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# DISSERTATION

Titel der Dissertation

## Monitoring of the environmental influence on stress regulated genes in oak

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**Doktorin der Naturwissenschaften (Dr. rer.nat.)**


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# Contents

Contents i

List of Figures v

List of Tables xi

I Main Part 1

1 General Introduction 3
1.1 Climate Change . . . 3
1.2 Oak . . . 3
1.3 Water deficit stress . . . 4
1.3.1 Factors determining the plant's response to water deficit stress/drought 5
1.3.2 Drought Avoidance and Tolerance . . . 5
1.3.3 Responses to drought: from molecular changes to physiological adaptations . . . 6
1.3.3.1 Signal perception and transduction . . . 7
1.3.3.2 Gene regulation . . . 9
1.3.3.3 Functional changes . . . 11
1.4 Objectives . . . 15

2 Morphology & Physiology 21
2.1 Introduction . . . 22
2.2 Material & Methods . . . 23
2.2.1 Plant Material and Growth Conditions . . . 23
2.2.1.1 Drought Stress Treatment . . . 23
2.2.1.2 Re-watering . . . 23

2.2.2 Determining the Leaf Developmental Stages . . . 23
2.2.3 Sampling . . . 24
2.2.3.1 First year (2006) . . . 24
2.2.3.2 Second year (2007) . . . 24
2.2.3.3 Third year (2008) . . . 25
2.2.4 Morphological Measurements . . . 27
2.2.5 Experimental Design . . . 27
2.2.6 Sample Preparation and Analysis of Stress Metabolites . . . 28
2.2.6.1 Carbohydrates & Polyols . . . 28
2.2.6.2 Proline and betaine analogues . . . 28
2.2.7 Statistical Analyses . . . 28
2.3 Results . . . 29
2.3.1 Plant Growth & Development . . . 29
2.3.1.1 Plant Development . . . 29
2.3.1.2 Shoot Growth . . . 30
2.3.1.3 Stem Diameter . . . 32
2.3.1.4 Summary . . . 32
2.3.2 Analysis of Stress Metabolites . . . 32
2.3.2.1 Sugars . . . 32
2.3.2.2 Polyols . . . 35
2.3.2.3 Proline and Betaine Analogues . . . 35
2.4 Discussion . . . 38
2.4.1 Plant Growth & Development . . . 38
2.4.1.1 Flushing Behaviour . . . 38
2.4.1.2 Shoot Growth . . . 39
2.4.1.3 Stem Diameter . . . 39
2.4.2 Stress Metabolites . . . 40
2.4.2.1 Sugars . . . 40
2.4.2.2 Polyols . . . 41
2.4.2.3 Possible function of carbohydrates and polyols in drought stress response . . . 41
2.4.2.4 Function of carbohydrates in drought stress relief (re-watering) . . . 42
2.4.2.5 Proline, Betaine and Analogues . . . 42
2.4.3 Conclusion . . . 42

3 Gene Expression 47
3.1 Introduction . . . 48
3.2 Materials and Methods . . . 49
3.2.1 Plant Material and Growth Conditions . . . 49
3.2.1.1 Water regime . . . 49
3.2.1.2 Sampling . . . 49
3.2.2 Experimental Design . . . 50
3.2.3 Sample Preparation and RNA-Extraction . . . 51
3.2.4 Construction of cDNA microarrays containing oak EST clones . . . 52
3.2.5 Preparation of fluorescent probes . . . 52
3.2.6 Hybridizations and microarray analyses . . . 52
3.3 Results . . . 53
3.3.1 Differentially expressed genes . . . 53
3.3.1.1 Drought Stress . . . 55
3.3.1.2 Rewatering . . . 55
3.4 Discussion . . . 59
3.4.1 Drought Stress . . . 59
3.4.2 Rewatering . . . 62

