Ainsworth & Long, 2005), therefore, Onoda *et al.* (2007) hypothesized that long-term growing under high CO_2 environment did not influence the photosynthesis feedback regulation at the ecosystem level. Lau *et al.* (2007) measured the morphological difference of a range of *Arabidopsis* recombinant inbred lines (RILs) growing under ambient/elevated [CO_2], and drew the conclusion that the biological differences detected in this experiment were primarily an ecological effect rather than an evolutionary effect of e[CO_2]. In work on *Populus*, when a mapping population was exposed to elevated CO_2(Rae *et al.*, 2006; Rae *et al.*, 2007),significant differences in the offspring of the cross in response to CO_2 were apparent, enabling QTL for response to CO_2 for several growth and morphological traits to

be mapped. These data suggest that there is a genetic component in response to elevated CO_2 that has yet to be fully elucidated.

a)

b)

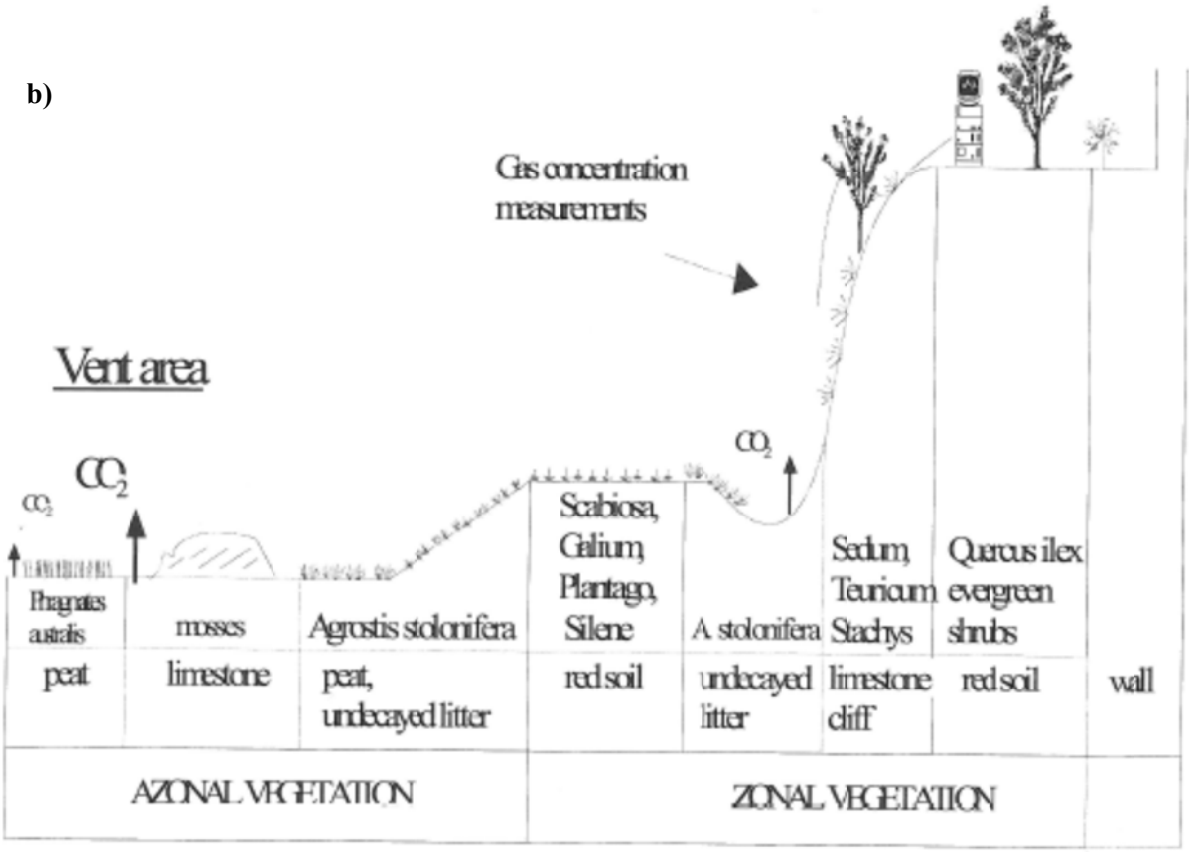


Figure 4.1.1 Natural carbon dioxide-rich spring in Italy.a) The Bossoleto spring site ground photos (Photos were taken by L. Graham). b) The Bossoleto spring site diagram, which illustrates the relation between topography and vegetation (From Schulte *et al.* (1999)).

Plant altered physiology to acclimate to the pressure of environment change, however, this is not detecting evolutionary response, which focuses on genetic change, to e[CO_2] (Hoffmann & Sgro, 2011). There were two approaches to understand the evolutionary adaptation in response to CO_2: the first is to detect the traits that are altered by e[CO_2] and also influence the plant fitness, for example tree productivity(Rae *et al.*, 2007), and the second is to determine whether the traits adjust by e[CO_2] are heritable to offspring (Leakey & Lau, 2012).The amplified fragment length polymorphism (AFLP) technique, which was invented at the beginning of 1990s, was widely used in measuring population-based genetic diversity, assessing genetic relationships between individuals and producing linkage maps with other sources for established mapping populations (Blears *et al.*, 1998; Meudt & Clarke, 2007; Agarwal *et al.*, 2008). This technique cut the plant genome into restricted fragments with restriction enzyme and two sets of complementary primers which contains corresponding adaptor and restriction site specific sequences, and indicated the genetic diversity by analysing the number or the size of amplified fragments (Vos *et al.*, 1995). Compared with other PCR-based common molecular marker techniques, including random amplified polymorphic DNAs (RAPDs), microsatellite and single nucleotide polymorphism (SNPs), AFLP can generate DNA fingerprints without any prior DNA sequence knowledge in any DNA source and can distinguish closely related individuals at the sub-species level (Althoff *et al.*, 2007; Agarwal *et al.*, 2008). AFLPs are dominant marker systems and are naturally abundant in the genome. These advantages mean that the AFLP technique is favoured when hybridization, polyploidy or high heterogeneity genomes are involved (Meudt & Clarke, 2007; Meudt, 2011). Kelly *et al.* (2003) successfully identified population segregation in birch acclimated to temperature change by using the AFLP technique, but to our knowledge, no AFLP studies have been completed for plant response to atmospheric CO_2.

This chapter studied *Plantago lanceolata* L. seeds which were collected from a high [CO2] spring and outside of the spring *i.e.*, ambient [CO2]. *Plantago lanceolata* L., which is a common forb in Europe, has been studied widely in physiological, ecological, and genetic studies (Bettarini *et al*., 1998; Klus *et al*., 2001; Sahin *et al*., 2007; Onoda *et al*., 2009) and grows naturally inside and

outside of natural high-CO_2 spring environment. It is a short-lived perennial rosette forb which favours long-day conditions with numerous leaves and fibrous stalks with spike inflorescences (Sagar & Harper, 1964; Fajer *et al.*, 1992). Flowering starts at early June and continues to mid-August and fruit maturation and subsequent seed dispersal occurs throughout the summer and autumn (Tonsor *et al.*, 1993). *Plantago* has a gametophytic self-incompatibility system therefore requires wind pollination (Ross, 1973), which potentially reduces the possibility of gene flow between plants from inside and outside of the CO_2 spring due to distance. It is also known to be capable of undergoing rapid evolutionary changes (Wu & Antonovics, 1976; Wolff & Vandelden, 1989; van Tienderen & van der Toorn, 1991; Klus *et al.*, 2001; Bischoff *et al.*, 2006) particularly on seed yield, biomass, leaf number and length and spike number. *Plantago* seeds were germinated and grown under 390ppm and 700ppm [CO_2] in eight chambers. The morphological and genetic results were compared to understand the response of *Plantago* to short and long-term exposure to a high [CO_2] environment and determine the potential covariation fitness-selected traits and the direction of genetic variation influenced by high [CO_2]. This should give us a preliminary understanding of the potential of e[CO_2] to act as a selection agent for evolutionary change. To our knowledge this is the first molecular study of long-term, multi-generational plant adaptation to increasedCO_2.

4.2 Materials and methods

4.2.1 Morphological measurements

4.2.1.1 Plant materials and growth conditions

Plantago lanceolata L.(Plantaginaceae) seeds were collected from the natural carbon dioxide spring (Bossoleto CO_2 spring) where the atmospheric [CO_2] is maintained above 600 ppm. *Plantago* grown in the elevated [CO_2] environment of the spring (spring site) were compared with the same species growing outside of the spring with ambient [CO_2] (control site), which were outside of the wall as seen on Figure 4.1.1.b. The seeds were collected from nine randomly-selected maternal plants in both sites on 12 May 2008 and stored in University of Southampton's cold room (0 ºC) until further study.

The seeds collected from the Bossoleto CO_2 spring were established in a controlled environment at the University of Southampton on 21st Sep 2009 (23°C/ 20°C, 16h day length). They were potted in 2cm compost (John Innes potting compost No.2, John Innes manufacturers association, UK), and topped with 1 cm fine sand. After three weeks germination, plants were moved into one of eight CO_2 chambers with four chambers at ambient [CO_2] (chamber 1 to 4,390 ppm) and four at elevated [CO_2] (Chamber 5 to 8, 700 ppm) (22°C /17°C, 16h day length, pipe nature air flow rate 3.4 m/s^2 and light intensity around 104-134 μmol $m^{-2}s^{-1}$) (Figure 4.2.1).

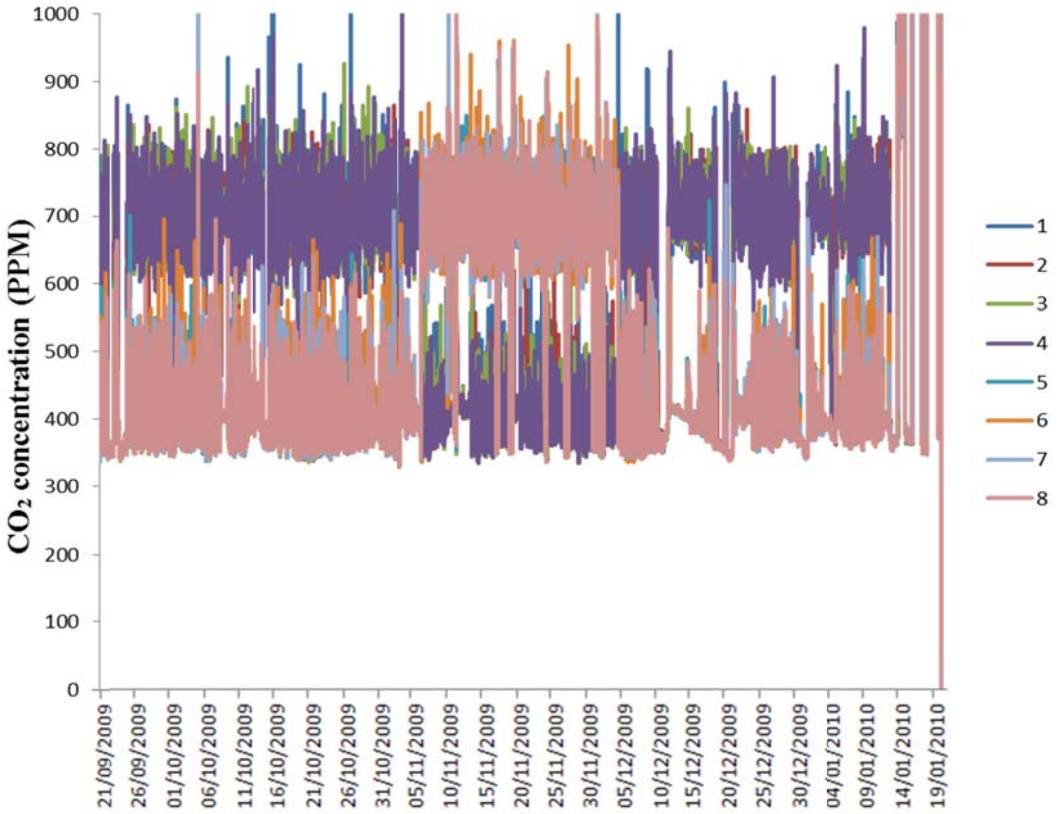
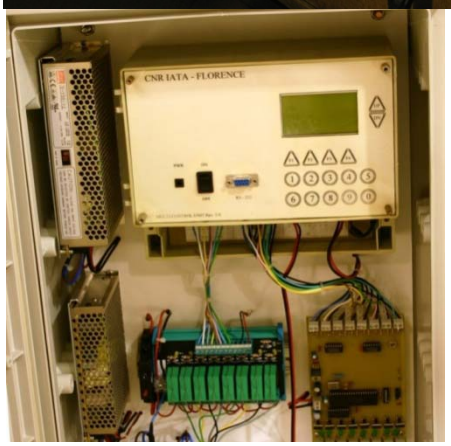


Figure 4.2.1 Carbon dioxide concentration of each chamber measured during the experiment. Chamber 1 to 4 were filled with e[CO_2] at the beginning and end of the experiment and chamber 5 to 8 were filled with a[CO_2] at the beginning and end of the experiment. During the experiment, to minimise the chamber effect on plants growth, plants in chamber 1 to 4 were switched with plants in chamber 5 to 8 as well as the CO_2 concentration. The [CO_2] drop of chambers with e[CO_2] is due to CO_2 supply running out temporarily or gas cylinder replacement. At end of the experiment, the control unit seems broken. However, all the leaf samples collected for DNA and RNA experiment were collected before; this should not affect our experiment results.

There was one plant from each of the nine maternal plants in each site growing in each chamber. The seedlings were transferred into small pots filled with sterilized vermiculite, and additional nutrients, particularly nitrogen (all-purpose soluble plant food, The Scotts Miracle-Gro Company, USA) were added twice per week and the positions of the pots were randomized within and between each CO_2 chamber every week. The wooden growth chambers measured 800mm(length) x 700mm (width) and 600mm (height) with a 10mm thick heat resistant glass developed by Dr K. Warwick at the University of Sussex (Tucker, 2006) (Figure 4.2.2).

a)

b)



c)

d)

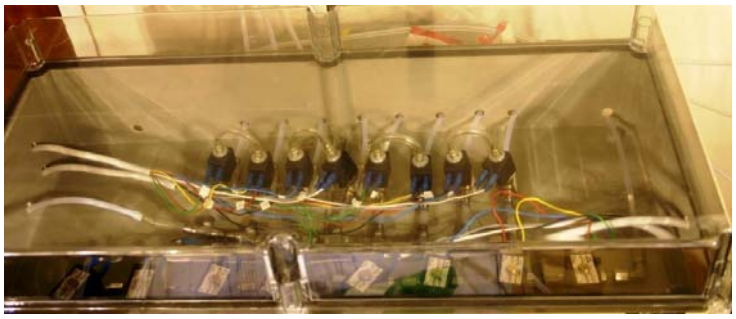


Figure 4.2.2 control components of the CO2 enrichment system. a) Chamber. Plants were isolated into each chamber for specialised [CO_2] treatment and daylight. b) Control unit. This calculates the amount of CO_2 needed by each chamber to reach the target [CO_2] by using the feedback from assessment unit. c) Assessment unit, the [CO_2] of each chamber was measured by unit c. d) Valve system for CO_2 delivery and monitoring. One set of valves controls the speed of CO_2 delivery to each chamber and the other set delivers CO_2 to assess unit for monitoring.

Table 4.2.1 The layout of *Plantago* chamber experiment. C represents the plants from the control site (outside of spring) whereas S represents the plant from the spring site. The first number is the label of the maternal plant and the second number represents the offspring of that maternal plant.

Chamber 1 a[CO_2]				Chamber 5 e[CO_2]		
S-8-7	S-2-2	S-10-7		C-7-10	S-8-1	C-9-3
C-6-12	C-3-2	C-1-13		C-4-10	S-7-1	S-4-2
C-9-5	blank	C-8-7		S-5-6	C-1-10	C-10-2
C-2-13	S-5-12	C-4-1		C-6-6	C-3-6	S-1-1
C-7-5	C-10-5	S-1-6		S-9-3	C-2-6	S-2-7
S-4-1	S-9-5	S-3-2		S-10-5	S-8-3	S-3-6
Chamber 2 a[CO_2]				Chamber 6 e[CO_2]		
S-1-3	S-7-3	C-6-3		S-2-5	C-2-5	S-8-6
S-5-14	C-10-13	C-3-4		blank	S-9-6	S-10-6
C-2-7	S-4-5	C-4-5		C-8-6	C-3-8	C-10-4
S-9-4	C-7-14	C-9-8		C-1-5	blank	C-6-7
C-8-9	S-10-3	S-2-9		S-5-7	C-4-2	C-9-6
C-1-15	S-8-9	S-3-4		S-4-3	S-1-5	C-7-8
Chamber 3 a[CO_2]				Chamber 7 e[CO_2]		
S-5-4	C-4-4	C-3-5		S-4-4	S-5-3	S-8-5
C-8-2	S-2-1	blank		S-3-3	C-6-4	C-7-12
C-9-7	S-10-1	C-2-10		C-1-14	S-2-10	C-2-11
S-3-5	S-1-8	C-7-15		C-9-4	S-1-2	blank
S-7-4	C-6-5	S-8-2		C-10-9	C-4-3	C-8-5
S-4-6	C-10-1	C-1-8		C-3-3	S-10-9	S-7-2
Chamber 4 a[CO_2]				Chamber 8 e[CO_2]		
blank	C-7-2	C-9-2		S-9-1	S-3-1	C-9-1
C-4-7	C-2-14	S-2-4		C-6-14	blank	C-3-7
C-6-1	S-9-2	blank		C-4-8	S-7-6	S-8-4
S-5-11	S-8-8	C-1-3		S-2-11	S-10-2	C-2-12
C-3-1	S-10-8	S-7-5		C-10-12	C-1-9	C-7-3
C-8-8	S-1-4	C-10-6		C-8-4	S-1-7	S-5-13

4.2.1.2 Leaf and cell measurements

Plantago were sampled on three occasions: on the 17th November 2009 (58 days after establishment), 17th December 2009 (87 days after establishment) and 21-22 January 2010 (123-124 days after establishment). In the first harvest, the most developed leaf (mature leaf) was harvested from each plant, frozen in liquid nitrogen and stored at -80 °C. At the second time point, the second or/and third leaves were harvested, representing a young expanding leaf, and one most developed leaf was harvested as a mature leaf. These leaves were also frozen in liquid nitrogen separately and stored at -80 °C. Whole plants were harvested at the last time point. The most mature leaf of each plant was removed and traced on a paper bag. The paper bags were then scanned into a digital format using a Scanner (HP Scanjet 4850, Hewlett-Packard development company, L.P), and analysed by ImageJ (Image J 1.42q, Wayne Rasband, USA) for single leaf area. The leaves were weighed separately after drying in an oven at 75°C for two days or until leaves no longer changed weight. The whole plant was cut at 1cm aboveground and all leaves were laid on white scaled A3 paper. Photographs were taken with a camera at a set distance of 120 cm for all leaf area images (Figure 4.2.3).

Figure 4.2.3 One of the total leaf area images.

Cell imprints were taken from the abaxial surface of the leaves, which were then harvested singly for single leaf area. A thin layer of nail polish was painted on the middle of the abaxial surface of the leaf (Ferris & Taylor, 1994). Once the nail polish dried, the surface of this area was removed by clear adhesive tape and directly placed on a clear area of glass slide. The cell imprints were stored at 4°C before microscope analysis using a Zeiss upright microscope. The number of stomata and epidermal cells were counted on microphotographs under fields of view of 1.3899mm^2 at 20x magnification. Epidermal cell size (ECS) was measured on microphotographs using ImageJ. Twenty randomly selected cells in each picture were traced by hand in the software, and the average of 20 measurements was used as final result for each plant. Specific leaf area, stomatal index, stomatal density and epidermal cell number per leaf were calculated using the following equations.

$$\text{Specific Leaf Area (SLA)} = \frac{\text{Single leaf area (mm}^2\text{)}}{\text{Single leaf biomass (g)}}.$$

$$\text{Stomatal Index (SI) 100\%} = \frac{\text{no. of guard cell}}{\text{(no.of guard cell + no.epidermal cells)}} * 100$$

$$\text{Stomatal Density (SD)} = \frac{\text{no. of stomata}}{\text{field of view (mm}^2\text{)}}$$

$$\text{Epidermal cell number per leaf} = \frac{\text{single leaf area (mm}^2\text{)}}{\text{average epidermal cell size (mm}^2\text{)}}$$

4.2.1.3 Statistical analysis

The Coefficient of Variation (CV) was calculated in order to quantify genotypic variation by using morphology measurements as described in Onoda *et al.* (2009). The morphology measurements were tested by Generalized Linear Models (GLM) in SPSS 16 (SPSS Inc., Chicago, IL, USA) with the model: Response = Location + Treatment + Treatment | Location + Treatment | Family (Location) + Location | Chamber (Treatment) (Doncaster & Davey, 2007) (Table 4.2.2). Traits were log-transformed before analysis if normalisation tests were rejected ($P<0.05$) with the *Kolmogorov-Smirnov* test. *Plantago* were not capable of self-fertilization, and the

plants have been moved between and within each chamber every week, so family and chamber can be regarded as random factors. The degree of freedom of component Chamber (Treatment) | Family (Location) is zero so there is no statistic test possible on this component. A planned post hoc Sidak's test was applied on the traits which were significantly influenced by location and treatment interaction to examine significant pairwise comparisons in SPSS.

Table 4.2.2 The statistic model table. In this table, A represents original site (location), B represents [CO_2] (treatment), C represents chamber number (chamber), S represents*Plantago* maternal family (family) and Q represents individual plants.

Y=S'(A)|C'(B)		Control Site Plants (A1)									Spring site plants (A2)								
		S1	S2	S3	S4	S5	S6	S7	S8	S9	S11	S12	S13	S14	S15	S16	S17	S18	S19
a[CO_2] (B1)	C1	Q1	Q3	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	Q11	Q12	Q13	Q14	Q15	Q16	Q17	Q18
	C2	Q19	Q20	Q21	Q22	Q23	Q24	Q25	Q26	Q27	Q28	Q29	Q30	Q31	Q32	Q33	Q34	Q35	Q36
	C3	Q37	Q38	Q39	Q40	Q41	Q42	Q43	Q44	Q45	Q46	Q47	Q48	Q49	Q50	Q51	Q52	Q53	Q54
	C4	Q55	Q56	Q57	Q58	Q59	Q60	Q61	Q62	Q63	Q64	Q65	Q66	Q67	Q68	Q69	Q70	Q71	Q72
e[CO_2] (B2)	C5	Q73	Q74	Q75	Q76	Q77	Q78	Q79	Q80	Q81	Q82	Q83	Q84	Q85	Q86	Q87	Q88	Q89	Q90
	C6	Q91	Q92	Q93	Q94	Q95	Q96	Q97	Q98	Q99	Q100	Q101	Q102	Q103	Q104	Q105	Q106	Q107	Q108
	C7	Q109	Q110	Q111	Q112	Q113	Q114	Q115	Q116	Q117	Q118	Q119	Q120	Q121	Q122	Q123	Q124	Q125	Q126
	C8	Q127	Q128	Q129	Q130	Q131	Q132	Q133	Q134	Q135	Q136	Q137	Q138	Q139	Q140	Q141	Q142	Q143	Q144

4.2.2 Genetic difference measurements

4.2.2.1 DNA extraction

The DNA of *Plantago* leaves harvested at the first time point were used for AFLP analysis. Leaf tissue was ground in liquid nitrogen using a pre-chilled pestle and mortar, and 50 mg of powdered leaf material was used for DNA extraction with the CTAB extraction method. 900 µl of pre-warmed (65 ºC) CTAB (2% hexadecyltrimethylammonium bromide (w/v), 2% polyvinylpyrrolidone (w/v), 100 mM Tris-HCl, 25 mM EDTA and 2 M NaCl) was added to each Eppendorf tube, and tubes were incubated at 65 ºC for 45 minutes. After incubation, 900 µl CHISAM (Chloroform/Isoamyl alcohol) was added into each tube and tubes were

centrifuged for 10 minutes at 12x g. The upper layer (aqueous phase) was transferred to a new tube and 50 µl 3 M sodium acetate and 333 µl cold isopropanol was added to each tube. After incubation at -20 ºC for 30 minutes, the samples were centrifuged for 10 minutes at 12x g to form a pellet. The pellet was then dissolved in a mix of 200 µl of TE (Tris-EDTA) buffer, 100 µl sodium aceteate and 1ml cold absolute ethanol and centrifuged at 12x g for 10 minutes. The liquid phase was discarded and the pellet was washed with 500 µl cold 70% ethanol. The samples were centrifuged again and remaining liquid were discarded. The pellet was air dried, then resuspended in 50 µl DEPC treated water. The DNA concentration was measured using a NanoDrop spectrophotometer (ND100, NanoDrop Technologies, Delaware, USA).

4.2.2.2 AFLP generation

The AFLP was carried with AFLP® Core Reagent Kit (Invitrogen, Life Technology, UK) following the combined protocol from the manufacturer's instructions and the "Maize Mapping Project" (http://www.maizemap.org/aflp.htm). DNA samples were diluted to a concentration of 500 ng/µl and digested with restriction endonucleases *EcoR* I and *Mse* I followed by the ligation of digested fragment. The pre-amplification was then performed with six pre-selective amplification primers (AACAT, AACTT, CTCAG, TCCCT, AACCT and CCCCT) separately, followed by amplification with EcoPRE and MsePRE primers. Six AFLP pre-amplification primers (primers ended with "e" were labelled with fluorescent dye HEX) were chosen from the Gaudet *et al,* (2008) paper and with experience of how AFLP primers worked on poplar from Dr Jennifer De Woody (former lab member). The sequences of each primer were presented in Table 4.2.2.

Table 4.2.2 The sequence for each primer code.

Primer code	Sequence (5' to 3')
AACATe	GACTGCGTACCAATTCAA
AACATm	GATGAGTCCTGAGTAACCAT
AACTTe	GACTGCGTACCAATTCAA
AACTTm	GATGAGTCCTGAGTAACCTT
CTCAGe	GACTGCGTACCAATTCCT
CTCAGm	GATGAGTCCTGAGTAACCAG
TCCCTe	GACTGCGTACCAATTCTC
TCCCTm	GATGAGTCCTGAGTAACCCT
AACCTe	GACTGCGTACCAATTCAA
AACCTm	GATGAGTCCTGAGTAACCCT
CCCCTe	GACTGCGTACCAATTCCC
CCCCTm	GATGAGTCCTGAGTAACCCT
EcoPRE	GACTGCGTACCAATTC
MsePRE	GATGAGTCCTGAGTAAC

4.2.2.3 AFLP scoring and analysis

The AFLP samples were sent to Source BioScience (Source BioScience, Nottingham, UK) for fragment analysis. Then the original data (fsa format) were input into PeakScanner v 1.0 (Applied Biosystems, USA) for peak scanning and scoring. AFLP bands were scored as present (1), absent (0) and failed (-1) for all plants used in this experiment, and only peaks with molecular weight greater than 50 bp were used for peak analysis. False peaks were removed by comparing with negative control (contains water instead of DNA) for each primer set. The non-robust peaks (if not present in at least four plants) were not scored and were also removed from AFLP result analysis. The statistics of AFLP peak scores were processed by GENALEX v 6.4 (Peakall & Smouse, 2006) for the analysis of molecular variance (AMOVA) and the principal coordinates analysis (PCoA). A dendrogram of AFLP results was produced by using Phylip v 3.69 (Joe Felsenstein, University of Washington; http://evolution.genetics.washington.edu/phylip.html) using the same peak score result based on the restriction fragments distance calculated using “Gendist” and

mapped by “Neighbor” with the unweighted pair group method with arithmetic mean (UPGMA) clustering method. The dendrogram was viewed using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) in rectangular cladogram format.

4.3 Results

Plantago lanceolata seeds collected from both spring site and outside spring site (control site) were exposed to ambient (390 ppm) and elevated [CO_2] (700 ppm). The morphological changes for both leaf and epidermal cell traits were measured (Table 4.3.1) with each trait described in section 4.3.1 and 4.3.2, respectively. The statistics of each trait are represented in table 4.3.2 and table 4.3.3 in the following chapter.

Table 4.3.1 Means and standard errors of *Plantago* leaf traits measured in this experiment. Percentage change = [(average in 700 ppm – average in 390 ppm) / average in 390 ppm]*100

Parameter	[CO2]	390 (ppm)		700 (ppm)		Percentage difference
		Average	SE	Average	SE	
	Sites					
Aboveground biomass (g)	Control	10.11	0.81	12.95	1.44	28.15%
	Spring	11.16	1.27	11.67	1.65	4.54%
Single leaf biomass (g)	Control	0.20	0.01	0.23	0.03	14.24%
	Spring	0.26	0.03	0.24	0.03	-10.02%
Single leaf area (mm^2)	Control	2871.45	103.88	3279.12	204.86	14.19%
	Spring	3595.86	270.43	3271.53	185.56	-8.97%
Specific leaf area (mm^2/g)	Control	16109.36	754.14	15257.25	703.77	-5.29%
	Spring	14523.00	588.56	15279.96	1587.51	8.31%
Epidermal cell size (μm^2)	Control	1456.89	98.91	1405.26	74.33	-3.54%
	Spring	1541.46	88.47	1349.97	69.76	-12.42%
Cell number per leaf (10^5/leaf)	Control	20906.04	553.04	25084.30	504.45	19.99%
	Spring	24550.37	1518.92	25340.62	697.76	3.22%
Stomatal density (No./cm^2)	Control	203.82	15.37	222.90	7.71	9.36%
	Spring	206.38	21.23	230.10	14.26	11.49%
Stomatal index (100%)	Control	42.72	1.00	41.20	1.45	-3.56%
	Spring	41.48	1.36	44.78	0.65	7.93%

4.3.1 Leaf biomass measurement

The single leaf dry mass of control site plants was increased by14.24% after growing in e[CO_2] chambers relative to a[CO_2] along with a 28.15% increase in total aboveground biomass (Figure 4.3.1). However, spring site plants decreased their single leaf dry mass by 10.02% when exposed to e[CO_2] compared with plants in a[CO_2], and showed only a 4.54% increase in total aboveground biomass, suggesting that *Plantago* selected from the spring site (and subjected to multi-generational high [CO_2]), may be somehow more adapted and less able to respond to the elevated CO_2 imposed in the chamber. It is interesting that plants taken from the control site, demonstrated a set of 'predictable' responses to high CO_2 at a growth and physiological level that have been found on many previous occasions – more biomass (Klus *et al.*, 2001) and larger leaves (Ainsworth & Long, 2005), whilst in contrast, those from the spring appear to lack this responsive ability. This is confirmed from the SLA data. The change in SLA for control site plants between e[CO_2] and a[CO_2] is as might be predicted from previous studies, particularly meta-analysis of very many datasets (Ainsworth *et al.*, 2002; Poorter & Navas, 2003) and declined by 5.29%. In contrast, the SLA of plants selected from spring actually increased in e[CO_2] compare with a[CO_2]. The reduction often observed in SLA in response to CO_2 is considered to reflect increased leaf thickness (Taylor *et al.*, 2003) and is also sometimes associated with the accumulation of starch (Lee *et al.*, 2005). It seems that the general higher single leaf biomass and single leaf area, as well as lower SLA in spring plants than control plants suggesting some adaptation to the high [CO_2], possibly reflecting different leaf anatomy or biochemistry which is yet to be elucidated.

The effect of original habitat on single leaf dry mass and single leaf area was significant, but not for either aboveground mass or SLA (Table 4.3.2), although there was a clear trend apparent for both of these traits. The site and CO_2 interaction effect ($P \leq 0.05$, post hoc Sidak's test) significantly influenced single leaf area indicating that individual leaf area may vary in response to growth CO_2concentration, depending on site or origin. Interestingly, there was no significant main effect of CO_2 detected on aboveground biomass but a clear trend

for larger plants was apparent e[CO_2] irrespective of whether plants originated from spring or control sites. Both aboveground biomass and SLA exhibited Family nested within site (Family (Site)) significance implying different families have different responses to growing conditions within control and spring sites. All leaf traits except above ground biomass respond significantly to CO_2 x family (site) suggesting that, again when maternal plants were considered, there is a carbon dioxide treatment and original [CO_2] concentration interaction effect on the plants physiology traits. There was also a significant Chamber (CO_2) effect for some of the traits but no significant difference on the level of Site x Chamber (CO_2). This suggests that when chamber effects were considered nested within site there were no significant interaction effects of CO_2 treatments and original environment [CO_2].

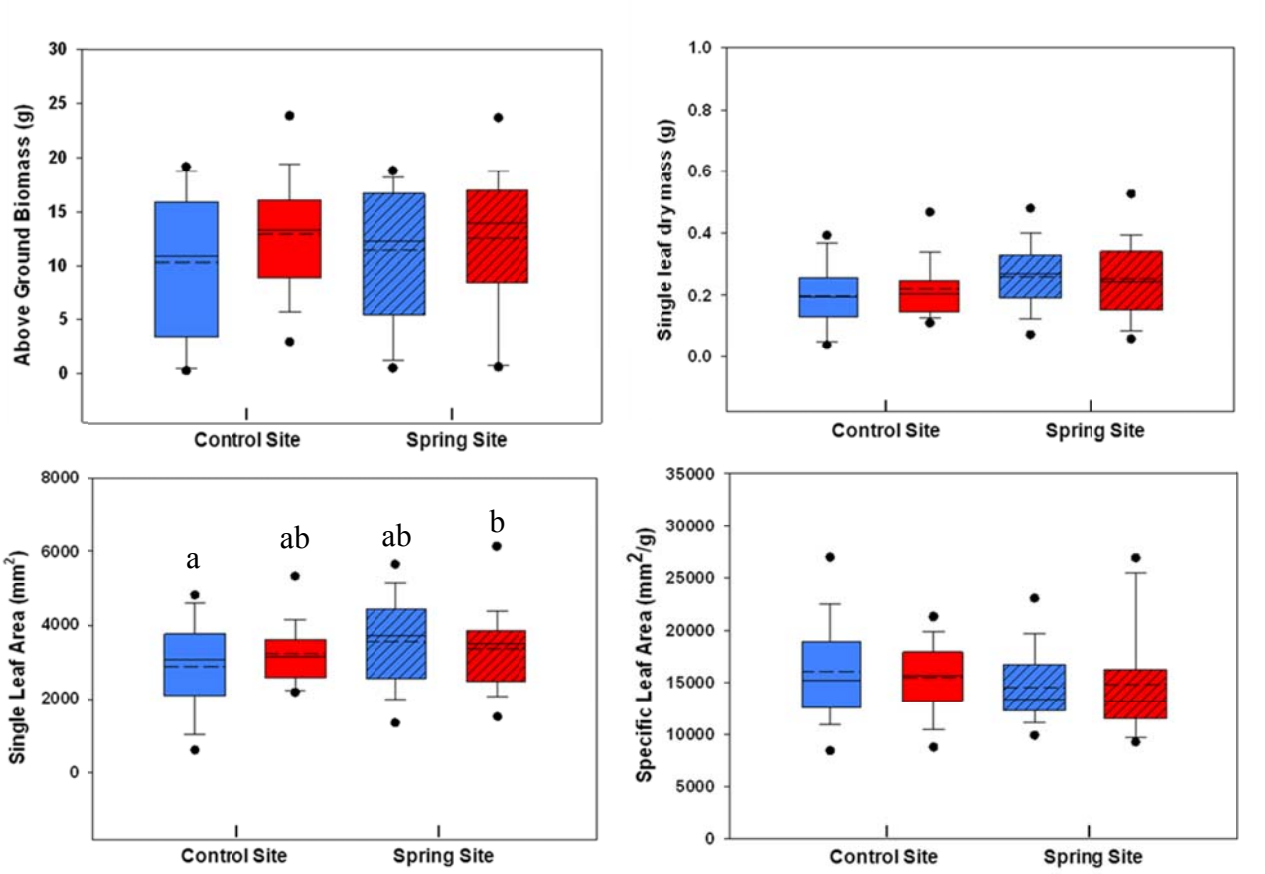


Figure 4.3.1 Leaf characteristics of *Plantago*: Aboveground biomass, single leaf area, single leaf dry mass and specific leaf area. *Plantago* control site in a[CO_2]; *Plantago* control site in e[CO_2]; *Plantago* spring site in a[CO_2]; *Plantago* spring site in e[CO_2]. The central line in each box plot shows the interquartile range and median, and the dash line shows the mean of whole data range; Whiskers indicate the 5^{th}/95^{th} percentiles. Each dot indicates the observation is an outlier data. Letters above each bar represented the post-hoc test results. Common letter indicates there is no significant difference (post hoc Sidak's test; $P \leq 0.05$).

Table 4.3.2 Statistical analysis of leaf mass traits shown in Figure 4.3.1

		Aboveground biomass		Single leaf biomass		Single leaf area		Specific Leaf Area	
Source	**d.f**	***T***	***P***	***T***	***P***	***T***	***P***	***T***	***P***
Site	1	0.002	0.963	6.126	**0.013 ***	4.426	**0.035 ***	1.263	0.261
CO_2	1	3.698	0.054	0.021	0.886	0.024	0.876	0.006	0.941
Site x CO_2	1	2.128	0.145	2.731	0.098	4.898	**0.027 ***	3.135	0.077
Family (Site)	16	31.031	**0.013 ***	20.016	0.220	14.190	0.585	33.503	**0.006 ****
CO_2 x Family (Site)	16	24.262	0.084	40.611	**0.001 *****	27.473	**0.037 ***	29.914	**0.018 ***
Chamber (CO_2)	6	5.020	0.541	15.770	**0.015 ***	13.160	**0.041 ***	17.455	**0.008 ****
Site x Chamber (CO_2)	6	1.274	0.973	1.601	0.952	1.956	0.924	3.161	0.788

Generalized Linear Models was used. The t value and the level of significant are represented.

Significance level: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$, where no * was reported data were not significant.

4.3.2 Epidermal cell measurement

There was no significant site effect detected in epidermal cell size (ECS), epidermal cell number per leaf and SD, however, there was a significant carbon dioxide treatment effect on epidermal cell number (Table 4.3.3). Control site *Plantago* ECS was reduced by 3.54% while cell number per leaf increased by 19.99% when exposed to e[CO_2] compared to a[CO_2] (Figure 4.3.2). Whereas, spring site *Plantago* showed a bigger reduction of ECS (12.42%) with less increase of cell number per leaf (3.22%) under e[CO_2] relatively to a[CO_2]. Once again, the acclimation to e[CO_2] observed in *Plantago*– increased cell production and reduced ECS – is similar to that observed very often before in previous experiments on a wide range of plant species (Ferris *et al.*, 2002; Tricker *et al.*, 2004). Very interestingly, we observed an intriguing stomatal development response to e[CO_2]. For both control and spring plants, SD were increased in response to elevated CO_2, which differs to the more frequently observed reduction in SD. Most important though was the site x CO_2 interaction for stomatal index ($P \leq 0.05$, post hoc Sidak's test) (Table 4.3.3) which reveals a difference in the response of this important developmental trait to e[CO_2] in the two groups of plants with a reduction and a stimulation observed in control and spring plants respectively. To confirm, the SI from different sites showed the opposite trend with a significant site effect on SI; the SI of control site *Plantago* decreased 3.53% in e[CO_2] compared with a[CO_2], whereas the SI of spring site *Plantago* was 7.93% higher in e[CO_2] (Figure 4.3.2). The cell imprints (Figure 4.3.3) clearly showed that ECS of plants grown under e[CO_2] was larger and fewer stomata in plants leaf compared to the plants grown under a[CO_2] irrespective of site of origin. Figure 4.3.3c & 4.3.3f are the cell imprints of the same plants in Figure 4.3.3b & 4.3.3e grown in a glasshouse (University of Southampton, Southampton, UK) under ambient environment after the experiment. The ECS of plants grown in a glasshouse under ambient environment was bigger and has lower SD compared with what was observed when they grew under e[CO_2] during experiment.

There was significant family (site) effect detected on leaf cell traits overall as well as the CO_2 x Family (Site) effect, implying that the genetic variation may respond

to the CO_2 selection on these traits (Table 4.3.3). There was also a significant chamber (CO_2) effect, including Site x Chamber (CO_2) detected on ECS and SD, which could be due to slightly different chamber temperature.

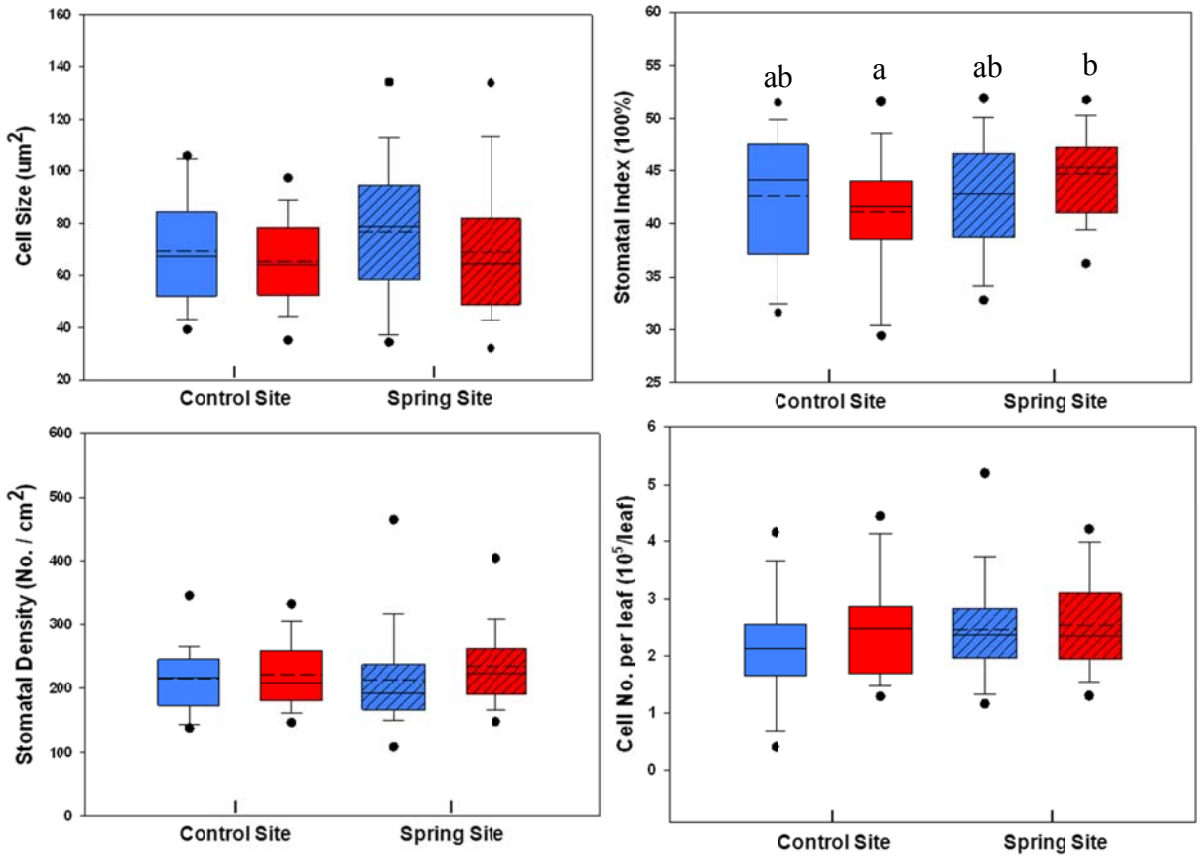


Figure 4.3.2 Cell characteristics of *Plantago*: Epidermal cell size, epidermal cell number per leaf, stomatal density and stomatal index. *Plantago* control site in a[CO_2]; *Plantago* control site in e[CO_2] *Plantago* spring site in a[CO_2] *Plantago* spring site in e[CO_2]. The central line in each box plot shows the interquartile range and median, and the dash line shows the mean of whole data range; Whiskers indicate the 5^{th}/95^{th} percentiles. Each dot indicates the observation is an outlier data. Letters above each bar represented the post-hoc testing. Common letter indicates there is no significant difference (post hoc Sidak's test; $P \leq 0.05$).

Table 4.3.3 Statistical analysis of leaf cell traits shown in Figure 4.3.2.

Source	d.f	Epidermal Cell Size		Cell Number Per Leaf		Stomatal Density		Stomatal Index	
		T	***P***	***T***	***P***	***T***	***P***	***T***	***P***
Site	1	0.386	0.534	1.379	0.240	0.131	0.717	5.539	**0.019 ***
CO_2	1	3.292	0.070	3.956	**0.047 ***	3.675	0.055	0.608	0.436
Site x CO_2	1	1.326	0.250	2.524	0.112	1.410	0.235	5.052	**0.025 ***
Family (Site)	16	46.403	**0.000 *** **	41.865	**0.000 *** **	38.267	**0.001 *** **	51.808	**0.000 *** **
CO_2 x Family (Site)	16	47.290	**0.000 *** **	34.482	**0.005 ** **	36.285	**0.003 *** **	27.755	**0.034 ***
Chamber (CO_2)	6	33.301	**0.000 *** **	6.958	0.325	15.755	**0.015 ***	2.726	0.842
Site x Chamber (CO_2)	6	39.697	**0.000 *** **	15.866	**0.014 ***	22.118	**0.001 *** **	9.242	0.160

Generalized Linear Models was used. The T value and the level of significant are represented.

Significance level: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$, where no* was reported data were not significant.

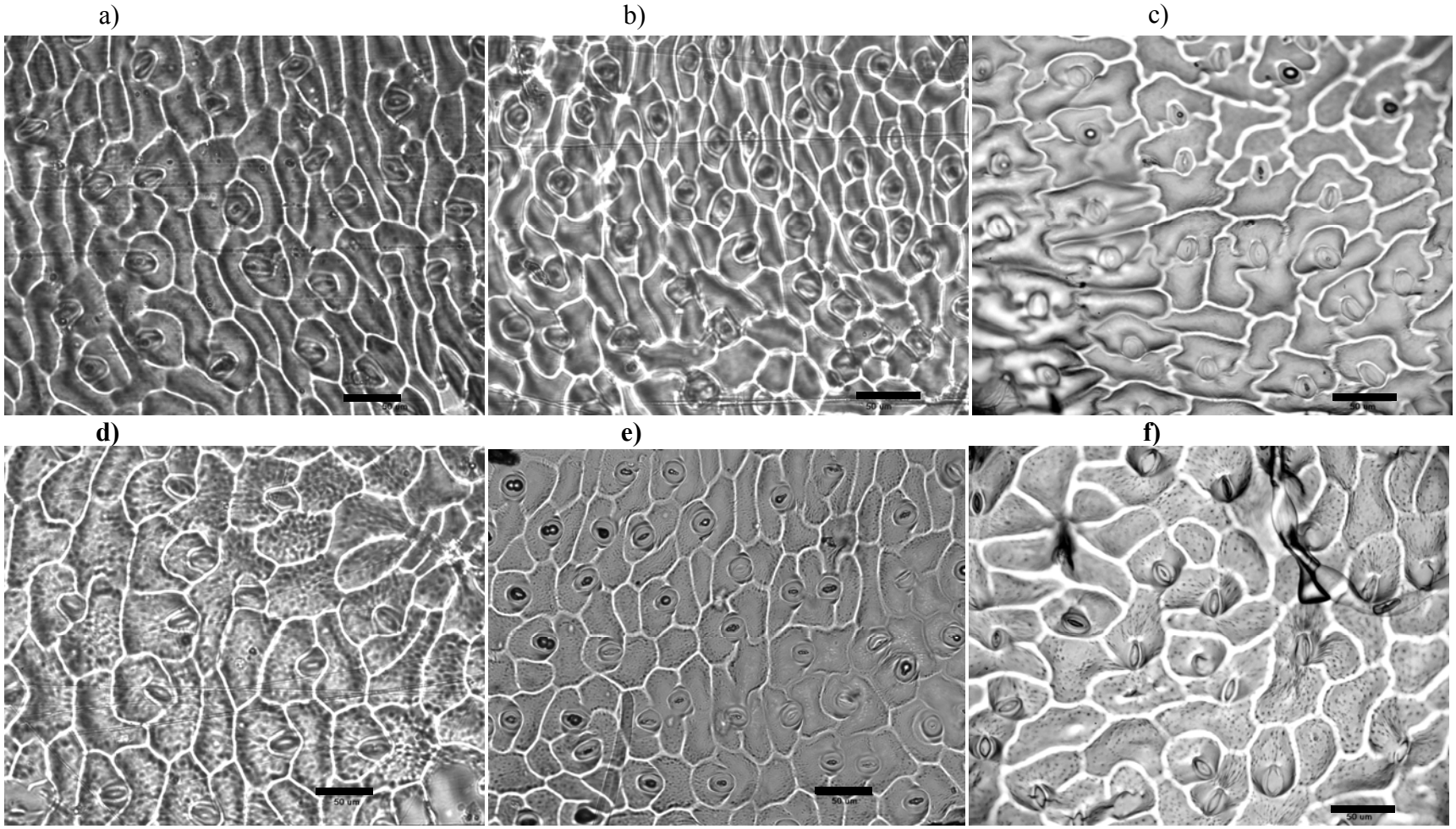


Figure 4.3.3 Cell imprints of Plantago from elevated and ambient CO_2 conditions from both sites. a) Cell imprint of control site *Plantago* grown under a[CO_2]. b) Cell imprint of same maternal Plantago grown under e[CO_2]. c) Cell imprint of the same plant as in figure b grown in glasshouse under natural air after experiment. d) Cell imprint of spring site Plantago growing under a[CO_2]. e) Cell imprint of same maternal Plantago grown under e[CO_2]. f) Cell imprint of the same plant as in figure f grown in glasshouse under natural air after experiment. The black bar in each picture on the right bottom represents a 50µm scale bar. Plants in image a and b were from same maternal mother, as were plants in image d and e.