4 Conclusion & Perspectives 69

II Technical Aspects 71

5 Technical Aspects of Microarray Analysis 73
5.1 From Raw Material to Microarray Images . . . 73
5.1.1 Extraction of RNA, labeling, hybridization, and scanning . . . 73
5.1.2 Selection of hybridization scheme . . . 73
5.2 From Image to Raw Data . . . 75
5.2.1 Image Analysis . . . 75
5.2.2 Quality Check . . . 78
5.3 From Raw Data to Results . . . 81
5.3.1 Data Preprocessing . . . 81
5.3.2 Statistical Analysis and Annotation of Results . . . 82

6 Measurement of Prolin and related Compounds 85
6.1 Introduction . . . 86
6.2 Experimental . . . 89
6.2.1 Chemicals and reagents . . . 89
6.2.2 Preparation of plant material . . . 89
6.2.3 Sample preparation . . . 90
6.2.4 Sample analysis by HPAEC-PAD . . . 90
6.2.5 Sample analysis by HPLEC-MS . . . 91
6.3 Results and discussion . . . 92
6.3.1 Analysis of osmolytes by HPAEC-PAD . . . 92
6.3.2 Analysis of osmolytes by HPLEC-MS . . . 93
6.3.2.1 Chromatographic separation . . . 93
6.3.2.2 MS parameters . . . 94
6.3.2.3 Sensitivity and linearity . . . 96
6.3.3 Effect of drought exposure . . . 97
6.4 Conclusion . . . 99

7 Greenhouse 105
7.1 Experimental Setup . . . 105
7.2 Water Regime and Leaf Sampling . . . 107
7.3 Irrigation and Soil Moisture Measurement . . . 108

Acknowledgments 111

III Appendix 113

A Supplemental Material 115

B Abstract / Zusammenfassung 127
B.1 Abstract . . . 127
B.2 Zusammenfassung . . . 129

C Curriculum Vitae 131

# List of Figures


1.1 Factors determining the response of a plant to environmental stress (figure from Buchanan et al. (2000)) . . . . . 5
1.2 Plant response to stress from stress recognition via signal transduction and gene expression to altered cellular metabolism, finally resulting in physiological and developmental changes (Buchanan et al., 2000, modified). 7
1.3 Drought stress inducible genes grouped into two categories - functional proteins and regulatory proteins - according to their presumed function (Yamaguchi-Shinozaki et al., 2002). . . . . 8
1.4 Calcium and protein kinase mediated signaling, respectively are two of the major signaling pathways in plants. The two pathways are connected via calcium-dependent protein kinases. V=vacuole, C=chloroplast, N=nucleus (figure from Buchanan et al. (2000)) . . . . . 10
1.5 Osmotic Adjustment. . . . . 13
1.6 A hydration shell of a protein being disrupted by ions such as $Na^+$ and $Cl^-$ but staying intact in the presence of osmolytes such as proline. Figure from Buchanan et al. (2000). . . . . 14

2.1 Leaf Development Stages of Oak: for the first flush leaf development stages (DS) were determined on a scale of 1-10, for subsequent flushes (second and third) a six stage scale was applied. . . . . 25
2.2 Shoot growth during $2^{nd}$ and $3^{rd}$ flush of *Quercus robur* plants. Individual flushes are indicated by arrows (red arrow = $2^{nd}$ flush, yellow arrow = $3^{rd}$ flush). . . . . 26

2.3 Experimental Design comprising a control group (C) of 14 plants in 2006 and 2007 which is reduced to 6 plants in 2008. The drought stress group (DS) with 30 plants in 2006 is split into two groups of 15 plants respectively in 2007. One of these groups is re-watered (DS/RW) the other group is kept under drought stress (DS/DS). In 2008 6 plants of the drought stress group (DS/DS) are kept for a re-watering treatment (DS/DS/RW). . . . . 27
2.4 a) control and b-e) drought stress symptoms: b) change in leaf angle, c) symptoms on newly developed shoots, d), leaf curling, e) sporadic leaf colouring. . . . . 29
2.5 Plant development of a) control (C) and drought stressed plants (DS). b) control, drought stressed plants (DS/DS) and re-watered plants (DS/RW) and c) control and re-watered plants (DS/DS/RW). In a) points marked with an "*" are significantly different ($p<0.05$) from the controls, in b) and c) all points are significantly different except the ones marked with parentheses. The lower dashed line (Development Stage 11) indicates the start of the second flush, the upper one (Development Stage 17) the start of the third flush. . . . . 31
2.6 Shoot growth of a) control (C) and drought stress treated plants (DS) during the 1st year (2006), b) control, drought stress treated plants (DS-DS) and re-watered plants (DS-RW) during the 2nd year (2007) and c)control and re-watered plants (DS-DS-RW) after two years of drought stress in 2008. In a) and c) Columns marked with an * are significantly different ($p <0.05$), in b) columns not marked with the same letter are significantly different. . 33
2.7 Stem diameter for all treatments from 2006 to 2008. Columns not marked with the same letter are significantly different. The columns with diagonal lines (DS/DS/RW) represent drought stress treatments in 2006 and 2007 but re-watering in 2008. The columns with cross hatch (DS/RW) represent drought stress in 2006 but re-watering in 2007. . . . . 34
2.8 Stem diameter increment for all treatments from 2006 to 2008. Columns not marked with the same letter are significantly different. The columns with diagonal lines (DS/DS/RW) represent drought stress treatments in 2006 and 2007 but re-watering in 2008. The columns with cross hatch (DS/RW) represent drought stress in 2006 but re-watering in 2007. . . . . 34

2.9 Monosaccharide concentrations in oak leaves of drought stress treated plants, re-watered plants, and controls. In 2007 samplings III and IV are missing in the drought stress group because the plants did not develop a second flush. Points not marked with the same letter are significantly different ($p < 0.05$). 37
2.10 Sucrose concentrations in oak leaves of drought stress treated plants, re-watered plants, and controls. Samplings III and IV are missing in the drought stress group because the plants did not develop a second flush. Points not marked with the same letter are significantly different ($p < 0.05$). 37
2.11 Quercitol and Mannitol concentrations in oak leaves of drought stress treated plants, re-watered plants, and controls. Samplings III and IV are missing in the drought stress group because the plants did not develop a second flush. Points not marked with the same letter are significantly different ($p < 0.05$). 38

3.1 Experimental design comprising a control group of 14 plants in 2006 and 2007 and a drought stress group of 30 plants in 2006 which is split up in two groups in 2007. One group (15 plants) is kept under drought stress, the other group (15 plants) is irregated regularly till the end of the experiment. Pictures show oak leaves during normal watering (control, rewatering) and during drought stress (change in leaf angle). . . . . 51
3.2 Differentially expressed genes during a) drought stress at sampling times I-IV in 2006 and samplings I, II and V in 2007. b) Drought stress in 2006 and rewatering in 2007 with samplings I, II, III, and V for rewatered plants. Times of sampling are given in days (d) and weeks (w) of stress exposure. . 54
3.3 Number of up-regulated genes for selected functional groups during a) stress in 2006 and 2007 and b) stress in 2006, rewatering (RW) in 2007. . . . . 54
3.4 Number of down-regulated genes for selected functional groups during a) stress in 2006 and 2007 and b) stress in 2006, rewatering (RW) in 2007. . . 55
3.5 a) Concentration of glucose in leaves of drought stressed plants at different sampling times in 2006 and 2007. Points not marked with the same letter are significantly different. At certain time points carbohydrate breakdown-related genes (encoding for amylases) were detected as being differentially expressed. b) Graphs indicate the log fold change of amylase encoding genes at different samplings of drought stressed plants in 2006 and 2007. . . . . 56

3.6 a) Shoot growth of control (C) and drought stressed plants (DS) during the second flush in 2006. Columns marked with an * are significantly different ($p<0.05$). b) Shoot growth of control, drought stressed, and rewatered plants (RW) during first and second flush in 2007. Columns not marked with the same letter are significantly different c) Log fold change of gene Expansin A8 at sampling time II (first flush) in 2006 and 2007 for drought stressed plants (DS) and at samplings I (first flush) and III (second flush) 2007 for rewatered plants (RW). . . . . 59