4.3.3 Coefficient of variation

The coefficient of variation (CV) ranged widely on the measured traits, from 10.7% in SI to 52.9% in single leaf biomass (Figure 4.3.4.a). The phenotypic variation of spring site did not show a consistent trend of being either higher or lower compared with the control site, indicating that growing in the high-CO_2 spring does not narrow the genetic variation. Single leaf area and total aboveground biomass have very similar CV implying carbon dioxide is not a strong selective agent on those traits. Whereas the CV of single leaf biomass and SD showed dramatic differences from the two sites suggesting those traits might show adaptation to long term CO_2 exposure over several generations.

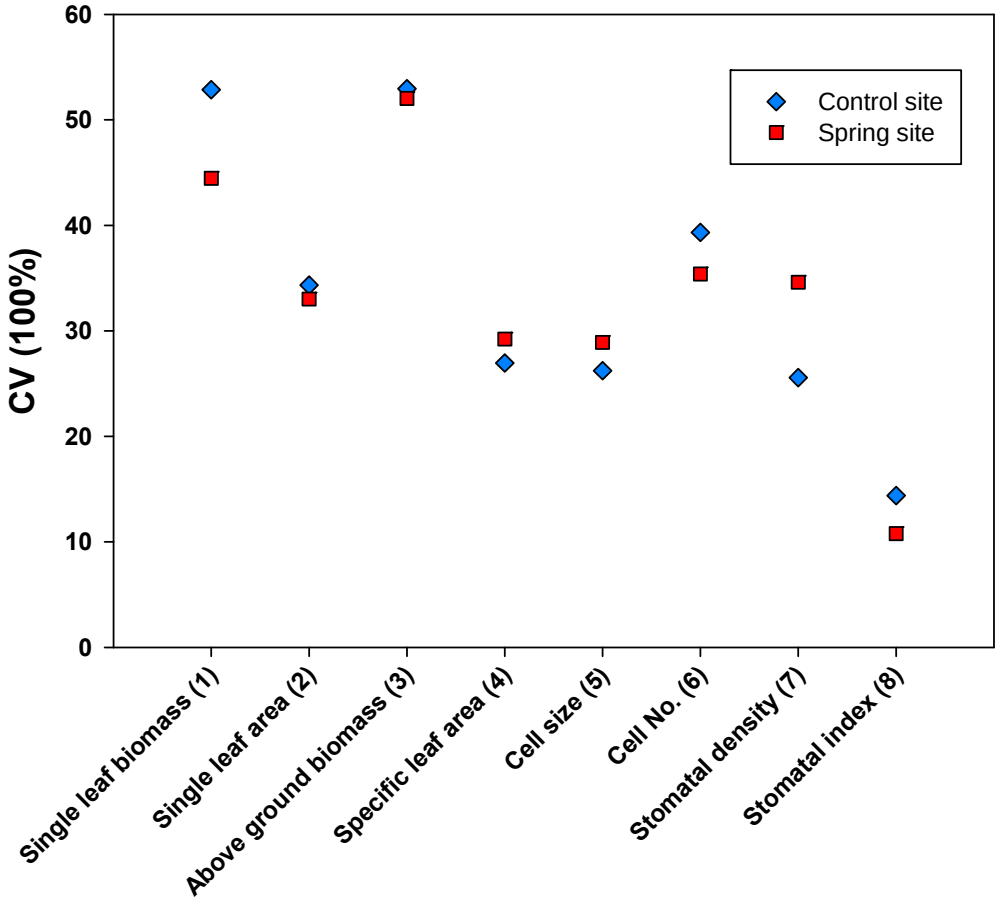


Figure 4.3.4 The Coefficient of variation of leaf and cell traits in *Plantago*. The coefficient of variation was calculated for each sites and the mean of physiology measurement for each sites were showed on the figure to identify whether there is restriction on variation induced by high [CO_2].

4.3.4 Genetic variation between samples based on AFLP analysis

Primer set CCCCT failed to be recognised by Peak Scanner therefore only five primer set results were used in this experiment. All 136 *Plantago* plants used in the morphological experiment were fingerprinted for five primer pairs individually and scored at 208 segregating loci in total. However, none of the five primer pairs uniquely fingerprinted all the *Plantago* plants. Plants grown from seeds collected from the same maternal plants were considered to be the same family; therefore, eighteen families in total were used for AFLP analysis.

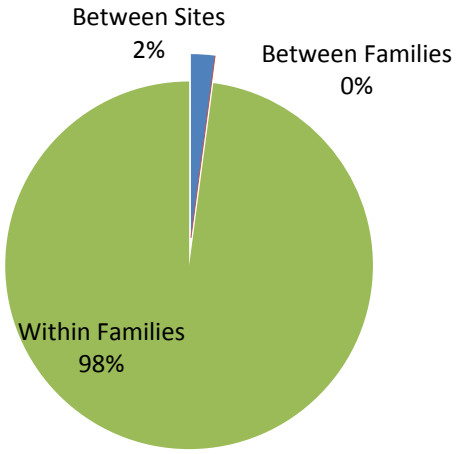
The genetic variation of plants grown in the spring sites and outside of the spring site were analysed by assessing the AFLP result, which identified the genetic variation. The analysis of molecular variance (AMOVA) indicated that most of the genetic variance (98%) fell into each family whereas 0% genetic variation was detected between families, and with a small amount (2%) of genetic variation detected between control site and spring site (Figure 4.3.5.a). None of the genetic variation detected with these five AFLP primer pairs was significantly different at the confidence level of 95% (The statistic of AMOVA is in Appendix II). However, the PhiRT which measures the comparison the genetic variance between sites with the total genetic variances within all plants showed there is a trend of genetic variation in response to e[CO_2].

The principal coordinate analysis (PCoA),which plot plants, based on the association among the genotypes(the AFLP results of each plant) and showed that the plants from control and spring site were not distinguishable from each other (Figure 4.3.5.b). However, spring site families 2, 3, 4, 7 and 8 and control site families 2, 4, 7 and 8 were grouped in opposite position in the analysis result, implying there is potentially genetic diversity between two sites induced by e[CO_2].

The UPGMA dendrogram represented the genetic distance between plants from the two sites (Figure 4.3.6). This result confirmed the AMOVA analysis that most of the genetic variation occurred within each family and did not show a clear

genetic diversity between the two sites of origin. We can conclude that the AFLP results did not resolve genetic structure between families within a site or structure between the spring and control sites. This could be due to gene flow between the sites that overcame the selective pressure caused by high [CO_2] in the spring site.

a)



b)

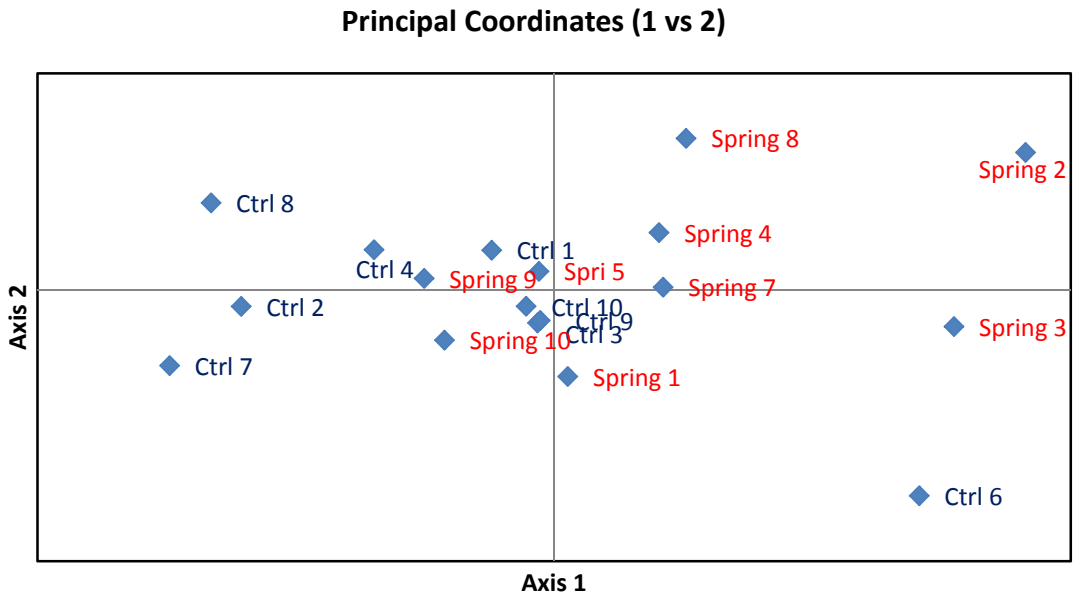


Figure 4.3.5 The statistic graphs of AFLP analysis. a) The pie chart summarizes AMOVA analysis and presented the partitioning of molecular variance within and between families and sites. b) The scatter plot presents PCoA analysis and indicates the genetic relationships between eighteen families. Variation explained by 75.10% PC1 and 12.55% PC2. (Ctrl represents plants collected from the control site and Spring represents plants collected from the spring site) (Data was collected by Carrie Marling (third year project student) and the author)

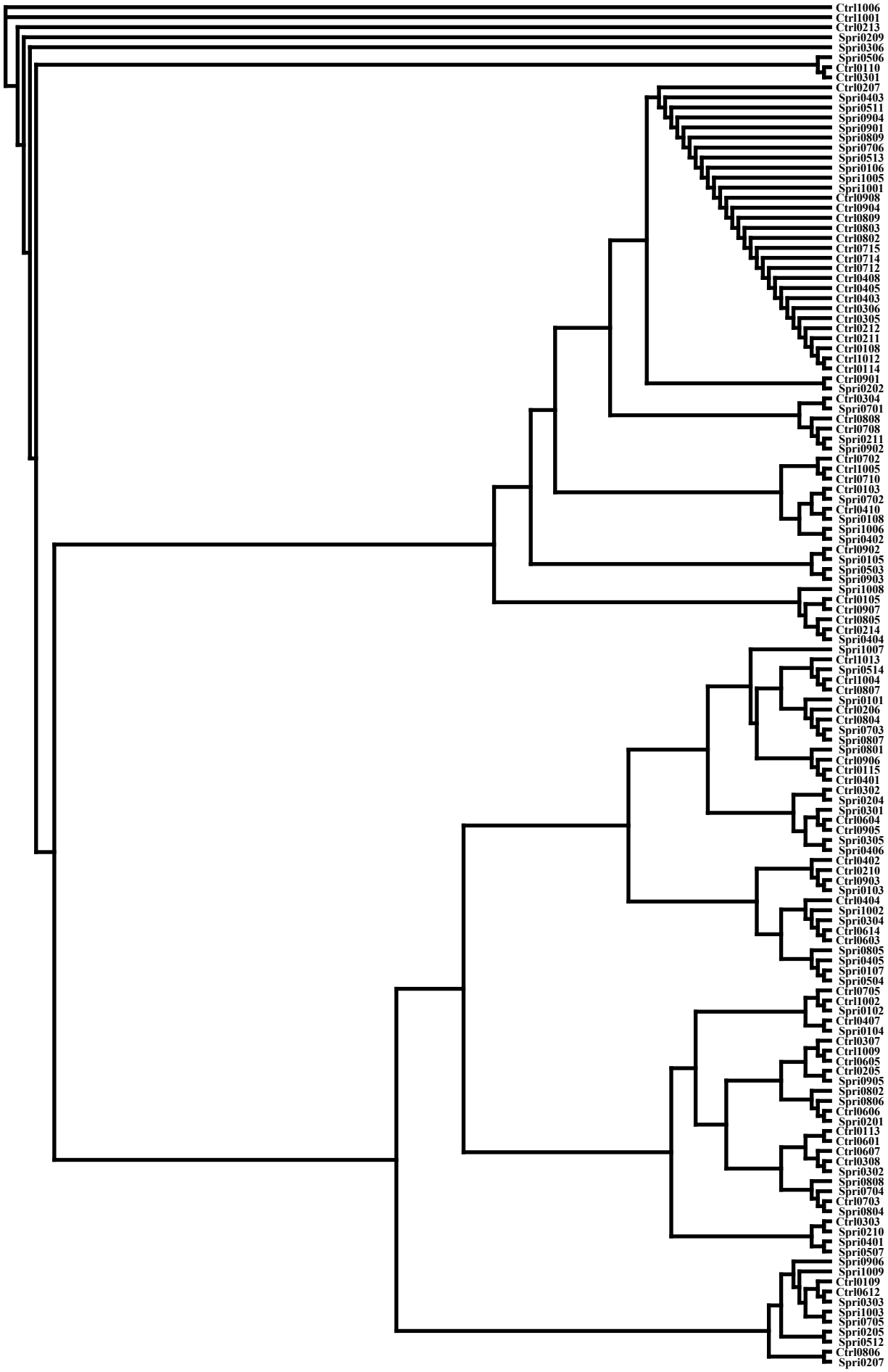


Figure 4.3.6 AFLP UPGMA dendrogram of *Plantago*. The Dendrogram is created based on restrict fragment distance value. Plants labelled with "Ctrl" were collected from the control site and "Spri" were collected from the spring site.

4.4 Discussion

Increasing atmospheric carbon dioxide concentration has a great effect on global climate and also on processes in the terrestrial environment, including a direct effects on plants, plant processes, plant assemblages and ecosystem functioning (IPCC, 2007). Comparing *Plantago lanceolata* from a naturally high-CO_2 spring in Italy with the same species which shares a similar habitat, but with a much lower CO_2 concentration has enabled us to deduce likely effects of adaptation (genetic change) to elevated atmospheric CO_2, resulting in a greater understanding of possible adaptive responses to this environmental change.

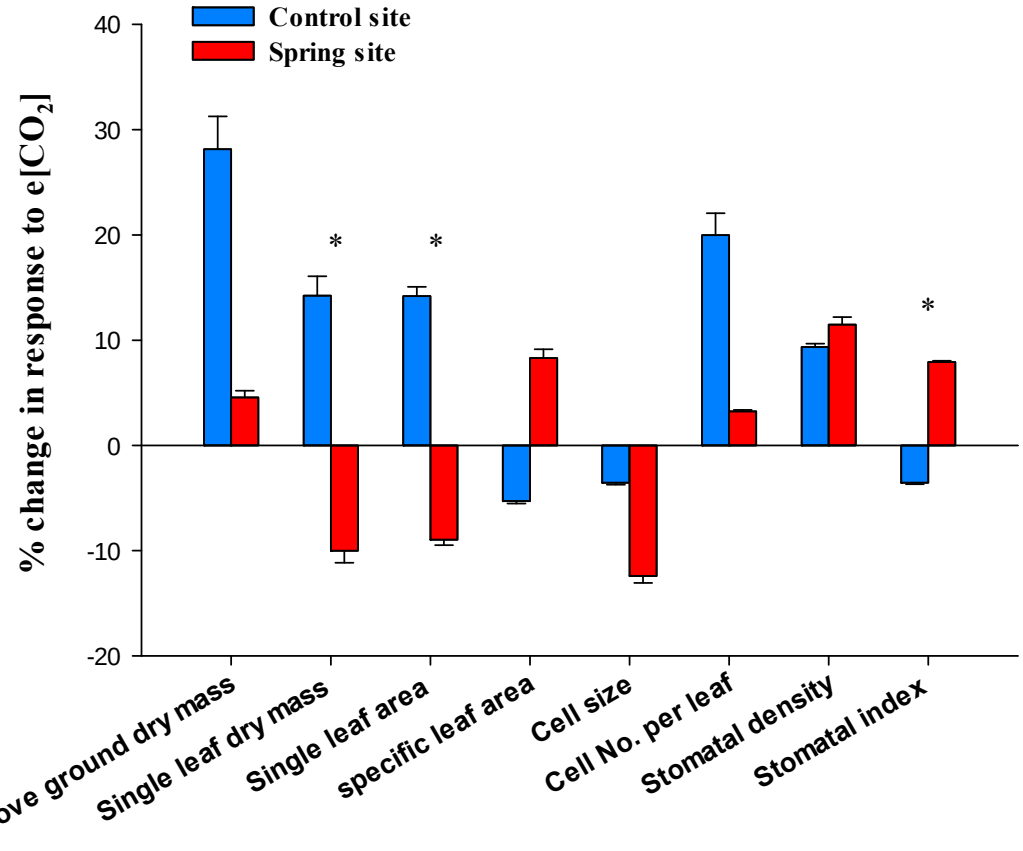


Figure 4.4.1 The percentage change of each *Plantago* morphological measurements. The percentage change were calculated by using the difference of each traits expression under e[CO_2] relative to a[CO_2]. * represents there is a significant site effect on morphological traits.

There was a carbon dioxide effect detected on total aboveground biomass in this experiment, with a 28.15% and 4.54% increase under e[CO_2] compared to a[CO_2] in control and spring site respectively (Figure 4.4.1). This follows the discovery by Klus *et al.* (2001) that e[CO_2] has significant effects on belowground mass, total mass and root:shoot ratio with higher means compared with a[CO_2] in a *Plantago* open-top chamber experiment. This aboveground biomass increase is generally observed in other experiments. The increased percentage shown by control site plants is similar to the average aboveground biomass increase in C_3 species (20%) observed from FACE experiments(Ainsworth & Long, 2005). In another study, a dramatic increase of 408% in *Arabidopsis* dry mass was observed when comparing plants growing at 710ppm to those growing under 250ppm [CO_2] (Tonsor & Scheiner, 2007). However, spring site plants showed much less increase in response to e[CO_2] (CO_2 effect, $P = 0.054$). Similar phenomena have been detected in *Arabidopsis thaliana*, *Bromus erectus* and *Chlamydomonas* that after many generations being grown under e[CO_2] (five generations for *Arabidopsis* and *Bromus*, and 1000 generations for *Chlamydomonas*), less or no increase in biomass in response to a rise in CO_2 was detected compared to the non-selected line (ones that were grown in the ambient present-day CO_2environment) (Ward *et al.*, 2000; Collins & Bell, 2004; Steinger *et al.*, 2007). This suggests that the dramatic increase of aboveground biomass following short-term e[CO_2] treatment is transient and in the long-term, following multi-generational exposure, plants will become adapted to high CO_2 with only a limited response.

Contrasting change in single leaf biomass, single leaf area and SLA were also apparent from two sites and these are intriguing (Figure 4.4.1). No significant effects of [CO_2] on leaf thickness (implied from SLA) at the population level were detected in this experiment, as was shown for paper birch in the AspenFACE experiment (Riikonen *et al.*, 2010). However, in this experiment, the significant family level site effect on SLA as well as total aboveground biomass indicated that original growth environment was more important than [CO_2] treatment for *Plantago*. Klus *et al.* (2001) discovered population or even family especially influence the traits associated with mass, regardless of the [CO_2] in the

atmosphere of growth, suggesting that when the genetic variation was fixed, applying CO_2 treatment will not restrict the genetic variation easily.
Control site plants showed a larger increase in total aboveground biomass than the increase in single leaf dry mass company with a reduced SLA. The thickness of poplar leaves grown under e[CO_2] have been found significantly increased compare to the poplar leaves grown under a[CO_2] in AspenFACE (Oksanen *et al.*, 2001; Oksanen *et al.*, 2004). The results observed in this experiment indicating that control site *Plantago* probably produced more and thicker leaves under e[CO_2]. Spring site plants generally showed lower single leaf dry mass with an overall increased total aboveground biomass under e[CO_2] compared with a[CO_2] implying that spring site plants also produced more leaves under e[CO_2] at the same as the control site plants. The leaf number were counted and confirmed that both control and spring site plants produced more leaves when grown under e[CO_2] relative to a[CO_2],with a significant interaction effect of CO_2 treatment and original environment (Appendix III).The studies of Wieneke *et al.* (2004)on the herb species, *Sanguisorba minor*, showed a similar phenomenon. In their study, plants that had been exposed for the long-term to high [CO_2] produced more leaves when growing under e[CO_2] than a[CO_2]. Increase in leaf number was also generally observed in FACE experiments with twelve species showing on average an 8% increase in leaf number under e[CO_2] compared with a[CO_2] (Ainsworth & Long, 2005). Control plants exhibited reduced SLA in e[CO_2], suggesting thicker leaves, or possibly more starch-filled leaves, as is seen in many species, including poplar (Taylor *et al.*, 2003). Spring plants showed a contrasting result with higher SLA in e[CO_2], suggesting thinner leaves or those with less starch. So although spring site *Plantago* leaves were thinner but bigger and heavier compared to control site *Plantago* leaves when grown in common a[CO_2] environment, both site plants developed similar leaf size and biomass and thickness under e[CO_2] (Table 4.3.1), indicating there might be a bottleneck effect with high [CO_2] induced evolutionary adaptation of *Plantago* leaf characteristics.

ECS consistently decreased in both sites under e[CO_2] with 3.54% (control) and 12.42% (spring) respectively(Figure 4.4.1), again suggesting that the magnitude of response to e[CO_2] may be influenced by original growth environment – spring or control site. A contrasting result was found with increasing ECS in e[CO_2] in

poplar (Taylor *et al.*, 2003) and paper birch from AspenFACE (Riikonen *et al.*, 2010), with Ranasinghe and Taylor (1996) suggesting that enhanced leaf growth in e[CO_2] occurred primarily because of an e[CO_2] effect on leaf cell size. In their study, cell division and expansion were temporally independently regulated by e[CO_2] on their own processes during different stages of growth. Despite this, however, cell number was also increased in *Phaseolus* leaves exposed to the high [CO_2] treatment. Sunflower leaves revealed smaller cells are associated with rapid rates of cell production (Granier & Tardieu, 1998; Taylor *et al.*, 2003), whereas trees such as poplar and paper birch, cell expansion and division are more determined by environmental and temporal conditions (Cockcroft *et al.*, 2000; Rose *et al.*, 2000; Taylor *et al.*, 2003). It now seems apparent that increased cell production may be considered as a general mechanism for enhanced growth in elevated CO_2 given the supporting data in a wide range of plant species including *Phaseoulus vulgaris*, *Glycine max* and wheat (Ranasinghe & Taylor, 1996; Masle, 2000; Ainsworth *et al.*, 2006).

Elevated [CO_2] also resulted in increased SD 9.36% and 11.49% for control and spring site plants respectively (Figure 4.4.1), which is in contrast to the general perception that stomatal numbers decline as atmospheric CO_2 rises (Ainsworth & Rogers, 2007). This result has been confirmed from both geological timescales (Franks & Beerling, 2009) and from more recent herbarium specimens (Lammertsma *et al.*, 2011) although the results for future concentrations of CO_2 are more varied (Woodward *et al.*, 2002). A review written by Casson and Gray (2008) explained that although the trend is a reduction of SD and SI associated with growth under increased concentration of atmospheric CO_2(Royer, 2001; Hetherington & Woodward, 2003), only three-quarters of the species analysed showed reductions in stomatal density in a study of 100 species. They also mentioned that species with higher initial stomatal densities tended to be more responsive to increased CO_2 irrespective of the habitat alteration (Woodward & Kelly, 1995; Casson & Gray, 2008) which may explain the greater increase in stomatal density in the plants from spring site. *Arabidopsis* mutants with reduced *HIC* (high carbon dioxide, controls CO_2 regulated stomatal development regulation) expression showed a similar response to increasing CO_2 with

increased SD and SI (Gray *et al.*, 2000) implying the ability of detecting CO_2 increase in *Plantago* might be weakened after long-term growth under high [CO_2].

The change in SI in *Plantago* for control- and spring-grown plants gives some insight into likely adaptive effects in this important developmental trait. A strong site x CO_2 effect was apparent – with a decrease of 3.56% and increase of 7.93% in control and spring site plants in e[CO_2] respectively (Figure 4.4.1). The same phenomenon was discovered from an experiment on *Agrostis canina*, which showed reduced SI on the abaxial surface under conditions of elevated [CO_2] only on plants from outside of CO_2 spring site (Haworth *et al.*, 2010). This provides some supporting evidence of a general phenomenon of adaptive response and since many of the genes regulating stomatal development and patterning are now known, these may be good targets for investigating functional gene diversity in our future research. Paper birch from AspenFACE also showed increased SI under elevated CO_2(Riikonen *et al.*, 2010) and so did *Zea mays* (Driscoll *et al.*, 2006).Different SI change in response to increased [CO_2] were observed with increase (Case *et al.*, 1998; Lawson *et al.*, 2002; Driscoll *et al.*, 2006), decrease (Case *et al.*, 1998; Hetherington & Woodward, 2003) and no alteration (Peñuelas & Matamala, 1990), but the mechanisms are still unknown.

Long-term exposure to elevated [CO_2] may cause adaptive modifications of stomatal numbers (Woodward, 1987; Paoletti & Gellini, 1993; Bettarini *et al.*, 1998), perhaps to improve the maximum *g*s (if stomatal numbers are increased),therefore benefiting from atmospheric [CO_2] change, higher SD and SI implies that more stomata were produced. *Plantago* from the spring site are associated with lower photosynthetic capacity and *g*s (Nakamura *et al.*, 2011), which might be the reason why *Plantago* produced more stomata under high [CO_2] to maintain or improve performance.

A different response was detected between the spring and control site *Plantago* exposed to high [CO_2] on SLA and SI in this experiment. It is widely believed that the microevolutionary response is that growth, development, and carbon accumulation of individual plants as well as the long-term dynamics of populations might fundamentally deviate from what would be expected from

short-term experiments that do not take selection into account and are largely documenting acclimation rather than adaptation (Schmid *et al.*, 1996; Ward *et al.*, 2000; Wieneke *et al.*, 2004). However, although there is considerable evidence to support microevolutionary effects in response to past, low CO_2 concentrations, there is limited evidence to suggest that adaptation occurs in response to elevated future CO_2, although experimental results in this area are limited (Leakey & Lau, 2012). This explains the different response between spring site plants – which have been selected by high atmospheric CO_2 after years of growing in a rich-CO_2 spring, and control site plants with short-term exposure to high [CO_2]. There is also a significant CO_2 x family (Site) effect throughout the whole experiment. The partitioning of variation between families and populations plays a critical role in determining the magnitudes of responses to selection (Klus *et al.*, 2001). This strong CO_2 selection is supported by the theory of Long *et al'*s (2004)that a direct interaction of the inner surfaces of the guard cells of stomata and the mesophyll with the atmosphere as they are the only exposed organs of plant.

Onoda *et al.* (2009) suggested that if high [CO_2] from the original sites acts as strong selective agent, there will be bottleneck selection induced by high [CO_2] on the traits which response to [CO_2], therefore reduce the genetic diversity on the particular traits and result in a smaller CV in plants from the spring site compared with plants from the control site. However, our CV results did not show a consistent trend of small CV on neither of the plants' morphological traits we measured in this experiment which is the same results found in the Japan CO_2 spring (Onoda *et al.*, 2009), suggesting that there might be other factors that counteract the selection effect of high [CO_2]. Both single leaf biomass and total aboveground biomass showed relatively high CV suggesting that they might have been selected by current or past [CO_2] or they have been influenced by various environmental heterogeneities (Onoda *et al.*, 2009).

Although the *Plantago* showed strong acclimation to original growing sites, our AFLP analysis results did not suggest a clear segregation at the genetic level between plants from two growth sites as seen in the CV analysis. 98% of genetic variation fell within families and only 2% of total variation identified between plants from two sites. Nakamura *et al.* (2011)noticed that there is much less gene

flow between *Plantago asiatica* grown in inside and outside of Japan CO_2 spring population than the gene flow that happened within *Plantago asiatica* located outside of spring which is further spatially separated. They believe it is due to the high percentage (more than 30%) of seeds produced by selfing within the spring that could not survive in ambient [CO_2] environment. In our experiment, there is no evidence of whether *Plantago* from control and spring sites were reproductively isolated from each other, *i.e*. they formed into two subspecies after hundreds of generations. Knowing that *Plantago lanceolata* is self-incompatible, using wind or insect as pollination media potentially increased the chance of gene flow between two populations from two sites. This gene flow theoretically would diminish the effect of environment selection pressures on local plant genetic differentiation and random drift (Rohlf & Schnell, 1971). However Bos *et al*(1986) showed that gene flow in *Plantago lanceolata* is also restricted due to its pollination method, wind-pollinated, which restricts the pollen flow distance compared to the plants that are insect pollinated. This could explain why there were five families from the spring site and four families from the control site that were more correlated in the PCoA results respectively, whereas, other families were mixed between spring and control site. These more correlated families might have less possibility of gene flow from the other site.

There are three ways for plants to response to environmental change: phenotypic plasticity if the change remains within the tolerance limit of the plant, genetic evolution to adapt to the long-term environment change; and movement to preferred environment in geography to avoid the change (Davis *et al.*, 2005; Jump & Penuelas, 2005; Chevin *et al.*, 2010). This experiment presents clearly significant phenotypic acclimation to [CO_2] after long-term exposure to different [CO_2] environments. However, there is not enough evidence ($P = 0.09$) on whether growing in high [CO_2] will induce genetic differentiation, although given the strikingly different phenotypic responses to e[CO_2], there is certainly some indication that spring plants have become adapted to high CO_2 and no-longer respond to this treatment in the same way as control plants (Woodward, 1999). Past studies have revealed rather limited contrasting responses to e[CO_2] in spring and control plants (Raschi *et al.*, 1999) although the stomatal responses have also been observed previously by Haworth *et al.* (2010). Fingerprinting the *Plantago*

samples with more AFLP primers or other molecular markers will help to provide stronger evidence on whether high [CO_2] acts as a selective agent on plant evolution and which direction of evolution is favoured by it. It could be that epigenetic rather than genomic change occurred in plants between two environments. This could also be the reason of the visibly opposite morphological response to e[CO_2] but no clear genetic diversity as what Nakamura *et al.* (2011) suggested.

4.5 Conclusion

This experiment has detected a significant CO_2 effect, site of origin effect and also interaction effect including family factor on plant morphologic data. Some traits respond differently to e[CO_2] for plants from the two sites which could be due to the bottleneck of high [CO_2] selection on leaf anatomy. The microenvironment where the plants originate from appears to have stronger effects on plant biomass over the carbon dioxide selection, whereas cell and stomatal morphology showed more adaptation to high [CO_2]. It is predicted that bigger plants with more branches/leaves will be produced in adaptation to future [CO_2], and on each leaf, ECS is getting smaller due to rapid cell division and production but higher stomatal index for optimum use of CO_2. However, the mechanism of adaptation is still unknown.

Genetic diversity between plants from two sites was quantified by AFLP technology. Although no significant genetic diversity was detected and the plants within each site showed high genetic variation in this experiment, PCoA results suggested some differentiation amongst families. Further research is required on this subject to understand the adaptation to future climate change.

Chapter 5: Transcriptomic responses of *Plantago* from spring and control sites subjected to elevated CO_2 investigated using RNA-seq

5.0 Overview

Following the findings in Chapter 4, transcriptome sequencing was carried out on young *Plantago* leaves. The leaves were collected from the chamber experiment to identify the transcriptomic change in response to different carbon dioxide concentration (390 ppm and 700 ppm) from both control site and spring site *Plantago*. *De novo* assembly was performed on the sequencing data using four species genome including *Arabidopsis*, *Oryza*, *Ricinus* and *Zea mays*as reference genomes in this experiment.

The *Arabidopsis* ortholog corresponding to each contigs generated in *de novo* assembly were then input into MapMan software (v3.5.1) for biological pathway analysis. There were several interesting pathways that identified exhibited different response to elevated [CO_2] compared to ambient [CO_2] between control site and spring site *Plantago*. These responses provided strong evidence of *Plantago* transcriptome in adaptation to high [CO_2] environment.

5.1 Introduction

Numerous studies have been carried out on plant morphological, physiological and biochemical responses to high carbon dioxide concentration ([CO_2]) but rather few have considered evolutionary adaptation(Ward & Kelly, 2004; Ainsworth & Long, 2005). Chapter four studied the possible morphological acclimation and genetic adaptation to high [CO_2], by using *Plantago* selected from two contrasting sites, a high CO_2 – spring site, and an ambient [CO_2] environment – control site. The research highlighted some important differences when these two groups of plants were exposed to elevated CO_2, with the implication that adaptive responses were apparent – plants originating from the spring did not respond to CO_2 as might be predicted from the literature and may have become 'genetically distinct' to those from the control site. Analysis of neutral molecular marker data failed to support this hypothesis, with a limited number of AFLP primers (AACAT, AACTT, CTCAG, TCCCT and AACCT), although there was some indication of genetic differentiation between some families of control and spring-grown plants. Here, for the first time, we investigate the functional genomic architecture of spring-grown and control plants and their response to elevated CO_2, using next generation high throughput RNA-Seq. The aim was to elucidate candidate or key genes that differ in expression between control and spring-grown plants and following this, to quantify the response of these genes to elevated CO_2 treatment in controlled conditions. No other RNA-seq data are currently available from plants in elevated CO_2 evolution adaptation has been studied on transcriptomic level by using transcriptome sequencing.

The RNA-seq was performed using massively parallel sequencing – the Illumina NGS technique – to identify and quantify the genes expression. Unlike the microarray technique (cDNA), RNA-seq requires a lower RNA amount to work on and produces a wider range of gene expression levels(Wang *et al.*, 2009). Furthermore, the RNA-seq data are highly replicable with little technical variation between technique replicates compared to both cDNA and Affymetrix microarray technique (Marioni *et al.*, 2008; Wang *et al.*, 2009). RNA-seq also can identify

more genes than the microarray technique on the same tissues. Kyndt *et al*(2012) detected ~2,610 more transcriptionally active regions on rice root tissue using Illumina RNA-seq technique compared to other experiments that used Affymetrix chips. This comparison also has been done on human tissues. Marioni *et al.* (2008) applied both Illumina RNA-seq and cDNA microarray on human liver and kidney tissues and found that only 57% of genes that were identified by RNA-seq were also identified in microarray. They believe it is due to the RNA-seq technique not requiring known probe sequences spotted onto array or synthesised on a chip.

The RNA-seq also allowed detection of detailed gene changes of species with non-model organisms, relatively cheaply, without the necessity of a reference sequence (Grabherr *et al.*, 2011; Schneeberger & Weigel, 2011; Xia *et al.*, 2011). The first study on transcriptomics in non-model species using NGS was performed on *Polistes metricus* (wasp) by Toth *et al.* (2007) and applied a related honey bee sequence to mapping the reads (454 sequencing), and very soon after that, *de novo* assembly method were raised and applied on *Melitaea cinxia* (Glanville fritillary butterfly) transcriptome sequencing without using any reference genome by Vera *et al.* (2008) (454 sequencing) which promoted the transcriptome study on non-model species using 454 sequencing (Ekblom & Galindo, 2010). Both Novaes *et al.* (2008) and Collins *et al.* (2008) published papers on non-model plants using *de novo* assembly in 2008. Novaes *et al.* (2008) applied 454 sequencing technology on *Eucalyptus grandis* generated 148 Mbp of EST sequences. For the first time, Collins *et al.* (2008) studied the transcriptome of *Pachycladon enysii* on Illumina sequencing platform using *Arabidopsis* as reference genome and *de novo* assembly tools. There have been a number of studies on transcriptome expression on non-model plants using the next generation sequencing (NGS) technique, including *Amaranthus tuberculatus* (Riggins *et al.*, 2010), *Scabiosa columbaria* (Angeloni et al., 2011), *Olea europaea* (Alagna *et al.*, 2009) and *Pisum sativum* (Franssen *et al.*, 2011), but none of them used Illumina platform (Strickler *et al.*, 2012). The 454 sequencing platform could provide relatively long reads which allow assembly without reference genome whereas Illumina platform provides deeper coverage as a result of large number of more accurate short reads (Ekblom & Galindo, 2010). A recent

research by Grabherr *et al.* (2011) presented the Trinity method which allowing *de novo* assembly on non-model plants without using a reference genome.

In this chapter, the RNA-seq of *Plantago lanceolata* was studied using the Illumina sequencing platform to understand the evolutionary adaptation to high CO_2 environment. *Plantago lanceolata* is an allogamous perennial herb, which is not capable of self-fertilising with diploid chromosome (2n=12, chromosome number) (Lumaret *et al.*, 1997; Bala & Gupta, 2011), and belongs to the Lamiales order (Figure 5.1.1). Due to the unavailability of *Plantago* genome sequence, the *Arabidopsis* genome, which has been well documented and fully annotated, was used as the reference genome in this analysis.

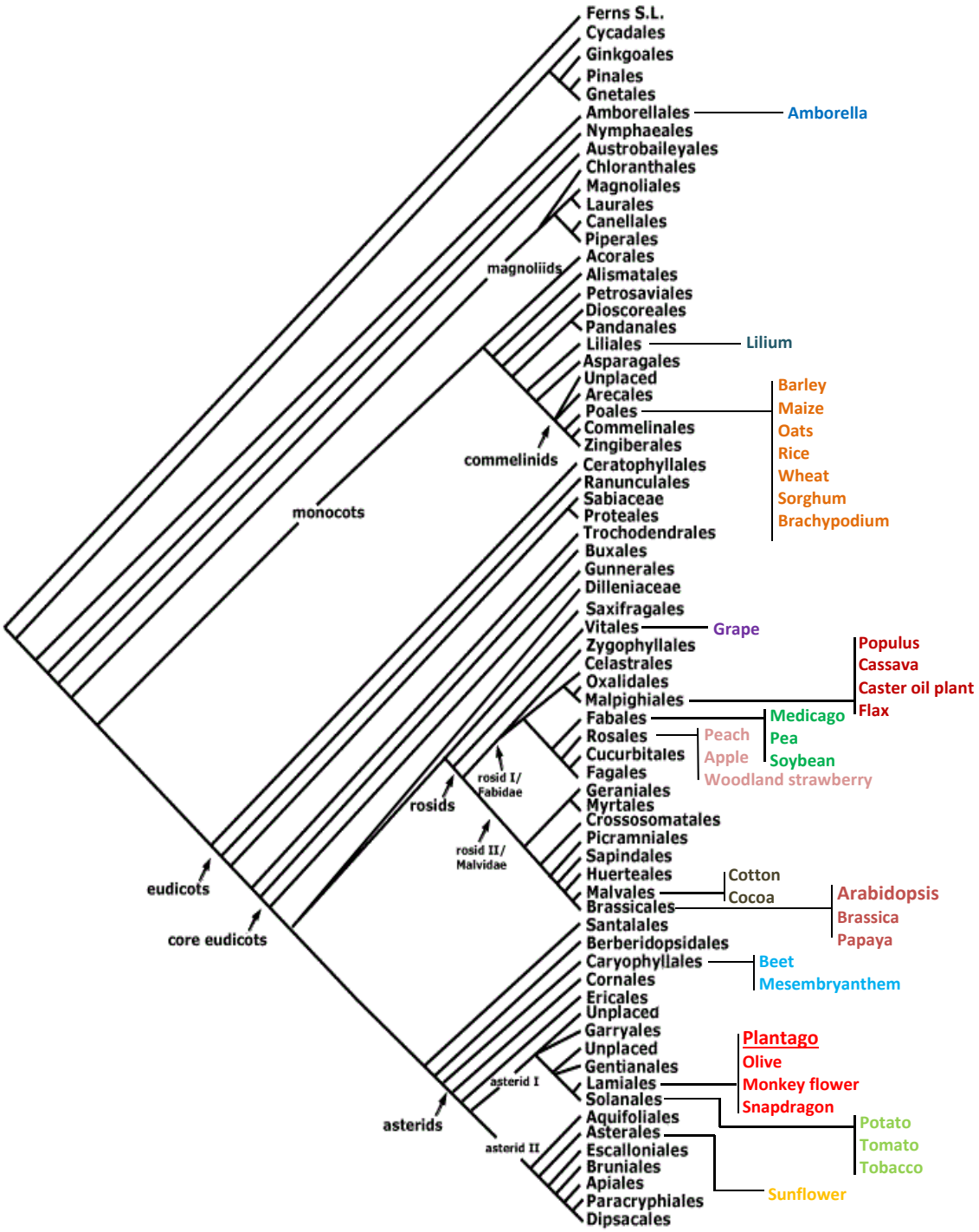


Figure 5.1.1 Angiosperm Phylogeny contains *Plantago* and other species with significant sequence information. Angiosperm phylogeny tree was taken from Angiosperm Phylogeny Website (http://www.mobot.org/MOBOT/research/APweb/welcome.html) (Feb 2012). The species which had their genome sequenced were pulled out from GoGepedia (http://genomevolution.org/wiki/index.php/Sequenced_plant_genomes, updated 26th Jan 2012) and Jansson and Douglas (2007). (Figure modified from Jansson and Douglas (2007))

5.2 Material and method

5.2.1 Plant material

The samples used for transcriptome sequencing were the newly developed leaves collected from the second time-point on 17th December 2009 (Chapter Four, 4.2.1.2). Three randomly chosen families (same maternal plants) out of nine spring families and nine control site families, respectively, were chosen in this experiment and in each family, two plants from a[CO_2] and e[CO_2] were randomly selected separately. Only the young (2-3 developing leaves) were selected from each plant and used for the RNA extraction. The samples were divided into four conditions: group CA is control site *Plantago* which were grown under a[CO_2] during experiment; group SA is control site *Plantago* which were grown under e[CO_2] during experiment; group CE is spring site *Plantago* which were grown under a[CO_2] during experiment and group SE is spring site *Plantago* which were grown under e[CO_2] during experiment (Figure 5.2.1).

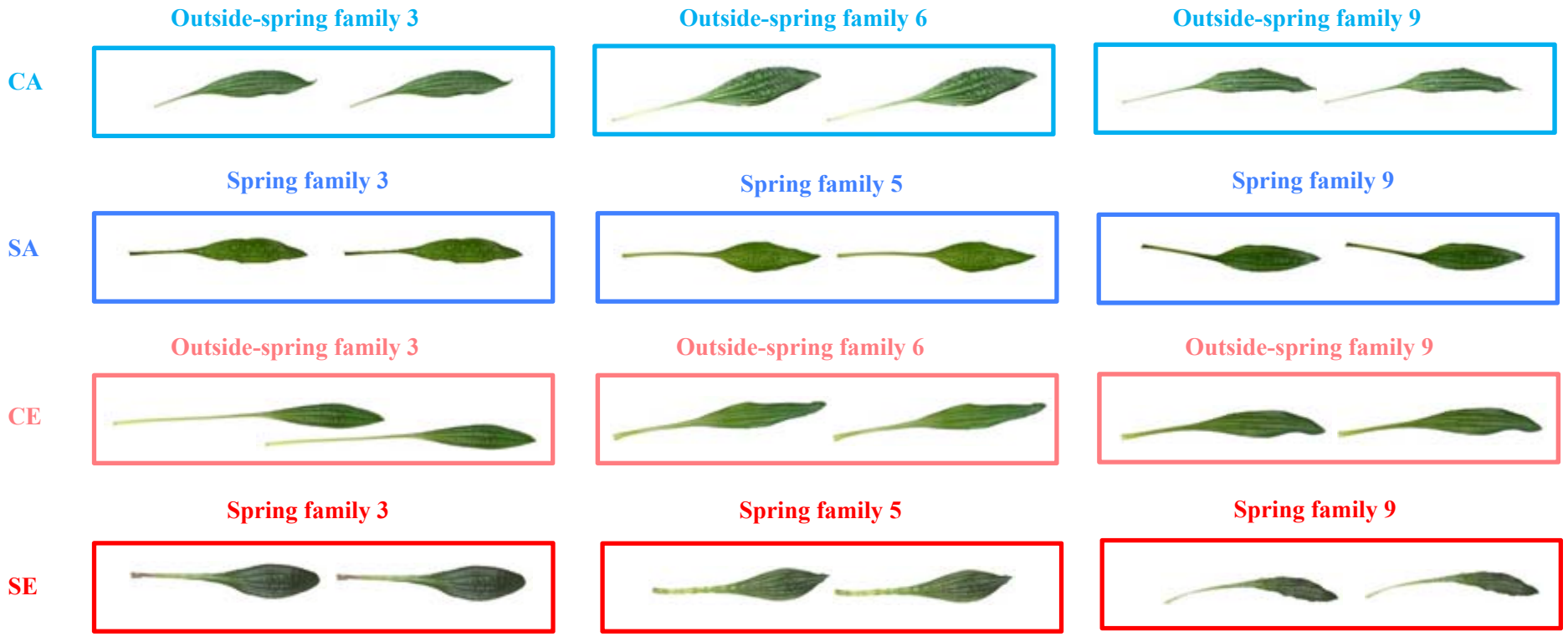


Figure 5.2.1 Illustration of the RNA-seq experiment design. In each group, three families were selected and the developing-leaves from two plants in each family were collected and used for the RNA-seq. Please note that same family number from outside-spring and spring are not from the same maternal plant. Group CA is control site *Plantago* grown under a[CO_2] during the experiment; group SA is control site *Plantago* grown under e[CO_2] during the experiment; group CE is spring site *Plantago*grown under a[CO_2] during the experiment and group SE is spring site *Plantago*grown under e[CO_2] during the experiment.

5.2.2 *Plantago* transcriptome sequencing

5.2.2.1 cDNA synthesis and library preparation

The RNA of all 24 samples was extracted using the CTAB protocol and measured by NanoDrop spectrophotometer as described in Chapter three (3.2.2). All RNA samples were then sent on dry ice to Istituto di Genomica Applicata (IGA, Italy). The IGA carried out the following library preparation, transcriptome sequencing and *de novo* assembly process. The RNA was each quantified by using RNA chips and an Agilent 2100 Bioanalyzer (Agilent technologies, USA). After the quantification, and quality check, two times poly(A) mRNA selections were carried out by oligo d(T) magnetic beads on each total RNA. mRNA were fragmented at 94 ºC using divalent cations and each fragment of mRNA was synthesised to cDNA. After double strand cDNA preparation, poly(A) were added to 3' ends of each fragment. Sequencing adapters were then added to each fragment and amplified by PCR.

5.2.2.2 Transcriptome sequencing

The deep transcriptome sequencing was carried out using an Illumina HiSeq 2000 machine (Illumina Inc., San Diego, CA, USA) with paired-end reads. The fragments of six samples, which were classed in one group were barcoded by 6-nucleotides on one of the adapters that enable identification of the samples when pooled together and loaded to the same lane of the paired-end flow cell. Once the fragments hybridised on the flow cell, each fragment was synthesised and amplified by bridge PCR to form a cluster which contains ~1,000 identical copies of single template molecule in the cBOT instrument (Illumina Inc.). The flow cell was then placed on the Illumina HiSeq 2000 to perform the sequencing. The fundamentals of Illumina sequencing technique are four-colour reversible termination methods which "comprises nucleotide incorporation, fluorescence imaging and cleavage" (Metzker, 2009). All four nucleotides are labelled with different fluorescence dyes. After four-colour images are taken, the fluorescent dyes were washed off (Figure 5.2.2).

a) b)

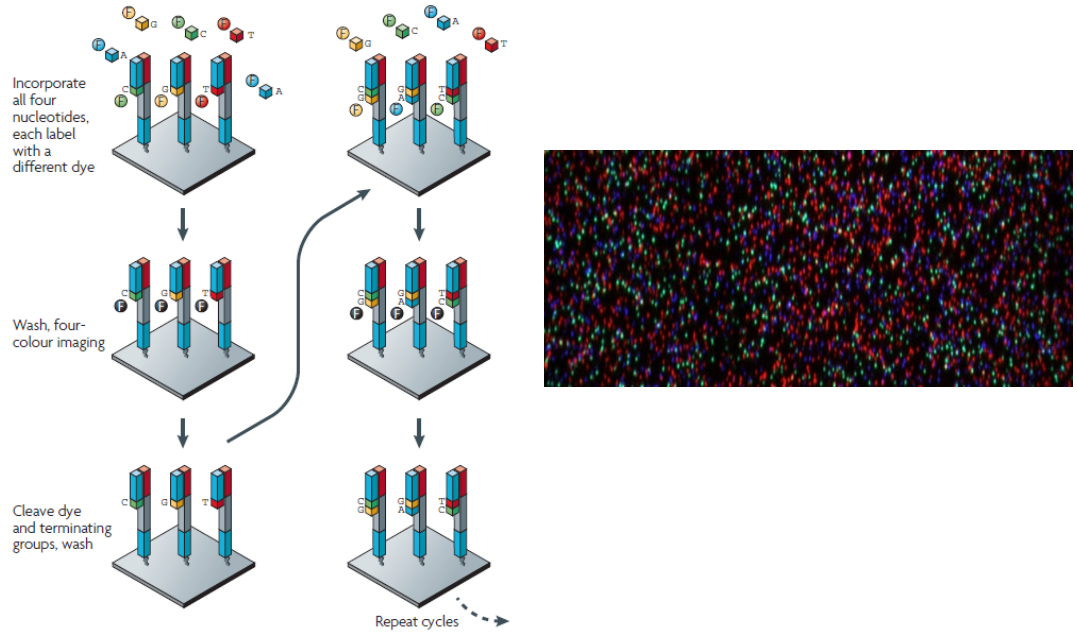


Figure 5.2.2 Illumina four-colour cyclic reversible termination methods. a) The procedure of four-colour reversible termination methods (Figure is from Metzker (2009)).b) An example of four-colour picture took during sequencing (Figure obtained by IGA).