5.1 Microarray construction and sample preparation for microarray hybridization. 74
5.2 M/A-plot of background corrected spot intensities in Genepix using the default local median background subtraction. Graph taken from Smyth et al. (2008). . . . . 76
5.3 M/A-plot of background subtracted spot intensities in Spot with method "morph". Graph taken from Smyth et al. (2008). . . . . 77
5.4 Diagnostic Plots for microarray quality control representing a microarray of good quality. . . . . 79
5.5 Diagnostic Plots for microarray quality control representing a microarray with uneven hybridization (3, 4, 1) which cannot be fully removed by normalization. . . . . 80
5.6 Boxplot of log-ratios (M) across eight arrays. Using this boxplot the need for between-array normalization can be assessed by displaying scale differences among arrays. . . . . 81
5.7 Overview of microarray analysis starting with image analysis followed by quality control, normalization, statistical analysis and multiple testing correction resulting in a list of differentially expressed genes. . . . . 83

6.1 Scheme of biosynthetic pathways of nitrogenous osmoprotectants (proline, hydroxyproline, N-methylproline, glycine betaine and trigonelline) in plants (SAM, S-adenosylmethionine; SAHc, S-adenosylhomocysteine; underlined compounds are analysed in this study). . . . . 87
6.2 Chemical structures of proline, glycine betaine and analogues analysed in this study. N-acetylproline (AP) was used as internal standard (IS). . . . . 92
6.3 HPLEC-PAD chromatogram of nitrogenous osmoprotectants standard solution at 100 µM (MP: N-Methylproline; P: Proline; HP: Hydroxyproline). . 93

6.4 HPLEC-MS chromatograms of nitrogenous osmoprotectants (AP: N-Acetylproline; HP: Hydroxyproline; GB: Glycine Betaine; MP: N-Methylproline; P: Proline and T: Trigonelline). a) Standard solution at 5 µM, b) Control sample, c) Drought stressed sample. . . . . 95

7.1 Five year old plants of *Quercus robur* clone P28 H9 in the greenhouse. . . . 105
7.2 Experimental Setup of oak plants distributed over two greenhouse cabins including irrigation system for controls (and rewatered plants) and drought stressed oak plants. . . . . 106
7.3 Volumetric soil moisture content (%) for controls (C), drought stressed (DS), and rewatered plants (RW) during the second year of the experiment (2007). Leaves were sampled during the a) first and b) second flush in the first (2006) and second year (2007) of the experiment. c) In 2007 an additional leaf sampling was included at the end of the growing season. . . 108
7.4 Irrigation system for controls and drought stressed plants. Water is applied at the soil surface and at 10 cm soil depth to facilitate homogenous/uniform water supply. . . . . 109
7.5 Measurement of volumetric soil moisture was conducted with a ThetaProbe ML2x FD-Probe at 10 cm and 15 cm soil depth as well as on the soil surface.109

A.1 Concentrations of Arabinose and Raffinose in oak leaves of drought stress treated plants and controls in 2006. Points not marked with the same letter are significantly different ($p < 0.05$). . . . . 115
A.2 Concentrations of Rhamnose, Raffinose and Maltose in oak leaves of drought stress treated plants, re-watered plants, and controls in 2007. Points not marked with the same letter are significantly different ($p < 0.05$). . . . . 116
A.3 Concentrations of Inositol, Trehalose, and Mannitol in oak leaves of drought stress treated plants and controls in 2006. Points not marked with the same letter are significantly different ($p < 0.05$). . . . . 116
A.4 Concentrations of Inositol, Sorbitol, and Galactinol in oak leaves of drought stress treated plants, re-watered plants, and controls in 2007. Points not marked with the same letter are significantly different ($p < 0.05$). . . . . 117
A.5 Concentrations of Proline, Methyl-, and Hydroxyproline in oak leaves of drought stress treated plants and controls in 2006. Points not marked with the same letter are significantly different ($p < 0.05$). . . . . 118