5.2.2.3 Transcriptome *de novo* assembly

Transcriptome *de novo* assembly was conducted using the software of CLC Genomics workbench (CLC bio, Denmark) with alignment using de Bruijn graphs as described in Zerbino and Birney (2008). Due to the unavailability of *Plantago*'s genome, the unique reads were aligned with reference genomes including *Arabidopsis*, *Oryza*, *Ricinus* and *Zea mays*from the National Center for Biotechnology Information database (NCBI, http://www.ncbi.nlm.nih.gov/). This step assigned the unique reads to known genes and also allowed detection of unknown genes (Figure 5.2.3). The expression of each contig, a set of overlapped reads on the same gene, took both molar concentration and gene length into consideration and calculated by reads per kilo base of exon model per million mapped reads (RPKM) (Mortazavi *et al.*, 2008).

$$\text{RPKM} = \frac{\text{Total exon reads}}{\text{Mapped reads (millions)} * \text{exon length (kb)}}$$

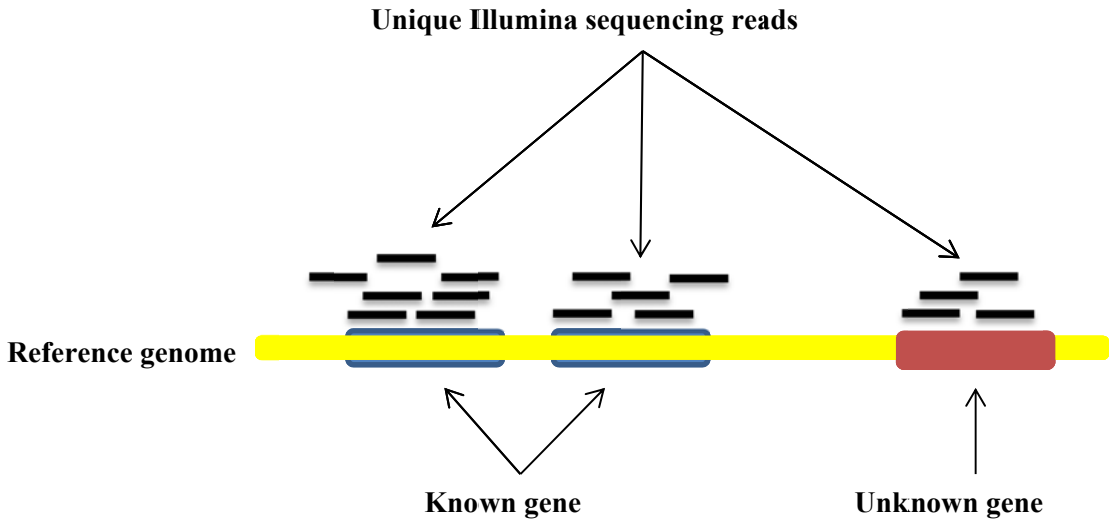


Figure 5.2.3 Illumina sequencing reads alignment to the reference genome. Each black line represented a unique sequencing read.

5.2.3 Normalisation, Statistics and Biological pathway analysis

Further normalisation was applied to the contigs expression. For each sample, the expression of each contig was divided by the summed contigs expression of that sample as showed below:

$$\text{Normalised contig expression} = \frac{\text{Contig RPKM}}{\text{Summed contigs RPKM}} * 1{,}000{,}000$$

The normalised contigs expression was used in the following biological pathway analysis. The contigs expression changes between CO_2 treatments were calculated separately for control site and spring site plants. For control site plants, each contig expression change was calculated by dividing the total normalised expression of six biological-replicate-contigs from group CE by the total normalised expression of six biological-replicates-contigs from group CA (for group explanation, refer back to 5.2.1). The same calculation was applied to spring site plants by using group SE and group SA respectively to the equation. At the condition when contig normalised expression was zero (there is no reads assigned to this gene in this particular plant), a substitute value 0.01 was used as the smallest detectable contigs expression was 0.0210.

A two-way ANOVA was applied to the contigs expression changes between CO_2 treatment from both spring and control site plants using R scripts (http://www.r-project.org/index.html). To understand the biological pathway shifts behind spring and control plant response to high [CO_2] environment, and to understand potential adaptive changes between control and spring grown plants when grown in identical conditions, all contigs were blasted against the *Arabidopsis* genome. This enabled Mapman (version 3.5.1) to be used in this study in order to display data on the metabolic pathways diagrams. The protein sequence translated from the *Plantago* contig sequence was blasted with *Arabidopsis* protein sequence to find out the best hit (cut-off value = 0.0001). Among all the contigs which hit the same *Arabidopsis* orthologue, only the most significant (the smallest *p* value out of three *p* values from the two-way ANOVA analysis) contigs expression were used for that *Arabidopsis* orthologue expression. The Wilcoxon rank sum test was applied to test that whether the genes in one BIN (functional class) displayed a significant pattern of expression different from other BINs. The R scripts of cross blasting the contigs gene with *Arabidopsis* gene, performing the principal component analysis (PCA), normalizing on contigs RPKM expression and ANOVA analysis were all written and performed by Dr R. Edwards (University of Southampton).

5.3 Results

5.3.1 *Plantago* transcriptome sequencing results

The transcriptome sequencing (RNA-seq) was applied on both the spring site and the control site *Plantago*, which have been grown under e[CO_2] and a[CO_2] for approximately three months (87 days, see section 4.2.1.2), to observe the global picture of transcriptome change in response to CO_2 treatment. The RNA-seq data for the 24 young leaves (newly developed leaves) were generated by Illumina HiSeq 2000 in four lanes with 6 plex per lane.

De novo assembly were then applied on the RNA-seq data and formed 51,284 contigs in total with size ranged from 100 bp to 15,730 bp. In which, over half of the contigs (28,283) successfully hit an NCBI protein ID by blastx with *Arabidopsis*, *Oryza*, *Riccinus* and maize. Although the expression of each contig is calculated and normalized by RPKM (Mortazavi *et al.*, 2008), the sum expression of each condition (the total contigs expression of six samples) did not reveal similarities in expression pattern (Figure 5.3.1). This could due to the different level of genes abundance in each condition. In order to be able to compare the gene expression difference across the experiment, further normalisation were applied on the contigs RPKM value. Each sample's contigs RPKM value was divided by the summed expression value of that sample which represented a proportion of the contigs expression value.

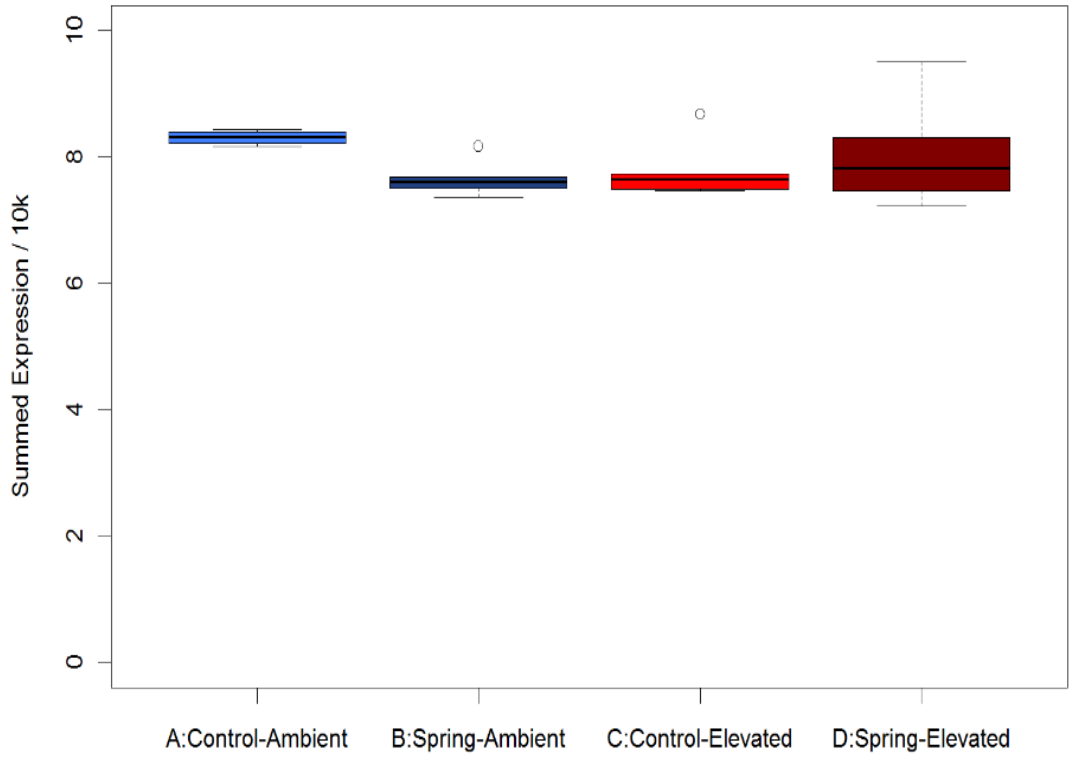


Figure 5.3.1 Sum expression of *Plantago lanceolata* transcriptome sequencing in four conditions. Original contigs sum expression in four conditions. (Figures produced by Dr R. Edwards).

The control site *Plantago* showed a dramatic change in contig expression patterns in response to CO_2 treatments, whereas the spring site *Plantago* showed an overlapped expression patterns between e[CO_2] and a[CO_2] (Figure 5.3.2), that somewhat mirrors the results for phenotypic trait characterisation reported in Chapter 4. This result suggested that the spring site *Plantago* were relatively insensitive to the [CO_2] change in a certain range.

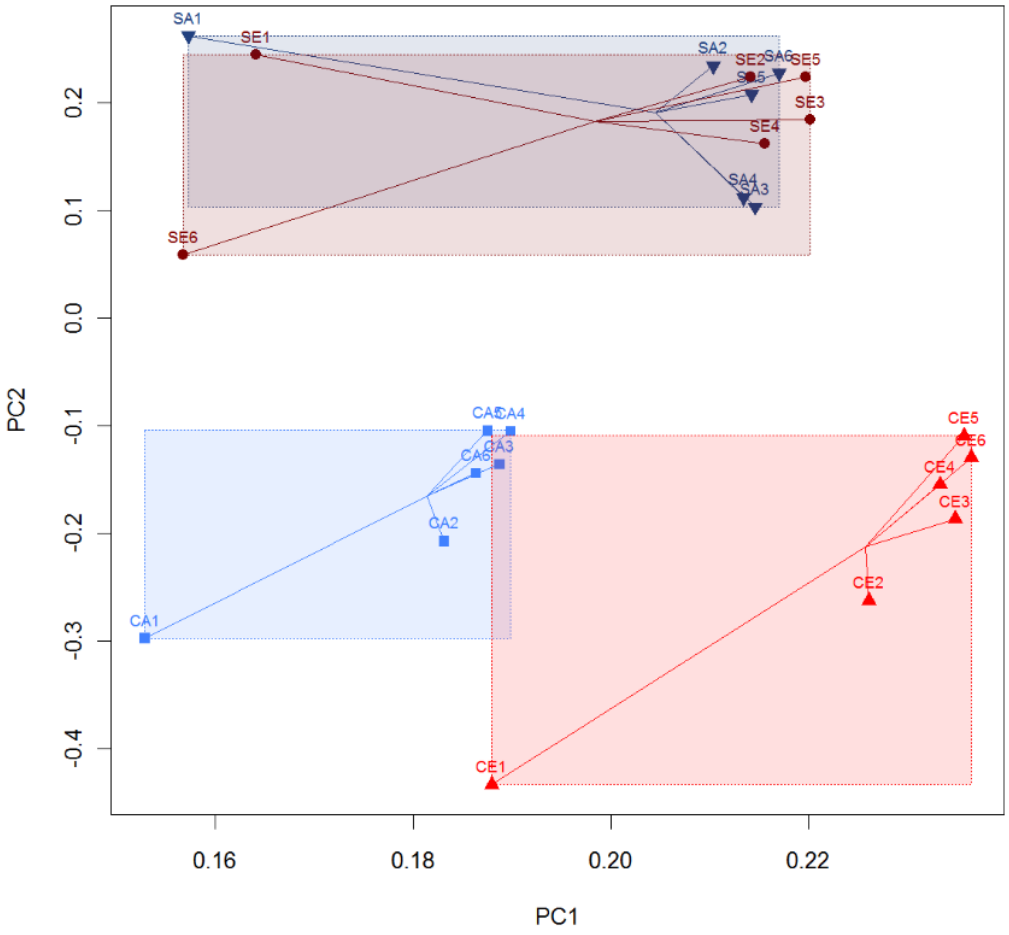


Figure 5.3.2 Principal component analysis of RNA expression patterns in four conditions. PC1 separated the ambient and elevated CO_2 treatments whereas PC2 separated the control and spring growth sites. Other details are described in Figure 5.2.1(Figure produced by Dr R. Edwards).

To explore the full gene expression changes in response to e[CO_2] compared to a[CO_2], which differ between *Plantago* from the spring site and the control site, all contigs' sequences were blasted to *Arabidopsis thaliana*, to obtain the best hit AGI number. The blast results contained 13,655 AGI numbers which represent 53% of the whole *Arabidopsis* genome (around 25,498 genes) (*Arabidopsis*, 2000). In this genes list, around 55% AGI number were hit by unique contig sequence and only 9% AGI number are hit by more than three contigs. There were 1,346 *Arabidopsis* orthologues (at FDR = 5%) which were significantly differentially expressed in response to either original growth environment, or experimental CO_2 treatments, or the interaction between CO_2 treatment and original growth environment (see Appendix VI for gene list).There was a high percentage of genes in cell death GO term (30.39%) that were significantly differentially expressed in response to either growth [CO_2] difference, original growth environment or the interaction in between, followed by immune system process (Table 5.3.1). There were no detected genes in biological adhesion, cellular component organization, nitrogen utilization, rhythmic process and viral reproduction that were significantly differentially expressed in this experiment.

The hieratical clustering figure clearly showed that control site plants exhibit larger gene expression change in response to temperate [CO_2] change compared to spring site plants as what the PCA figure showed (Figure 5.3.3). However, the hieratical clustering of significant genes did not present a clear pattern of gene expression change in each group. To understand the acclimation and adaptation change in response to high [CO_2], full gene lists were studied in the following section.

Table 5.3.1 The secondary level of GO terms in biological process and the percentage of significantly differentially expressed genes in overall gene list. The GO terms annotation were obtained using singular enrichment analysis tool from Agrigo (http://bioinfo.cau.edu.cn/agriGO/analysis.php).

GO terms: biological process	No. of significant genes	No. of genes in all	Percentage
Death	31	102	30.39%
Immune system process	19	122	15.57%
Positive regulation of biological process	19	127	14.96%
Multi-organism process	48	373	12.87%
Response to stimulus	213	1823	11.68%
Negative regulation of biological process	23	197	11.68%
Multicellular organismal process	117	1043	11.22%
Reproductive process	63	572	11.01%
Establishment of localization	96	873	11.00%
Reproduction	63	577	10.92%
Localization	98	903	10.85%
Developmental process	116	1081	10.73%
Cellular process	500	4925	10.15%
Metabolic process	443	4446	9.96%
Biological regulation	152	1610	9.44%
Regulation of biological process	127	1360	9.34%
Cellular component biogenesis	25	294	8.50%
Growth	13	176	7.39%
Biological adhesion	0	10	0.00%
Cellular component organization	0	606	0.00%
Nitrogen utilization	0	8	0.00%
Rhythmic process	0	31	0.00%
Viral reproduction	0	7	0.00%

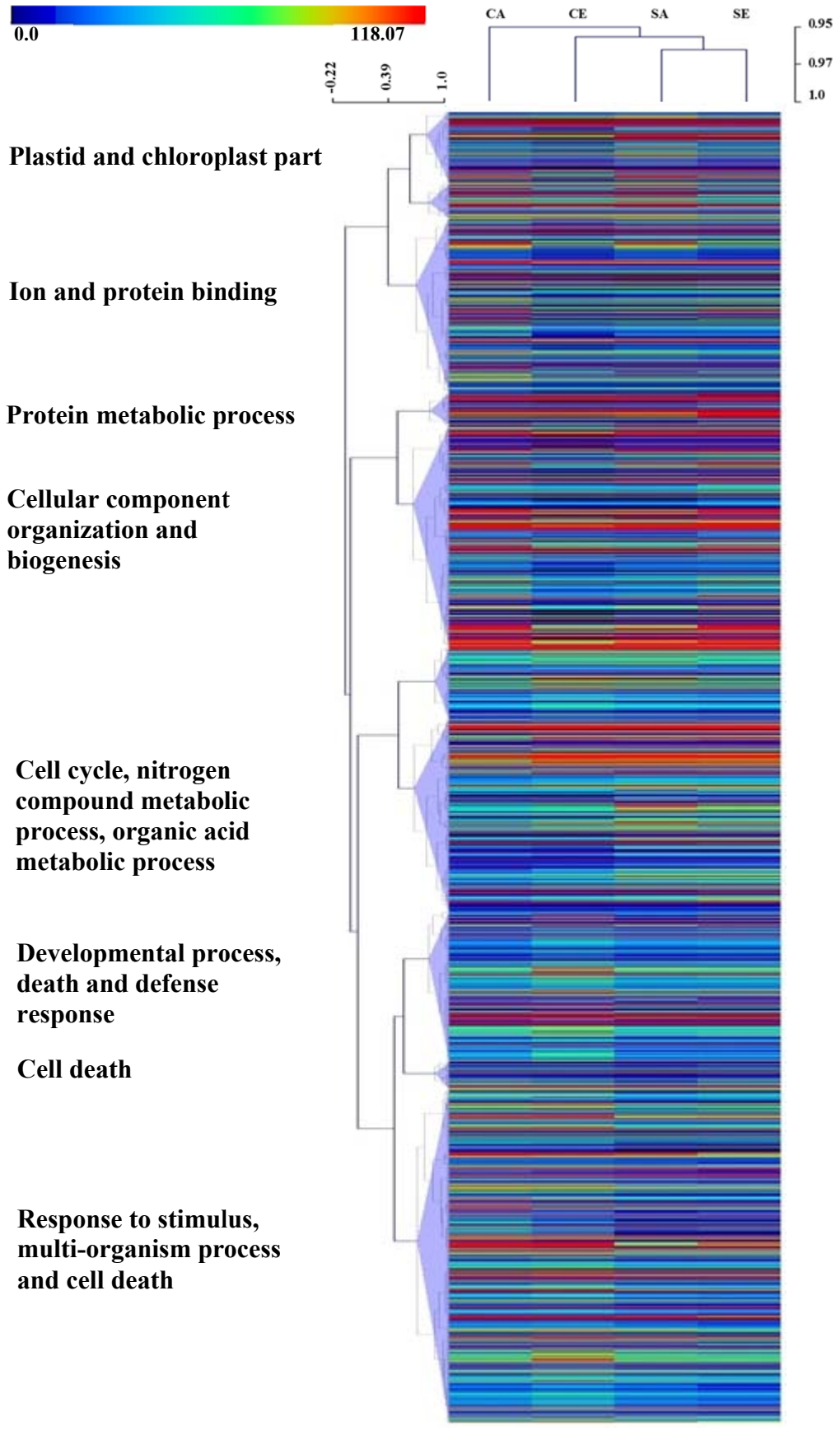


Figure 5.3.3 Hieratical cluster figure of the genes that response significantly different to [CO2] change. The light blue triangle represented the clustering based on gene expression (distance threshold = 0.43). The text by each cluster is the significant gene ontology for that cluster. The cluster without text means there is no significant gene ontology.

5.3.2 Stomatal development

There were four gene functioning in stomatal development that showed significant changes in response to either CO_2 treatment, original growth environment [CO_2] or the interaction effect in between, including two LRR receptor-like serine/threonine- protein kinase (*ERECTA* (*ER*) and *ERECTA LIKE-1* (*ERL1*)), *MYB124* (*FLP*) and *STOMAGEN* (*EPFL9*) (Table 5.3.2). Both control site and spring site plants exhibited increased *ER* expression (53% and 9% respectively, P=0.09) in response to e[CO_2] compared to a[CO_2], whereas an opposite expression change of ERL1 in response to e[CO_2] was observed in control (75% decrease, P=0.03) and spring (17% increase, P=0.03) site plants. The ER-family work together to negatively regulated the stomatal development before the meristemoid stage (Shpak *et al.*, 2005). In this experiment, the ER-family expressed less in e[CO_2] compared to a[CO_2] in control site plants implying higher amount of stomatal were produced which has been observed by morphological data (Figure 4.3.3 in chapter four). Interestingly, spring site plants exhibited a small increase of ER-family in response to e[CO_2]. The puzzling different gene expression change between control and spring site plants observed here might be a consequence of spring site plants adaptation to high [CO_2] as ER-family were also suggested playing important roles in growth and development (Shpak *et al.*, 2004).

FLP and *STOMAGEN* were both significantly increased under e[CO_2] in both control (51% and 19% respectively) and spring (16% and 5% respectively) site plants. *FLP* encodes a R2R3 MYB protein which drives guard mother cell differentiation as well as restricts to one division (Bergmann & Sack, 2007). Higher expression of *FLP* implying more stomata were produced under e[CO_2] in both sites plants. *STOMAGEN* has been reported to positively regulate stomatal density in *Arabidopsis*(Sugano et al., 2009), therefore, again, this highly expressed STOMAGEN under e[CO_2] approved the morphological we have observed. There were a few other genes involved in stomatal development that were not identified in this RNA-seq including *MYB88*, *HIC* (*HIGH CARBON DIOXIDE*) and *bHLH33* (*SCREAM*).

Table 5.3.2 The genes involved in stomatal development and patterning and their expression obtained from RNA-seq. The two-way ANOVA results presented in p.Site (the original [CO2] effect), p.CO2 (temperate CO2 treatment effect) and p.Interaction (the original CO2 effect and interaction effect). * means $p \leq 0.05$. The function of each gene was obtained from TAIR website (http://www.arabidopsis.org/index.jsp). The gene expression in each group is the average expression of six biological replicates. Gene list was extracted from Bergmann and Sack (2007) and Sugano *et al*. (2009).

	Gene name	Arabidopsis othology	CA	SA	CE	SE	p.Site	p.CO_2	p.Interaction	Function
Early-acting genes	Too many mouths gene (TMM)	AT1G80080	2.93	4.32	4.20	4.86	0.52	0.39	0.88	Encode putative cell-surface receptor, repress divisions
	ERECTA (ER, ERL1) Quantitative resistance to plectosphaerella 1	AT2G26330 (ER)	184.49	205.01	281.49	223.05	**0.02***	0.09	**0.04***	Promote above ground organ growth, ER, ERL1 and ERL2 together govern the initial decision of protodermal cells to enter proliferative division to produce pavement cells or asymmetric division to generate stomatal complexes; ERL1 is important for maintaining stomatal stem cell activity and preventing terminal differentiation of the meristemoid into the guard mother cell
		AT5G62230 (ER1)	470.31	90.79	118.02	106.29	0.06	**0.03***	0.21	
	YODA (encode MAP kinase kinase kinase 4)	AT1G63700	40.91	42.91	48.98	38.33	0.08	0.07	0.12	Alter stomatal density and spacing, possess a long N-terminal extension with negative regulatory activity
	Stomatal density and distribution 1(SDD1)	AT1G04110	135.44	181.66	164.15	178.51	0.10	0.19	0.16	Expressed in meristemoids and guard mother cells (GMCs), represses stomatal divisions and also causes arrest of meristemoids and GMCs
Late-acting genes	**MYB124 Four lips (FLP)**	AT1G14350	13.59	15.63	20.54	18.08	0.97	**0.03***	0.49	Encodes an R2R3 MYB protein, halt proliferation directly by regulating the expression of cell cycle gene, and /or indirectly by promoting the developmental transition to a terminal cell fate
	FAMA Transcription factor	AT3G24140	11.80	12.42	12.02	10.59	0.95	0.34	0.98	Encodes a basic helix-loop-helix protein, limits symmetric divisions at the end of the stomatal cell lineage, required for proper cell specification and differentiation
Response to environment	Phytochrome-interacting factors (PIF4)	AT2G43010	22.43	18.36	25.70	20.69	0.11	0.48	0.78	Play rules in several light regulated responses, PIF4 might be involved in regulating stomatal development in response to light
Others	MUTE (bHLH45)	AT3G06120	2.47	3.90	3.50	4.64	0.46	0.31	0.85	Control the transition from a meristemoid to GMC
	SPEECHLESS (SPCH)	AT5G53210	1.14	2.87	2.89	3.52	0.34	0.15	0.37	bHLH transcription factor
	Epidermal Patterning Factor family	AT2G20875 (EPF1)	14.62	10.52	11.63	12.41	0.43	0.74	0.06	Express in stomatal lineage cells and presumably provide positional information in order to enforce the one-cell spacing rule
		AT1G34245 (EPF2)	4.90	5.10	3.52	5.20	0.13	0.31	0.21	
		AT4G12970 (STOMAGEN)	38.98	41.31	46.58	43.45	0.62	0.41	**0.01***	Positive intercellular signalling factor, inducing the stomatal density

5.3.3 Mapman analysis of interesting GO terms

The AGI number and genes expression change were imported into Mapman (v3.5.1) for biological pathway analysis. The overview of expression change in response to e[CO_2] relative to a[CO_2] between control and spring site confirmed what was observed from the PCA analysis. The control plants showed larger expression change between e[CO_2] and a[CO_2], whereas less genes expression change was observed in spring site plants. The comparisons were performed in two ways; the first approach is to compare the gene expression change under e[CO_2] compared to a[CO_2] in both sites' plants, revealing the different acclimation system; the second approach is to compare the control and spring site plants' gene expression under identical environments to discover the potential genomic change selection by high [CO_2].

5.3.3.1 Photosynthesis pathway

There were numerous genes involved in light reactions and Calvin cycle that exhibited different expression change patterns in response to two CO_2 treatments in spring site plants compared to control site plants (Figure 5.3.4). The control site plants showed a generally down-regulated PS II light-harvesting complex (LHC-II) ($P = 0.046$, Wilcoxon rank sum test) and PS I polypeptide subunits ($P = 0.005$) with up-regulated electron transporters ($P = 0.018$) in response to e[CO_2] compared to a[CO_2] (Figure 5.3.4.a), whereas the spring site plants light reactions did not change much, except the ATP synthases in the light reaction ($P = 0.040$) were down-regulated in e[CO_2] relatively to a[CO_2] (Figure 5.3.4.b). The genes encoding Rubisco small subunits (rbcSs) (rbcS-1A and rbcS-1B) involved in the Calvin cycle showed completely opposite expression change in response to e[CO_2] compared to a[CO_2] between control site plants (down-regulated, $P = 0.020$) and spring site plants (up-regulated, $P = 0.016$), whereas, the genes encoding Rubisco activity including Rubisco activase, Chaperonin-60α (CPN60α), CPN60β,Rubisco large subunits (rbcLs) N-methyltransferase, Plastid transcriptionally active 14, were generally up-regulated in response to e[CO_2] in control site plants ($P = 0.032$) with no significant change in spring site plants. This down-regulation of the light

reaction and Rubisco genes in response to e[CO_2] in control plants were commonly observed in many other species due to current atmospheric [CO_2] is insufficient for the Rubisco catalyse. Therefore, the highly induced genes in light reactions in response to e[CO_2] detected in spring site plants suggest the ability of Rubisco catalyse might be reduced when grown under high [CO_2] over a long term, leading to a lower efficiency of photosynthesis system emerging in *Plantago*.

The gene expression differences between control and spring site plants under identical environments were then compared. There was no significant difference in expression pattern between spring and control site *Plantago* under a[CO_2] except the up-regulated photosynthesis electron chain ($P = 0.009$) and ATP synthase ($P = 0.040$) (together named redox chain) (Figure 5.3.4.c). Under elevated CO_2 environment, the majority of the genes involved in photosynthesis, including LHC-II ($P = 0.002$), PSI polypeptide subunits ($P = 0.003$) and photosystem I light harvest complex (LHC-I) ($P = 0.018$), were highly expressed in spring site plants compared to control site plants (Figure 5.3.4.d). When comparing spring site plants to control site plants, the rbcSs were less-expressed ($P = 0.049$) under a[CO_2], while higher expressed ($P = 0.017$) under e[CO_2].The genes encoding Rubisco activity ($P = 0.003$) were significantly up-regulated in control site plants in response to e[CO_2] compared to a[CO_2] ($P = 0.003$), as well as when comparing SA plants to CA plants. This is in contrast to generally believed experiment results that less content of *rbcLs* and *rbcSs* were observed in plants in response to increased [CO_2] (Cheng *et al.*, 1998).

a) Control site **b) Spring site**

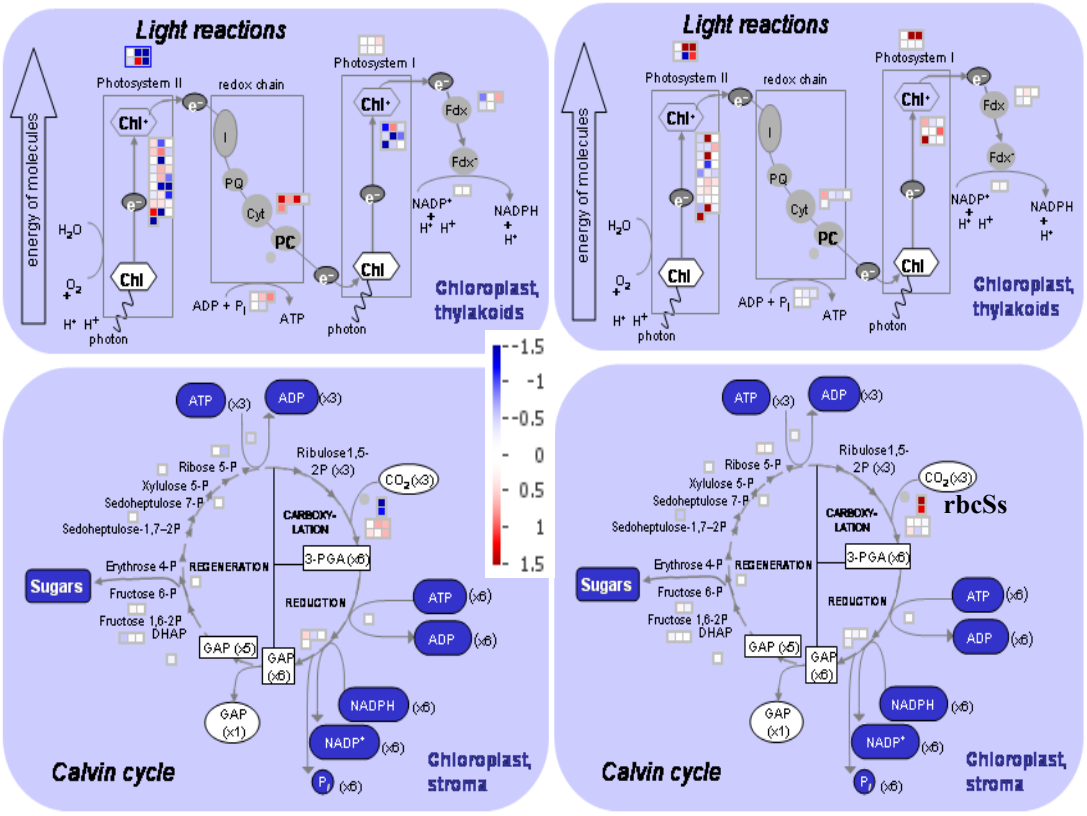


Figure 5.3.4 The photosynthesis genes expression change in response to two [CO_2] treatments in control and spring site plants. a) The expression difference between CO_2 treatments in control site plants. b) The expression difference between CO_2 treatments in spring site plants. c) The gene expression difference between two site plants under ambient CO_2. d) The gene expression difference between two site plants under elevated CO_2. Each square represents a unique *Arabidopsis* orthologue. The colour of squares represents the log fold change of each gene. Red represents highly expressed in spring site plants compared to control site plants, whereas blue represents less expressed in spring site plants compared to control site plants. The scale of gene expression is shown in the middle of the figure.

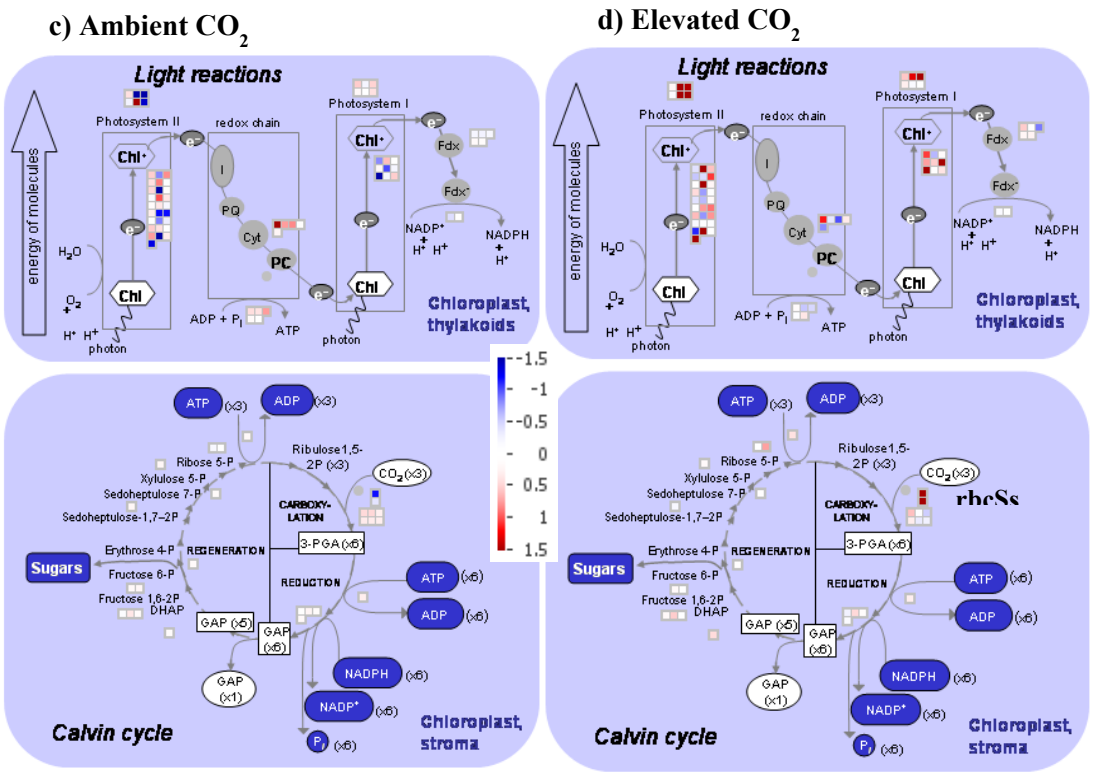


Figure 5.3.4 Continued.

5.3.3.2 Carbonic anhydrase

The *β-CARBONIC ANHYDRASE 1* (*βCA1*), which has been suggested to impair the stomatal aperture affected by [CO_2] change regardless of ABA influence (Hu *et al.*, 2010), is highly reduced along with increased [CO_2] in control site plants, whereas only little change was detected in spring site plants in response to [CO_2] change (Table 5.3.3). This *βCA1* expression decrease in control site plants implying the young leaves might have higher stomatal conductance (g_s) under e[CO_2] compared to a[CO_2]. In a[CO_2] environment, spring site plants expressed less *βCA1* compared to control site plants (Table 5.3.3), which suggests that the young leaves of spring site plants might have higher g_s. The g_s changing subtlety in response to increasing CO_2 in spring site plants implied the ability of stomatal aperture in response to [CO_2] change might be diminished after long-term growth under high [CO_2]. Both control and spring site plants showed decreased *βCA2* in response to increased [CO_2] and spring site plants generally expressed less compared to control site plants. *βCA2* is targeted to the cytosol and is suggested to be involved in CO_2 membrane transport (Uehlein *et al.*, 2003; Fabre *et al.*, 2007).

The *α-CARBONIC ANHYDRASE*4 (*αCA4*) showed different gene expression change in response to increased [CO_2] from the control site (down-regulated) and the spring site plants (up-regulated) (Table 5.3.3). No research has identified the function of *αCA4* to the author's knowledge.

Table 5.3.3 The carbonic anhydrase genes expression change in two way comparison. e/a means the gene expression change in e[CO_2] relative to a[CO_2] (E-A/A * 100%). Control and spring represented the control site and spring site. s/c means the gene expression difference between spring and control site plants. Ambient and elevated [CO_2] are represented by a[CO_2] and e[CO_2]. The data presented in this table were log_2 transformed.

AGI number	Gene annotation	Control e/a	Spring e/a	Ambient s/c	Elevated s/c
AT3G52720	α-carbonic anhydrase 1 (αCA1)	0.10	0.30	-0.20	0.01
AT4G20990	α-carbonic anhydrase 4 (αCA4)	-0.80	0.68	-1.26	0.21
AT3G01500	β-carbonic anhydrase 1 (βCA1)	-1.74	-0.06	-0.57	1.10
AT5G14740	β-carbonic anhydrase 2 (βCA2)	-0.22	-0.20	-0.18	-0.16

5.3.3.3 Cell division and cell cycle

There were a significant number of genes involved in cell division and cell cycle that were up-regulated in response to increasing CO_2 in control site plants (Figure 5.3.5, Table 5.3.4). Again, the spring site plants exhibited less expression changes of cell division and cell cycle genes in response to [CO_2] increase compared to the control site plants in cellular response function groups (Figure 5.3.5). The up-regulation observed in cell division under e[CO_2] in both control and spring site plants imply more cells or stomata were produced. There were also a significant number of genes involved in cell division that were highly expressed in spring site plants compared to control site plants under a[CO_2] (Table 5.3.4) suggesting there might be more cells/stomata produced in spring plants which was confirmed by an

earlier physiology measurement (Figure 4.3.2, Chapter 4). There are significant amount of genes that highly expressed when comparing plants from CA group to CE group and when comparing plants from CA group to SA group. This might be the consequence of more cells being produced and therefore higher gene expression of cell cycle genes were observed. The genes involved in the cell cycle did not show a clear pattern of whether they were up or down regulated in response to increasing CO_2, and there is no expression change pattern in both cell division and cell cycle when comparing spring to control site plants under e[CO_2].

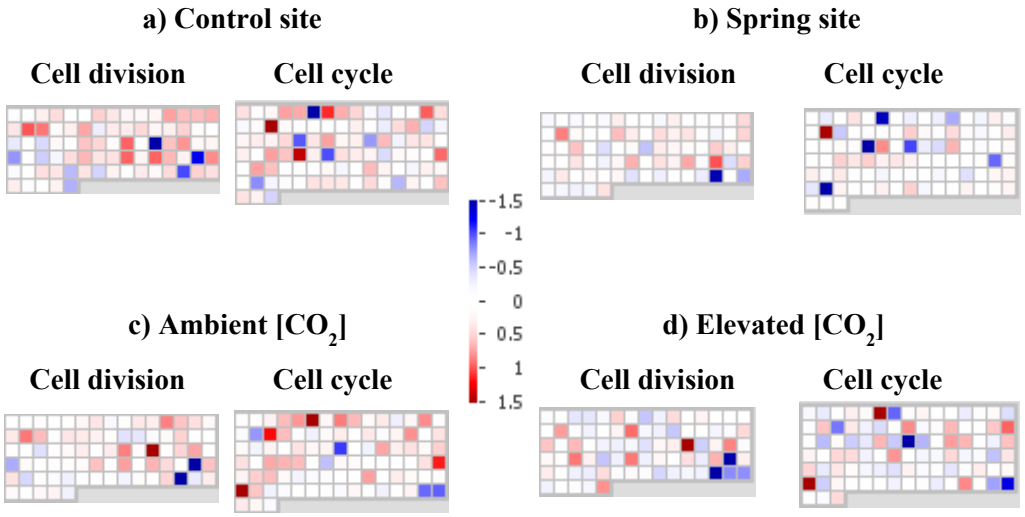


Figure 5.3.5 The cell division and cell cycle genes expression change in response to two [CO_2] treatment in control and spring site plants. a) The expression difference between CO_2 treatments in control site plants. b) The expression difference between CO_2 treatments in spring site plants. c) The gene expression difference between two site plants under ambient CO_2. d) The gene expression difference between two site plants under elevated CO_2. Other details are described in figure 5.3.4.

Table 5.3.4 The Wilcoxon rank sum test of gene expression change involved in cell division and cell cycle. P-values were obtained from Mapman. (+) represents the genes were generally up-regulated in comparison. (-) represents the genes were generally down-regulated in comparison. Other details are described in Table 5.3.3.

		Wilcoxon rank sum test P-value			
BIN	**No. of genes**	**Control e/a**	**Spring e/a**	**Ambient s/c**	**Elevated s/c**
Cell division	80	**<0.001 (+)**	**0.015 (+)**	**<0.001 (+)**	0.12
Cell cycle	93	**0.018 (+)**	0.32	**0.002 (+)**	0.48

5.3.3.4 Carbohydrate metabolism

There was no clear trend of gene expression change involved in sucrose and starch metabolism in response to increasing CO_2 in control plants except the down-regulated α and β-amylase family functioning starch degradation (P = 0.046,Wilcoxon rank sum test) (Figure 5.3.6.a). The down-regulated starch degradation pathway suggested the starch might be stored for respiration during the night (Leakey *et al.*, 2009b). The spring site plants exhibited a clearer trend of down-regulation of carbohydrate metabolism in response to e[CO_2] (Figure 5.3.6.c), including α and β-amylase family (P<0.001), sucrose phosphate synthases (P = 0.002), starch synthases (P = 0.005) and SUSs (P = 0.041). The generally down-regulated sucrose and starch synthesis and degradation in response to increased [CO_2] observed in this experiment does not follow what was observed in other experiments, that the carbohydrate metabolism pathway is up-regulated in response to e[CO_2] compared to a[CO_2] (Taylor *et al.*, 2005; Ainsworth *et al.*, 2006). The reason why spring site plants carbohydrate metabolism genes were less expressed in response to e[CO_2] is not very clear. It might be due to spring site plants becoming adapted to high [CO_2] environment, therefore, growing under lower [CO_2] environment (relatively to spring site plants original growth [CO_2]) will induce them to produce more starch and sucrose for energy use.

Although spring site plants showed genes that were down-regulated to a larger extent by e[CO_2] compared to a[CO_2], there is no significant gene expression change when comparing spring site plants to control site plants under e[CO_2] (Figure 5.3.6.d). In contrast, the spring site plants showed positively regulated carbohydrate metabolism in response to a[CO_2] compared to control site plants (Figure 5.3.6.c), including sucrose phosphate synthases (P = 0.003), α and β-amylase family (P = 0.014) and starch synthases (P = 0.020). This approved the hypothesis that spring site plants were adapted to high [CO_2] environment and that when growing under a[CO_2], higher gene expression involved in sucrose and starch metabolism pathway were required to maintain normal growth.

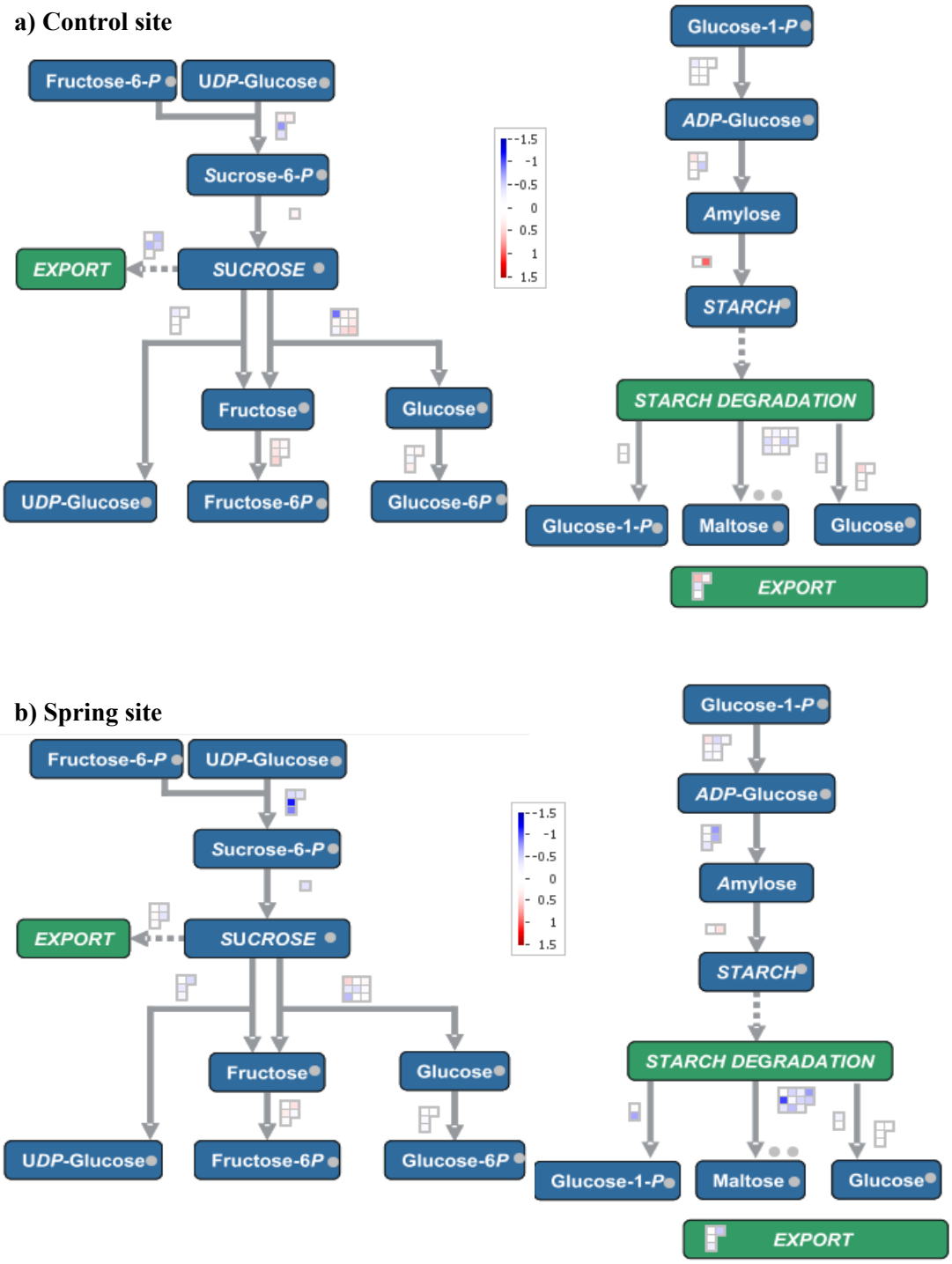
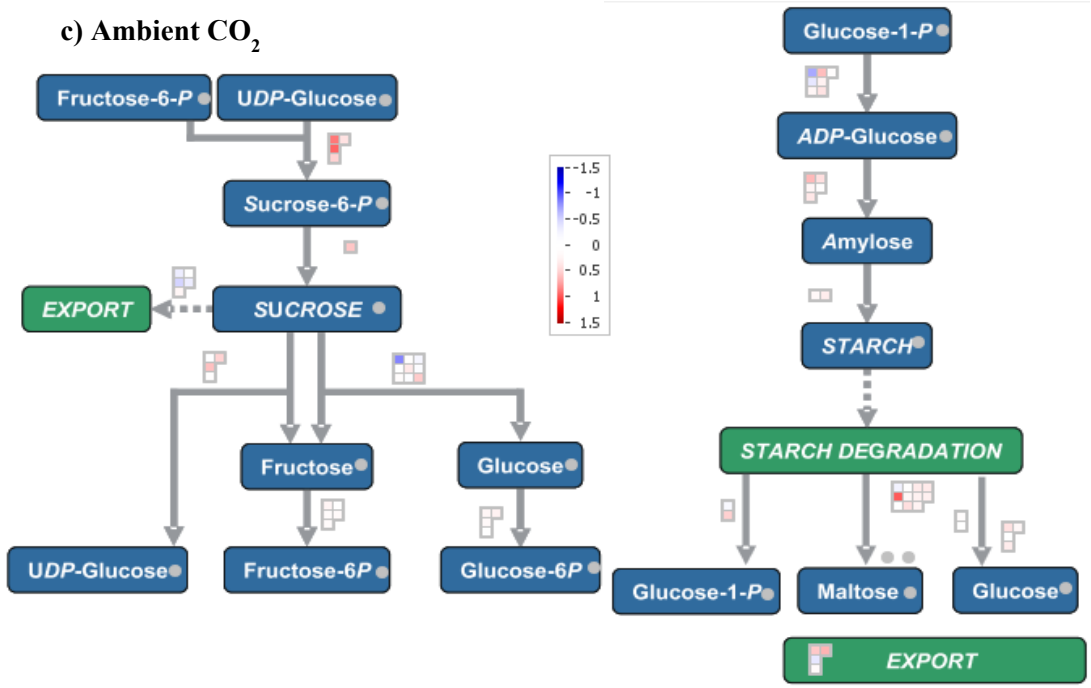


Figure 5.3.6 The sucrose and starch pathway genes expression change in response to two [CO_2] treatments in control and spring site plants. a) The expression difference between CO_2 treatments in control site plants. b) The expression difference between CO_2 treatments in spring site plants. c) The gene expression difference between two site plants under ambient CO_2. d) The gene expression difference between two site plants under elevated CO_2. Other details are described in figure 5.3.4.

c) Ambient CO_2



d) Elevated CO_2

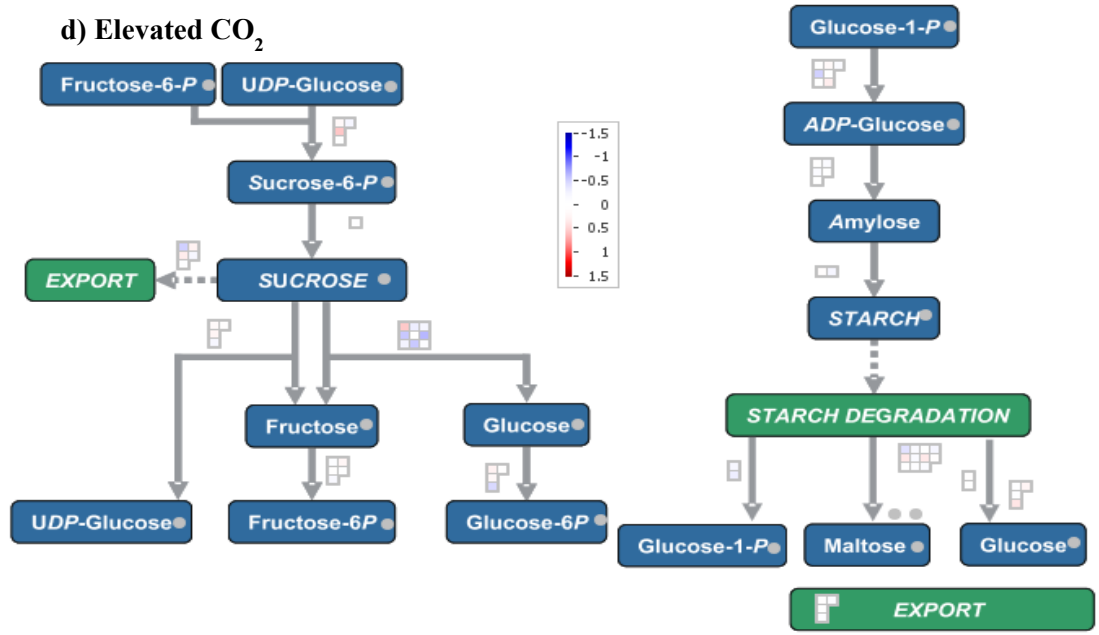


Figure 5.3.6 Continued.