A.6 Concentrations of Betain and Trigonelline in oak leaves of drought stress treated plants and controls in 2006. Points not marked with the same letter are significantly different (p <0.05). . . . . 118
A.7 Concentrations of Proline, Methyl-, and Hydroxyproline in oak leaves of drought stress treated plants, re-watered plants, and controls in 2007. Points not marked with the same letter are significantly different (p <0.05). . . . 119
A.8 Concentrations of Betain and Trigonelline in oak leaves of drought stress treated plants, re-watered plants, and controls in 2007. Points not marked with the same letter are significantly different (p <0.05). . . . . 119

C.1 Poster at the Plant Abiotic Stress Conference 2008. . . . . 136
C.2 Poster for Poster Competition at the ARC Seibersdorf. . . . . 137
C.3 Poster for BOKU Student Seminar. . . . . 138
C.4 Poster at the SHE Conference 2008 in Vienna. . . . . 139

# List of Tables

2.1 Time schedule of leaf sampling (year, date, flush, days of stress) and volumetric soil moisture [%] for controls (C), drought stressed (DS) and re-watered (RW) plants at different sampling times in all experimental years. 26

3.1 Time schedule of leaf sampling (year, date, flush, days of stress) and volumetric soil moisture (%) for controls (C), drought stressed (DS) and re-watered (RW) plants at different sampling times in 2006 and 2007. Samples in brackets were not used for microarray-analysis . . . . . 50
3.2 Differentially expressed genes (adjusted p-values <0.05) of selected functional groups at sampling times I-V in 2006 and I, II and V in 2007 for drought stressed plants (DS) and sampling times I, II, III, and V in 2007 for rewatered plants (RW). For each gene accession number, annotation and log-fold change are given. Continued in table 3.3. . . . . 57
3.3 Continued from table 3.2. . . . . 58

6.1 Quantification parameters of proline, glycine betaine and analogues. . . . . 94
6.2 Calibration data (unweighted linear regression without offset) of target analytes. . . . . 96
6.3 Within-day recovery of N-acetyl proline added to oak leave extracts . . . . 97
6.4 Effect of drought exposure on endogenous proline (P), glycine betaine (GB), N-methylproline (MP), hydroxyproline (HP) and trigonelline (T) levels in leaves of *Quercus robur*. . . . . 98

A.1 Differentially expressed genes (adjusted p-Values <0.05) allocated to functional groups at sampling times I-V in 2006 and I, II and V in 2007 for drought stressed plants (DS) and sampling times I, II, III, and V in 2007 for rewatered Plants (RW). For each gene accession number, annotation (est2uni) and log-fold change are given. Continued in table A.2. . . . . 120

A.2 Continued from table A.1. Continued in table A.3. . . . . 121
A.3 Continued from table A.2. Continued in table A.4. . . . . 122
A.4 Continued from table A.3. Continued in table A.5. . . . . 123
A.5 Continued from table A.4. . . . . 124
A.6 Sample R results table. . . . . 125

# Part I

# Main Part

# CHAPTER 1

## General Introduction

### 1.1 Climate Change

During the past 100 years the climate has warmed by approximately 0.6 °C. In this period the rate of warming from 1976 to today was twice as high as between 1910 and 1945 (Walther et al., 2002). With continuing increases in greenhouse gas concentrations the temperature of the atmosphere is expected to increase by another 1-3.5 °C till 2100. Based on these increasing temperatures it is expected that global hydrologic budgets will be modified in a way that - depending on location - winter precipitation at high latitudes, the number of droughts and floods, and days with high temperature will increase. Despite uncertainties the severity of future drought conditions is assumed to increase (Hanson and Weltzin, 2000, and references within). While in Austria the precipitation in winter months is expected to increase a decrease in precipitation by 10-50% during the summer months, especially in August, is expected (Niedermair et al., 2007). Drought stress in the summer months however, has considerable effects on tree growth and seedling/sapling survival (Pessarakli, 1999) and measures should be taken to avoid economical losses. One recommendation is to choose local trees which show a broad ecological range and are well adapted to the expected climate change (Niedermair et al., 2007). This could be realized by transforming the now predominating coniferous forests into mixed forests with drought adapted oak, especially on dry sites (Nicolussi et al., 2006; Schüler et al., 2007).