5.3.3.5 Cell wall

The genes involved in cell wall modification were significantly influenced by [CO_2] change in both control (down-regulated) and spring (up-regulated) site plants. Even under identical environments (a[CO_2] and e[CO_2] respectively), a significant number of genes' expression changed between control and spring site plants (Figure 5.3.7, Table 5.3.5). The cell wall modification genes, including xyloglucan endotransglucosylase/hydrolase family and expansin family, were responsible for cell wall loosening which allows the cell wall enlarging for cell growth (Cosgrove, 2005). The down-regulated cell wall modification genes under increasing [CO_2] followed the cell size change as described in that last chapter, that is smaller cells were produced under e[CO_2] in control plants. However, the up-regulation of cell wall modification genes along with increased [CO_2] in spring site plants suggest that the spring site plants produced bigger cells in new leaves under e[CO_2] relative to a[CO_2]. This is contrast to what was observed in mature leaves, that smaller cells were produced under e[CO_2] compared to a[CO_2] (Table 4.3.1, Chapter 4). Under a[CO_2], the cell wall modification genes were down-regulated in response to e[CO_2] meaning smaller cells were produced in spring site plants compared to control site plants. In contrast, the gene expression of cell wall modification implies, that under e[CO_2], spring site plants developed bigger cell on new leaves compared to the new leaves of control site plants. This is also in contrast to what was observed on mature leaves of control and spring site plants under e[CO_2]. It seems that spring site plants did not increase much cell size under e[CO_2] during growth (from new leaf to mature leaf) compared to the ones grown under a[CO_2] or to the control plants under the same environment. There were significant amounts of cellulose synthesis genes, including cellulose synthase (CESA) and COBRA, positively responding to increased [CO_2] for control site plants (Table 5.3.5). The CESAs were involved in primary and secondary cell wall formation by forming microfibrils, and the microfibrils belonging superfamily– cellulose synthase-like (CSL)– were involved in encoding the backbone of hemicelluloses (β-D-GLYCAN) (Cosgrove, 2005). This increase in cellulose synthase genes along with increased [CO_2] suggested the cell wall might be stiffer and thicker under e[CO_2] for control site plants which affects cell elongation. The spring site plants also showed a significant number of genes

coding arabinogalactan proteins (AGPs) and Leucine-rich repeat/extension family proteins (LRXs) that were up-regulated in response to increased [CO_2] (Table 5.3.5). Both AGPs and LRXs were suggested to be involved in signalling intermediates during cell wall development and maintaining cell wall integrity (Ringli, 2010). This might be related with the activated cell wall modification genes that allowed cell elongation under e[CO_2] in spring site plants at the early development stage. The down-regulated LRXs in spring site plants compared to control site plants under a[CO_2] also corresponds to down-regulated cell modification genes (Table 5.3.5). The pectate lyases and polygalacturonase, responsible for pectin degradation, were down-regulated under e[CO_2] in spring site plants compared to control site plants.

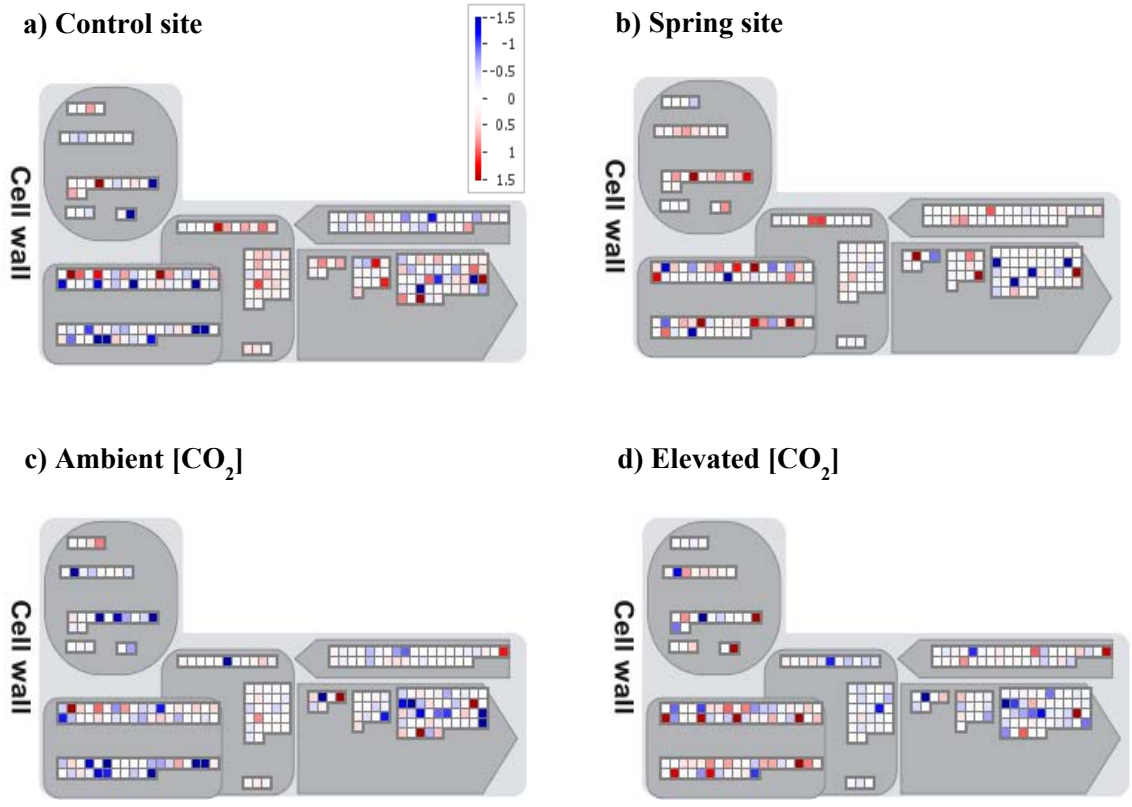


Figure 5.3.7 The cell wall metabolism genes expression change in response to two [CO_2] treatments in control and spring site plants. a) The expression difference between CO_2 treatments in control site plants. b) The expression difference between CO_2 treatments in spring site plants. c) The gene expression difference between two site plants under ambient CO_2. d) The gene expression difference between two site plants under elevated CO_2. Other details are described in figure 5.3.4.

Table 5.3.5 The Wilcoxon rank sum test of gene expression change involved in cell wall synthesis and degradation. P-values were obtained from Mapman. (+) represents the genes were generally up-regulated in comparison. (-) represents the genes were generally down-regulated in comparison. Other details are described in Table 5.3.3.

		Wilcoxon rank sum test P-value			
Cell wall Bins	**No. of genes**	**Control e/a**	**Spring e/a**	**Ambient s/c**	**Elevated s/c**
Hydroxyproline-rich glycoproteins	4	0.690	0.940	0.280	0.600
Arabinogalactan proteins	8	0.120	**0.002 (+)**	0.170	0.059
Leucine-rich repeat/extensin proteins	12	0.790	**< 0.001 (+)**	**0.016 (-)**	0.600
Reversibly glycosylated polypeptides	3	0.230	0.950	0.950	0.270
Proline rich protein	2	0.390	0.320	0.560	0.240
Pectin esterases	36	0.180	0.390	0.390	0.240
Modification	29	**0.007 (-)**	**0.001 (+)**	**0.003 (-)**	**0.023 (+)**
Hemicellulose synthesis	11	0.091	0.220	0.790	0.150
Cellulose synthesis	27	**0.043 (+)**	0.760	0.890	0.078
Pectin synthesis	3	0.230	0.290	0.130	0.470
Precursor synthesis	36	0.064	0.820	0.180	0.150
Cellulases and beta-1,4-glucanases	6	0.051	0.670	0.390	0.670
Mannan-xylose-arabinose-fucose	13	0.970	0.230	0.260	0.950
Pectate lyases and polygalacturonase	45	0.790	0.410	0.190	**0.044 (-)**

5.4 Discussion

Comparing the gene expression changes under different CO_2 treatments (390 ppm and 700 ppm) and between control and spring site plants has enabled us to elucidate an understanding of both acclimatory and adaptive responses to high [CO_2]. The principal component analysis revealed distinct differences between global gene expression changes in response to elevated [CO_2] for the control and spring site *Plantago*. The data reveal a large change in gene expression when control-grown plants were exposed to elevated CO_2 whilst for spring-grown plants, rather few changes in transcriptome expression were apparent. This is an intriguing finding and suggests that spring-grown plants do not respond to elevated $CO_2$to the same extent as control plants, supporting the findings for growth and morphology documented in Chapter 4.When comparing gene expression between control- and spring-grown plants, when grown in identical conditions of either ambient or elevated CO_2, the data reveal that spring site *Plantago* did not exhibit the same as the control site *Plantago* under either [CO_2] treatment suggesting there is evolutionary adaptation selected by high [CO_2] on spring site *Plantago*.

The down-regulated LHC-II and PS I polypeptide subunits in response to increased [CO_2] detected in control site *Plantago* was observed in chapter three during the growth season and other experiments on *Arabidopsis*, poplar and legumes (Li *et al.*, 2008; Cseke *et al.*, 2009; Leakey *et al.*, 2009b; Tallis *et al.*, 2010).The up-regulated electron transport chain under e[CO_2] in control plants suggested more energy was produced, which will benefit the following Calvin cycle. Much fewer genes of spring site plants showed big expression changes influenced by different [CO_2] in the light reaction, in which ~55% of the genes showed opposite gene expression change compared to control site plants. This was especially true in genes in the redox chain suggesting the light reaction in spring site plants might not respond much to [CO_2] change compared to control site plants. The genes involved in the redox chain expressed higher in spring site *Plantago* compared to control site *Plantago* when both grown in a[CO_2] but not in the e[CO_2] environment suggesting that spring site plants have a high cost-

effective light reaction system relatively to control site plants as a consequence of adapting to high [CO_2] environment, therefore higher gene expression in the redox chain were required in a[CO_2] to supply enough energy for the following reaction.

Despite that most of the genes involved in the Calvin cycle showed no large expression change in response to different CO_2 treatments in both control and spring site *Plantago*, two rbsSs, which are rbcS1α and rbcS1β, were significantly differentially expressed in e[CO_2] compared to a[CO_2] in control (down-regulated) and spring (up-regulated) site plants. This decrease of rbcSs in control site plants is a straight forward phenomena, which was observed previously in many studies on plants growing under e[CO_2](Gesch *et al.*, 1998; Leakey *et al.*, 2009b) due to current atmospheric [CO_2] not saturating to Rubisco activity, therefore higher [CO_2] would require less Rubisco to maintain normal growth. The generally up-regulated genes, which enable Rubisco activity to respond to e[CO_2] in control site plants proved the theory given by Long *et al.* (2004), that a higher concentration of CO_2 induced a higher rate of Rubisco carboxylation and suppressed the formation of Rubisco. The contrasting rbcSs expression change in response to e[CO_2] relative to a[CO_2] in spring site *Plantago* suggested the spring site plants produced more Rubisco, rather than increase the carboxylation rate as the control site plants do, in response to increased [CO_2]. Spring site plants, relative to control site plants, showed less Rubisco rbcSs gene expression with slightly higher Rubisco activity genes in a[CO_2] and a higher rbcSs gene expression in e[CO_2], suggesting spring site plants maintained a high Rubisco carboxylation rate irresponsive to [CO_2] change but do adjust the concentration of Rubisco. The review by Leakey and Lau (2012) explained that the Rubisco is a very inefficient enzyme and the optimum catalytic rate of modern C_3 plant is when atmospheric [CO_2] is around 200 ppm which was the average [CO_2] over the last 400,000 years. They believe the evolution of Rubisco was constrained by its fundamental manner as Rubisco activity is still the limiting step of C_3photosynthesis in many conditions currently. However, the phenomenon observed in spring site plants implied the possibility of Rubisco evolution, a higher but non-flexible Rubisco carboxylation rate, in adaptation to CO_2 selection after long-term growth in high [CO_2].

Both control and spring site plants exhibited significantly down-regulated starch degradation genes, α-amylase and β-amylase, in response to increased CO_2. Erbs *et al.* (2010) found that under saturated N environment, both α-amylase and β-amylase were significantly reduced in e[CO_2] compared to a[CO_2] in winter wheat. This degradation in both sites' plants observed in this experiment suggesting starch was mainly accumulated in leaves during the day and will be consumed during photorespiration at night. *Plantago* was classed as a starch-accumulating species which maintains high starch synthesis in photosynthesising leaves (Ivanova *et al.*, 2008). The highly expressed sucrose and starch synthesis genes in spring site plants under a[CO_2] could provide a similar high starch environment in leaves under an environment in which [CO_2] is lower than its original growth environment. The sucrose and starch synthesis and degradation pathways in spring site plants compared to control site plants were generally enhanced in a[CO_2] but none were significantly differentially expressed in e[CO_2] proving that spring site plants were adapted to high [CO_2] environment and therefore initiated higher carbohydrate metabolism to maintain the normal growth when they were growing in e[CO_2].

The β-carbonic anhydrases family (especially *βCA1* and *βCA4*) is suggested to be the key enzyme upstream of plants signalling pathway in response to [CO_2] change and regulate stomatal closure under high [CO_2] (Hu *et al.*, 2010). The βCAs are positive regulator of CO_2-induced stomatal closure (Kim *et al.*, 2010), by binding CO_2 and converting CO_2 and water into HCO_3^-(bicarbonate) and H^+(Pillitteri & Torii, 2012). They are also one of the proteins that participated in C_3 plants and evolved in the C_4 pathway which is the advantaged photosynthesis pathway compared to C_3 (Aubry *et al.*, 2011). The subtle CAs expression change in response to increased [CO_2] in spring site *Plantago* confirmed the hypothesis that spring site *Plantago* has somehow evolved insensitive to the [CO_2] change. But it is unclear whether the adaptation occurred on βCA catalyse or another part of the CO_2 signalling pathway. Lower expression of *βCA1*detected with higher [CO_2] treatment in control site plants indicated that there is even higher stomatal conductance (g_s) in response to e[CO_2] in control site plants. The reason of this lower-expressed *βCA1* is still unclear. The CAs were expressed lower in spring site *Plantago* compared to control site *Plantago* under a[CO_2], proposing a higher

*g*s in spring site plants. This phenomenon implying that spring site plants might have a lower water-use efficiency under a[CO_2] environment compared to control plants.

There was also much less expression change on genes that are responsible for stomatal formation in spring site plants compared to control site plants (Figure 5.4.1). The *ER* and *ERL1* were homologues that are both negative regulators of stomatal formation by promoting asymmetric division of the meristemoid and suppress the formation of guard mother cells(Bergmann & Sack, 2007). The highly induced *ER* in both control and spring site plants and *ERL1* in spring site plants under e[CO_2] confirmed the morphological data collected in the last chapter that more epidermal cells were produced under e[CO_2] in both control and spring site plants. It is intriguing that *ERL1* were largely down-regulated in control site plants under e[CO_2]. This large reduction of*ERL1* in response to e[CO_2] in control site plants, but increased in spring site plants, also explained the larger increase of epidermal cell density in spring site plants compared to control site plants. Both *FOUR LIPS* and *STOMAGEN* were up-regulated in e[CO_2] in control and spring site plants. The *FOUR LIPS* is responsible for regulating the cell cycle, therefore restricting the second symmetric division on the same guard mother cell (Bergmann & Sack, 2007). The significantly highly expressed *FOUR LIPS* in response to e[CO_2] suggests there were more stomata formed under e[CO_2], therefore more genes were required to determine any second symmetric division. The function of STOMAGEN on stomatal formation was discovered by Sugano *et al.* (2009). They overexpressed *STOMAGEN* in wild type *Arabidopsis* and the plant exhibited higher stomatal density. The up-regulation of STOMAGEN observed in our experiment also confirmed the higher stomatal density in response to e[CO_2] in both site plants. Interestingly, there is a bigger change in stomatal density but smaller gene expression in spring site plants compared to control site plants.

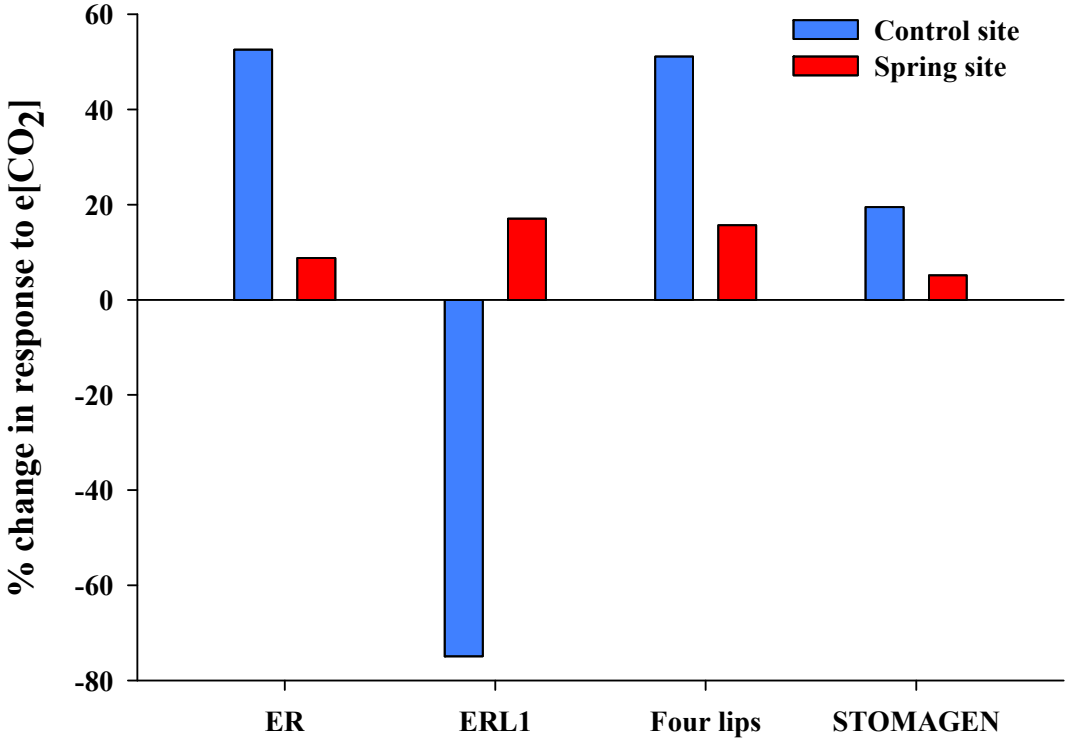


Figure 5.4.1 The percentage change of genes which regulate stomata formation that showed significantly differentially expression in a two-way ANOVA test. The percentage change was calculated by using the difference of each gene expression under e[CO_2] relative to a[CO_2].

The significantly up-regulated cell division and cell cycle genes in response to e[CO_2] in control site plants suggested a highly activated cell proliferation and growth under e[CO_2].In addition to the highly activated cell division and cell cycle, the cellulose synthesis which synthesises cellulose, the component of cell primary and secondary wall structure, were also up-regulated along with down-regulated cell wall modification genes in response to e[CO_2] compared to a[CO_2] in control site plants. The gene expression change suggesting there were more, and smaller, cells produced with stiffer/thicker cell wall along with the increased [CO_2] in control site plants. Research on *Quercus ilex* and *Arabidopsis* also found increased foliar cellulose concentration which might be caused by highly activated cellulose synthase in e[CO_2] (Gibeaut *et al.*, 2001)suggesting thicker cell walls were formed (Staudt *et al.*, 2001; Teng *et al.*, 2006). The spring site plants cell division genes were up-regulated under e[CO_2] compared to a[CO_2] along with increased genes that encode arabinogalactan proteins and LRXs suggesting a higher rate of cell division which requires constant cell wall breakdown and

formation under e[CO_2] in spring site plants. Spring site plants also exhibited higher gene expression related to cell division and cell cycle but reduced gene expression that encodes LRX and cell modification in response to increased CO_2 compared to control site plants. There is no significantly different expression pattern between spring and control site plants when growing under e[CO_2] on genes involved in cell division and cell cycle. The cell wall modification genes were generally up-regulated especially the *XTR7* (1.30 $\log_2$ fold), *EXPANSIN A13* (1.34 $\log_2$ fold) and *XTH21* (5.56 $\log_2$ fold) and genes encoding pectate lyases (response for pectin degradation) and polygalacturonase (involved in cell expansion) (Geisler-Lee *et al.*, 2006)were generally down-regulated in spring site plants compared to control site plants in e[CO_2]. The highly activated cell wall modification could be the result of higher epidermal cell production in spring site plants relative to control site plants in e[CO_2] observed from morphological data and the reduced pectin degradation genes, suggesting the spring site *Plantago* converts the carbon gained from the high [CO_2] environment into the cell wall for storage.

In this experiment, another level of normalisation was added to the RNA–seq contigs expression besides using RPKM methods, due to the imbalance of summed genes expression. A recent method pointed out the bias problem of using only RPKM as normalisation (Robinson & Oshlack, 2010). They believe the RNA composition should also being taken into consideration along with expression level and gene length when estimating the contigs expression; otherwise the information of highly–expressed gene groups particular to one condition will be lost. Thus the new normalisation method solved the bias contigs expression when comparing across conditions.

5.5 Conclusion

The different gene expression changes in response to CO_2 treatment between control site and spring site *Plantago* were studied in this experiment by using a transcriptome sequencing technique. This experiment provided the essential evidence that spring site *Plantago* has evolved to CO_2 selection on transcriptome level compared to control site *Plantago*. The spring site *Plantago* exhibited generally less gene expression change under e[CO_2] compared to a[CO_2]. Potentially in the future, *Plantago* might adapt to high [CO_2] with an inefficient photosynthesis metabolism and insensitive CO_2 signalling pathway, along with an efficient primary metabolism pathway and pathogen resistance system. This implying that the bigger plants induced by high [CO_2] established a more suitable carbon assimilation pathway in adaptation to the high [CO_2] and may improve the current pathogen resistance system. This data has the potential to yield more information, which will be further discussed in Chapter 6.