### 1.2 Oak

*Quercus* (oak), with about 200-450 species (Krahl-Urban, 1959; Krüssmann, 1978; Neger and Münch, 1950), comprises the largest genus of the Fagaceae (beech family). The genus

is divided into three groups

- red oaks (*Quercus* section *Lobatae*),
- white oaks (*Quercus* section *Quercus*) and
- intermediates (*Quercus* section *Protobalamus*).

All of them comprise tree and shrub species. In the intermediate group only evergreen oaks are found whereas the red and white oaks also include deciduous species. The intermediate group - with only five species - is limited to the southwestern United States and northwestern Mexico, while the red oaks are present in the Western Hemisphere, and the white oaks are widely distributed over the Northern Hemisphere (Johnson et al., 2002). In Europe more than 10 million ha are covered by native oaks comprising 24 species subdivided into 15 deciduous and nine evergreen species (van Loo, 2003; Kleinschmit, 1993). The most abundant species found in Central Europe are *Q. petraea* L. (sessile oak) and *Q. robur* [Matt.] Liebl. (pedunculate oak), both deciduous tree species belonging to the subgroup of white oaks. In Austria, oaks cover 2% of the total forest which corresponds to an area of 66,000 ha. This fraction shall be increased in future by replacing Norway spruce with deciduous trees such as oaks in order to obtain/create stable mixed forests (Schüler et al., 2007) being more resistant to ecological challenges such as wind and drought. While adult oak trees can withstand periods of drought the seeds of *Quercus robur* L. are desiccation-sensitive (recalcitrant). Still, the acorns of oak possess some adaptations to dehydration including the accumulation of abscisic acid (ABA) and dehydrins (LEA-proteins) during maturation (Prewein et al., 2006; Sunderlíková et al., 2009).

## 1.3 Water deficit stress

Water deficit stress can result from periods without rainfall - drought periods - causing soil-water deficit and finally plant-water deficit. Another cause for dehydration stress can be the formation of ice crystals in intercellular spaces due to freezing temperatures. However, water deficit stress can also occur when water is not limiting such as in saline habitats where high salt concentrations impede the plant roots from extracting water from the soil. Water deficit is characterized as a stress when inducing injury in the plant. It can disrupt many cellular functions, alter gene expression and slow down growth (Buchanan et al., 2000; Bray, 2001). Drought can reduce growth of adult trees by 30-70% and stem diameter increment by 70-80%. Besides, water deficit stress is a major cause of seedling

mortality in trees causing as much as 60% of the first-year mortality in pine plantations (Pessarakli, 1999; Lyr et al., 1992).

### 1.3.1 Factors determining the plant's response to water deficit stress/drought

The response of a plant to environmental stress is determined by a multitude of factors (Figure 1.1). Responses differ not only depending on the characteristics of the stress such as severity, duration, number of exposures or a combination of multiple stresses. They also vary with respect to the plants stage of development, genotype and organ or tissue. Furthermore, plants may apply different strategies to avoid and/or tolerate stress enabling them to survive and grow even under unfavourable conditions (resistance) while plants which lack these strategies will die (susceptibility).