Chapter 6: General discussion – Genomics of plant response to elevated atmospheric CO_2 – elucidating plastic and adaptive mechanisms

6.0 Thesis overview

Atmospheric carbon dioxide is essential for higher plants and ecosystem functioning. Plants assimilate carbon dioxide from the atmosphere supplying energy to the terrestrial ecosystem and potentially regulate atmospheric carbon dioxide concentration ([CO_2]) (Leakey & Lau, 2012). The rapidly increasing atmospheric [CO_2] and the serious changes in climate caused by this increase also alter the timing of plant life traits (Hoffmann & Sgro, 2011), *i.e*. early bud setting (caused by global warming) (Menzel *et al.*, 2006) and delayed natural autumnal senescence (influenced by increased [CO_2]) (Taylor *et al.*, 2008). Many studies have now focussed on physiological, morphological and biochemical plant responses to elevated atmospheric CO_2 but rather fewer have considered adaptive and evolutionary changes, or those at the functional genomic level for plants grown in realistic conditions, which has been the focus of this thesis. This is an important omission since green plants in the terrestrial biosphere contribute to the largest global carbon sink – they fix 120 petagrams of carbon each year by photosynthesis(Beer *et al.*, 2010) and understanding likely adaptation is relevant to future predictions of biogeochemical cycles and how atmospheric CO_2 is likely to rise in future. Secondly, plants in a high CO_2 world will still be required for food, fuel and fibre and understanding genomic and genetic change, which will inform how we engineer and breed plants for these roles in a future climate (Ainsworth *et al.*, 2008).

This thesis focused on two topics. Firstly identifying suites of candidate genes sensitive to elevated CO_2 and secondly, an attempt to relate these key genes to adaptive responses to elevated CO_2 following multi-generation exposure in a high CO_2 spring. Secondly global gene expression changes were studied using an evolving set of microarrays and synthesised chips in two different experimental systems and two different species of tree within the genus *Populus.* Gene expression changes studied in realistic field conditions remain extremely limited (Taylor *et al.*, 2005; Ainsworth *et al.*, 2006), and this study extended our early finding for both actively growing and senescing *Populus* trees in POPFACE/EUROFACE and AspenFACE. *Populus* is a model genus (Taylor,

2002) which has shown responses to e[CO_2] in previous studies (Ferris *et al.*, 2001; Rae *et al.*, 2006), for both *Populus* species (*Populus x euramericana* and *Populus tremuloides*). These include increased leaf expansion which is associated with the highly activated cell wall-loosening enzyme xyloglucan endotransglycosylase, reduced specific leaf area and delayed leaf senescence. More recently, (Tallis *et al.*, 2010), in a novel piece of research, demonstrated that elevated CO_2 could directly influence autumnal senescence with a closer look at gene expression changes associated with delayed autumnal senescence in elevated CO_2. Although this identified a suite of key genes that were sensitive to CO_2 treatment, trees are long-lived and difficult to study in an adaptive and evolutionary framework and so to test our hypothesis on plant adaptation to CO_2 further, a different experimental and plant system was employed. *Plantago lanceolata* is the common forb that is found in several high atmospheric [CO_2] sites, and it is therefore a suitable species for study for detecting rapid evolutionary change (Bischoff *et al.*, 2006). For example, Zheng *et al.* (2002) grew *Plantago major*, which was classified as O_3-sensitive plant, in an elevated ozone environment and observed a series of reactions including inhibited photosynthesis, reduced g_s and reduction of Rubisco activity. Hikosaka's group (Onoda *et al.*, 2007; Onoda *et al.*, 2009; Nakamura *et al.*, 2011) have also studied *Plantago asiatica* in several natural high-CO_2 springs and successfully identified evolutionary adaptation to different [CO_2] environments by comparing the spring site plants with plants collected close to the site but in a[CO_2] environment. They found that under e[CO_2] both stomatal conductance and starch accumulation is reduced in spring site plants compared to control site plants, and the spring site plants have a higher WUE, photosynthetic N use efficiency along with reduced maximum rate of RuBP carboxylation (V_{cmax}) and maximum rate of electron transport driving RuBP regeneration (J_{max}).

The FACE results (Chapter 2 and 3), from studies of *Populus* in Italy (*P. x euramericana*) and the USA (*P. tremuloides*) both showed very clear delays in autumnal senescence associated with increased primary productivity and leaf growth (Cseke *et al.*, 2009; Tallis *et al.*, 2010). Gene expression pattern analysis identified clear mechanisms underpinning early delayed senescence in POPFACE, whilst a later phase of senescence was studied in AspenFACE highlighting

changes in gene expression compared with those from the earlier senescence phase in POPFACE. The spring site studies (Chapters 4 and 5) revealed changes in growth and morphology as well as gene expression that may go some way to explain different acclimatory and adaptive responses to elevated CO_2. Several interesting biological pathways that exhibited opposite transcriptomic change in response to e[CO_2] relative to a[CO_2] were identified. In particular, acclimatory responses to CO_2 including single leaf biomass, single leaf area, SLA and SI. At the same time, when gene expression was studied for control and spring grown plants subjected to identical environments, the adaptive differences were revealed including changes in epidermal cell formation (stomatal formation, cell division, cell cycle, cell wall), photosynthesis and carbon metabolism. The morphological changes under e[CO_2] compared to a[CO_2] also showed some difference between spring and control plants (Chapter 4). It would appear that spring plants have indeed become adapted to high CO_2 and this research provides the first insight into a suite of genes associated with that response.

6.1 Different mechanism but same delayed senescence phenomena influenced by e[CO_2]

Although both *P. x euramericana* and *P. tremuloides* (aspen) showed delayed autumnal senescence under e[CO_2] compared to a[CO_2], the transcriptome change from two experiments showed different mechanisms behind this phenomena, and this may be related to the timing of autumnal events at the two sites and the date at which samples were taken for transcriptome analysis. The latitude of the two sites varied considerably (Taylor *et al.*, 2008) and it is certain that senescence was initiated far earlier at AspenFACE than POPFACE. From differences in latitude and from consideration of the phenology data it is likely that senescence was initiated at the end of October for POPFACE and early October for AspenFACE. The consequences of this are important, as POPFACE leaves showed a dramatic increase in the carbon assimilation pathway, including photosynthesis, glycolysis and the TCA cycle, under e[CO_2] compared to a[CO_2] at late senescence stage, for gene expression. This ties together with previous reports for photosynthesis from this site (Bernacchi *et al.*, 2003) which was shown to be consistently higher it

elevated compared to the ambient CO_2 treatment. This increase in carbon assimilation enhanced the carbohydrate content, but this occurred after bud set (Walter *et al.*, 2005b), which then induced the flavonoids production (Teng *et al.*, 2005) under e[CO_2] that scavenged ROS (Gould, 2004), which led to delayed autumnal senescence. Whereas in aspen (Chapter 3), at late senescence stage, the carbon flux was abundantly in glycolysis rather than carbohydrate synthesis under e[CO_2] compared to a[CO_2], along with decreased secondary metabolism including the flavonoids biosynthesis pathway. It is suggested that other antioxidant enzymes and non-enzymes including thioredoxin, ascorbic acid and glutathione rather than anthocyanin took the role of scavenging ROS. The interesting thing is aspen showed similar expression changes at an early senescence stage to that of *P. x euramericana*, when sampled much later in the Italian senescence season. It is possible that the senescence response pathways were induced at an earlier stage of senescence under e[CO_2] in aspen compared to *P. x euramericana*. Plants allocate assimilated carbon between growth, defence and storage (Chapin *et al.*, 1990) following the growth-differentiation balance hypothesis (Herms & Mattson, 1992), which explained the different strategies behind the same phenomena that *P. x euramericana* allocated the carbon to defence whereas the aspen allocated the carbon to early growth.

Growing *Arabidopsis* mutants or poplar transformations with the candidate gene, that identified might be involved in inducing delayed senescence under e[CO_2], in a[CO_2] environment would allow us to test the hypothesis concluded from Chapter 2 and 3. The genetic engineering technique has been widely applied on studies of plants response to other environment factors including temperature change and pest hazard (Paoletti & Pimentel, 2000; Iba, 2002). It allowed the study of the effect of one gene or pathway without influencing the rest of plant. *Arabidopsis thaliana* has proven to an excellent and easy to genetically engineer model plants to study ecological interaction and underlying mechanism on various studies (Jackson *et al.*, 2004; Kappers *et al.*, 2005). Future work could concentrate on producing transgenic plants (RNAi silence or overexpress) with key genes that identified from Chapter 2 and 3, and grow them under control environment with a[CO_2] (Table 6.1.1).

Table 6.1.1 Potential genes and mutants which play important roles in e[CO_2] delayed autumnal senescence. Annotations were obtained from TAIR (http://www.arabidopsis.org/index.jsp).

AGI number	name	Annotation	Reference
AT5G13930	CHS	Chalcone synthase, a key enzyme involved in the biosynthesis of anthocyanin	(Solfanelli *et al.*, 2006)
AT5G42800	DFR	Dihydroflavonol reductase. Catalyses the conversion of dihydroquercetin to leucocyanidin in the biosynthesis of anthocyanins.	
AT4G22880	LDOX	Leucoanthocyanidin dioxygenase, involved in proanthocyanin biosynthesis.	
AT1G56650	PAP1	MYB 75;Encodes a putative Myb domain containing transcription factor, Encodes a putative MYB domain containing transcription factor involved in anthocyanin metabolism and radical scavenging. Essential for the sucrose-mediated expression of the dihydroflavonol reductase gene.	(Pourtau *et al.*, 2006) (Solfanelli *et al.*, 2006)
AT1G66390	PAP2	MYB90;Myb family transcription factor, putative/production of anthocyanin pigment 2 protein	
AT1G62300	WRKY6	Transcription factor, nucleus, regulation of transcription, DNA-dependent, response to chitin, transcription factor activity	(Miao *et al.*, 2004; Guo & Gan, 2006; Balazadeh *et al.*, 2008)
AT4G23810	WRKY53	Transcription factor, acts as an upstream control element in a transcription factor signalling cascade leading to leaf senescence	
AT1G69490	AtNAP53	Transcription factor, have been shown to control leaf senescence	
AT5G45890	SAG12	Senescence-associated gene 12 encoding a cysteine protease	(Pourtau *et al.*, 2006)
AT4G29130	Hexokinase	Encodes a HEXOKINASE1 in the plant glucose-signalling network. Functions as a glucose sensor to interrelate nutrient, light, and hormone signalling networks for controlling growth and development in response to the changing environment.	(Wingler & Roitsch, 2008)

Table 6.1.1 Continued.

Mutant Name	Description	Reference
Sweetie mutant	Sweetie accumulates high levels of endogenous sugars and most of the phenotype were the same as in the wild-type	(Veyres *et al.*, 2008)
Jar1-1/ coi1-1	JA-insensitive mutant	(Loreti *et al.*, 2008)

6.2 Phenotypic plasticity versus genetic change under high [CO_2]

Populations subjected to climate change are likely either to move/spread to favourable habitats, perform plastic changes or undergo evolutionary adaptation to minimise the climate change influences, which at some extreme conditions could cause extinction (Hoffmann & Sgro, 2011). The transcriptomic expression changes under e[CO_2] and a[CO_2] from both FACE rings and natural CO_2 spring were studied in order to understand the plants response to future atmospheric [CO_2] and whether there will be evolutionary adaptation or plastic changes selected by high [CO_2]. Chapter 3 detected the plastic changes by comparing *Populus* which have been grown under e[CO_2] for at least five years, with same species which were grown under a[CO_2] at the same experiment sites. The genetic evolutionary changes were studied by comparing the rapid physiological response (reversible response) (Helmuth *et al.*, 2005) to e[CO_2] compared to a[CO_2] between *Plantago* from spring and outside (Chapters 4 and 5).

The phenotypic plasticity response to high [CO_2] did not follow the same trend of genetic change in response to high [CO_2]. Aspen exhibited a down-regulated photosynthesis pathway during growth season under e[CO_2] (Chapter 3). In contrast, spring site *Plantago* showed up-regulation of the photosynthesis pathway under e[CO_2] compared to a[CO_2] which was the opposite results of observations in control site *Plantago* (Chapter 5). The AFLP results in Chapter 4 suggested there is genetic variation occurring between spring and outside *Plantago* populations which suggested the observation from Chapter 5 is what we would expect to occur after hundreds years of growing under high [CO_2]. The results imply that the plant photosynthesis transcripts are expressed less under e[CO_2] due to unsaturated carbon uptake from a[CO_2] currently. However, after long-term growth under high [CO_2], the photosynthesis pathway and related genes, including carbonic anhydrase ([CO_2] signalling gene) (Hu *et al.*, 2010), would lose sensitivity to different [CO_2] (within a certain range).

There has not been much research on plant evolution or adaptation that has successfully detected the high [CO_2] induced evolutionary adaptation (Klus *et al.*, 2001; Collins & Bell, 2006). The transcriptome sequencing technique does not only detect the expression of known transcripts, it allowed the detection of single nucleotide polymorphisms (SNPs) and gene fusions between each sample (Morozova *et al.*, 2009). Future work could focus on the SNPs detection to test the high [CO_2] selection at the gene (DNA) level. This will allow discovery of more convincing evidence that plants experience genetic evolution in response to high [CO_2].

Both Affymetrix microarray (Chapter 3) and transcriptome sequencing (Chapter 5) results detected interesting gene expression in cell wall synthesis and the cell wall modification pathway. Studies on the cell wall of plants grown under e[CO_2] compared to a[CO_2]showed there were abundant transcripts involved in cell wall loosening, which benefit cell elongation (Ranasinghe & Taylor, 1996; Ferris *et al.*, 2001), and that the cell wall is also thinner under e[CO_2] (Oksanen *et al.*, 2004). The morphological study (Chapter 4) detected there was an increase in cell number and these cells were smaller under e[CO_2] compared to a[CO_2] in both spring and outside plants.

The cell wall structure is closely related to cell elongation and division; therefore, it is not clear whether the transcripts change in cell wall metabolism is purely induced by e[CO_2] or if it is a consequence of more cell division and the activated cell cycle. Future studies on cell wall modification as a consequence of high [CO_2] could focus on the effect in leaves by using cell-specific dyes to highlight the different cell wall components for examination under a microscope.

6.3 Morphological traits favoured by high [CO_2]

Chapter 4 presented a series of morphological traits including single leaf dry mass, single leaf area, specific leaf area and stomatal index, which showed the opposite response to high [CO_2] relative to a[CO_2] between spring and control site *Plantago*. The suggested plant evolutionary direction, smaller, thin but more

leaves with smaller ECS while grown under high [CO_2], does not follow the physiological plasticity change from other CO_2 studies – bigger leaves with larger ECS(Taylor *et al.*, 2003) and reduced SD under e[CO_2] (Casson & Gray, 2008). The phenomena of small and more brunch/leaves has also been observed in *Populus nigra* genotype which were adapted to drought (Viger, 2011), suggesting the water-use efficiency will be improved in *Plantago* as a benefit from high [CO_2] adaptation.

The stimulated growth by high [CO_2] is one of the traits that is favoured by high [CO_2], bigger plants with more branches/leaves were observed in Chapter 4 as well as in other studies (Long *et al.*, 2004). Higher SE and smaller ECS in forb plants might also be the traits that were selected by high [CO_2] (Figure 4.3.3). The problem of identifying the traits that are favoured by high [CO_2] is that there might be multiple traits or pathways involved with relatively subtle effects (Leakey & Lau, 2012).

Future work could focus on the traits that have been identified that evolved in adaptation to low [CO_2] from prior palaeoecological studies (Leakey & Lau, 2012) including Rubisco and SD and other photosynthesis related traits or genes, as they are directly related to carbon assimilation from atmospheric carbon. The photosynthesis rate of spring and control site *Plantago* should also be measured during growth to support the theory of inefficient photosynthesis rate in spring site plants as an adaptation to e[CO_2]. The soluble sugar and starch content should also be measured from both site plants to support the hypotheses in chapter 5.

It would be interesting to compare the leaf vein density of *Plantago* grown inside of the spring to the ones grown outside of the spring as it will reduce the leaf hydraulic conductance that would not be able to sustain greater g_s, therefore more stomata would be needed

6.4 Conclusion

This study provided insights into plant responses to future atmospheric carbon dioxide concentration (higher [CO_2]) on both acclimation and adaptation to such conditions. Different mechanisms of delayed senescence in response to e[CO_2] were studied in two *Populus* species, indicating that antioxidants were stimulated by e[CO_2] ,which are likely to be part of the causative mechanism leading to delayed autumnal senescence. The morphological, genetic and genomic studies on *Plantago* which are native to a high [CO_2] environments compared to the *Plantago* from a[CO_2] in the same area indicated potential genetic evolution selected purely by high [CO_2]. Together, these results revealed the interesting pathways and traits that will be influenced by increasing [CO_2] and the evolutionary direction, allowing a better understanding of future ecosystems.

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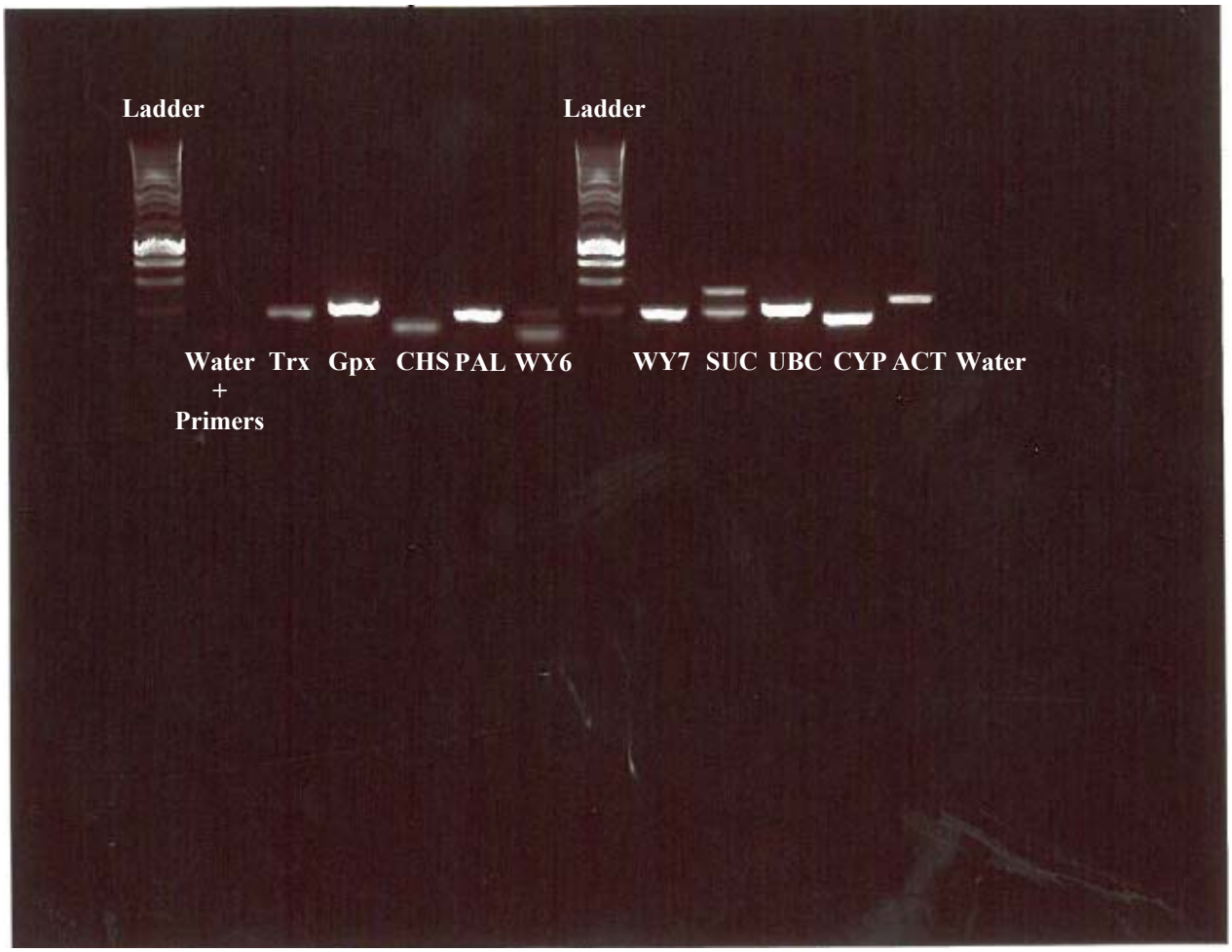
Appendices

Appendix I. The electrophoresed PCR result of primers with Aspen DNA



The electrophoresed PCR result of each primer pair tested with Aspen DNA. Trx represents *THIOREDOXIN*. Gpx represents *GLUTATHIONE PEROXIDASE*. CHS represents *CHALCONE SYNTHASE*. PAL represents *PHENYLALANINE AMMONIA LYASE*. WY6 represents *WRKY6*. WY75 represents *WRKY75*. SUC represents *SUCROSE SYNTHASE*. UBC represents *UBIQUITIN-CONJUGATING ENZYME E2*. CYP represents *CYCLOPHILIN*. ACT represents *ACTIN*. The primer pairs of *WRKY6* formed primer dimer but still used in this experiment. This was already the second pair of WRKY 6 primer and still had primer dimer and no better primer can be designed based on poplar WRKY 6 sequence and will not hit other sequences. The primer of SUC hit two fragments therefore was not used in this experiment.

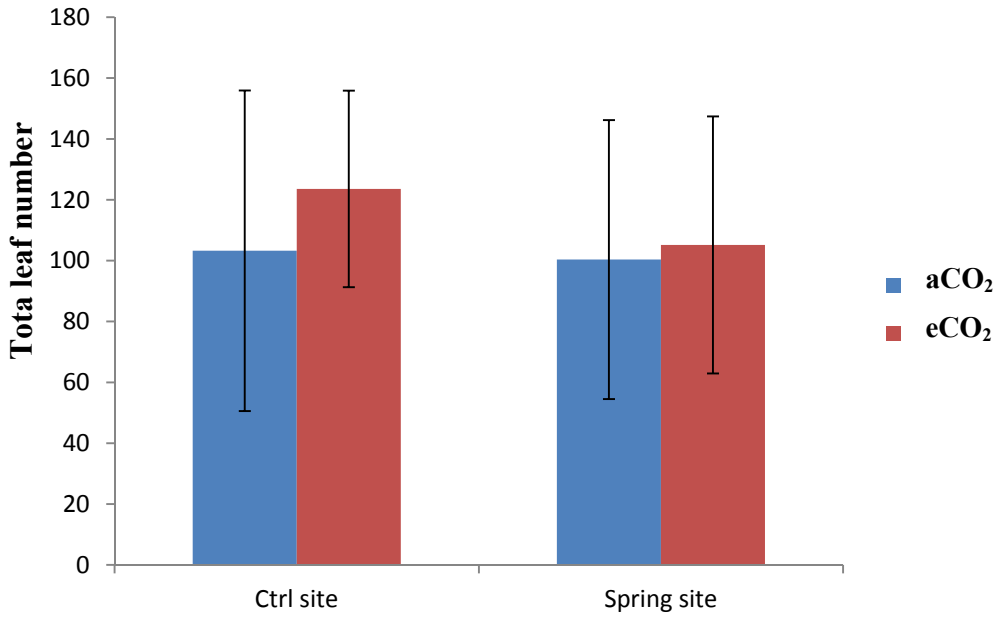
Appendix II. Statistical data for *Plantago* AFLP AMOVA analysis

Source	df	SS	MS	Est. Var.	%
Between Sites	1	400.970	400.970	3.733	2%
Between Families	16	2370.613	148.163	0.000	0%
Within Families	118	20497.792	173.710	173.710	98%
Total	135	23269.375		177.444	100%

Stat*	Value	P(rand >= data)
PhiRT	0.021	0.090
PhiPR	-0.020	0.700
PhiPT	0.002	0.430

*The P value were calculated as PhiRT= AR/ (WP + AP + AR) = (AR / Total); PhiPR = AP / (WP + AP); PhiPT = (AP + AR) / (WP + AP + AR) = (AP + AR) / Total. AR represented estimated variance between sites. AP represented estimated variance between families. WP represented estimated variance within families.

Appendix III. The total leaf number of *Plantago lanceolata* and the statistic



		Total leaf number	
Source	**d.f**	***T***	***P***
Site	1	1.988	0.159
CO_2	1	3.270	0.071
Site x CO_2	1	5.408	**0.020 ***
Family (Site)	16	36.848	**0.002***
CO_2 x Family (Site)	16	14.601	0.554
Chamber (CO_2)	6	2.662	0.850
Site x Chamber (CO_2)	6	2.644	0.852

Generalized Linear Models was used. The T value and the level of significant are represents. Significance level: * $P \leq 0.05$, where no* was reported data were not significant.