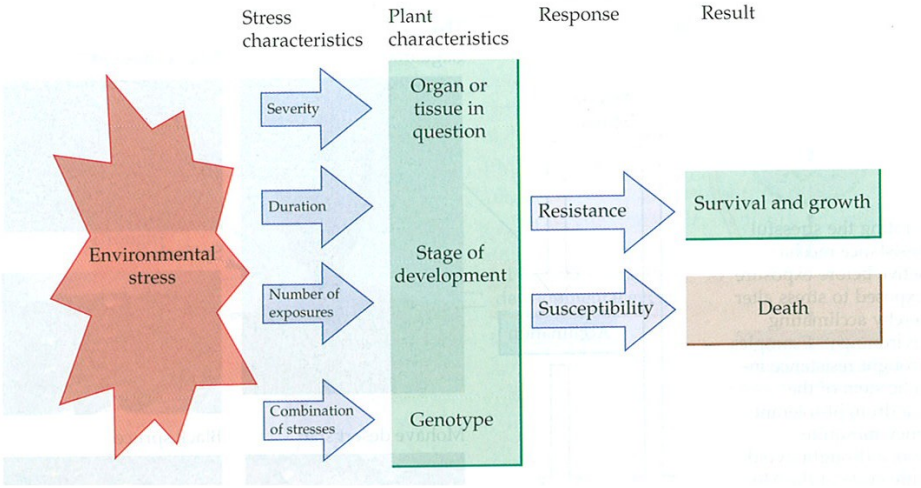


**Figure 1.1:** Factors determining the response of a plant to environmental stress (figure from Buchanan et al. (2000))

### 1.3.2 Drought Avoidance and Tolerance

Resistance to drought can be achieved by two approaches: avoidance or tolerance. Drought avoidance is characterized by mechanisms which prevent the exposure to stress (Buchanan et al., 2000). According to Chaves et al. (2003) these include strategies to

1. minimise water loss
2. maximise water uptake.

Water uptake is maximised by deep rooting to improve access to groundwater and thus survival of long periods without rainfall. Strategies to minimise water loss comprise the

reduction of transpiring surface through small leaves and even leaf shedding under severe stress, sunken stomata, a thick cuticula, hairs or trichomes on the leaves, the ability to orientate the aerial plant parts in a way that the effect of sunlight on transpiration is minimal (e.g. circadian rhythm of leaf movement, leaf rolling) and specialized photosynthetic pathways (C4, CAM) (Chandler and Bartels, 2003). Yet another mechanism would be to escape water-deficit by completing the life cycle while water is available (e.g. desert ephemerals). On the other hand, plants evolved tolerance mechanisms which allow them to withstand drought. This means that plants can tolerate low water potential while maintaining turgor pressure. Strategies to tolerate cellular dehydration include biochemical mechanisms such as osmotic adjustment and/or morphological adaptations such as smaller cells and more rigid cell walls.

### 1.3.3 Responses to drought: from molecular changes to physiological adaptations

When plants are exposed to environmental stresses such as drought the first step in the response is the perception of the stress signal followed by signal transduction via different pathways depending on the nature of the stress. The gene expression changes elicited by these signals then lead to the production of regulatory and functional proteins, thus transmitting the plant's response from the cellular to the whole plant level resulting in physiological and developmental changes and adaptations to the stress (Figure 1.2).

Under water deficit stress a multitude of genes is induced: i) regulatory genes being involved in signal transduction and the regulation of gene expression and ii) genes being directly involved in stress response (e.g. through osmotic adjustment) (Figure 1.3). The first group contains transcription factors, protein kinases and enzymes of the phospholipid metabolism. The second group comprises proteins such as water channel proteins, proteases, chaperones, late embryogenesis abundant (LEA) proteins, mRNA binding proteins, enzymes involved in osmolyte biosynthesis, sugar and proline transporters and detoxification enzymes (Yamaguchi-Shinozaki et al., 2002). These groups of proteins can further be distinguished by their expression profile. While genes of the first group with signalling and regulatory functions are expressed rapidly within several hours and then are down-regulated again, the second group of genes - encoding functional proteins - is induced slowly within 10 hours after the stress treatment (Yamaguchi-Shinozaki and Shinozaki, 2006).

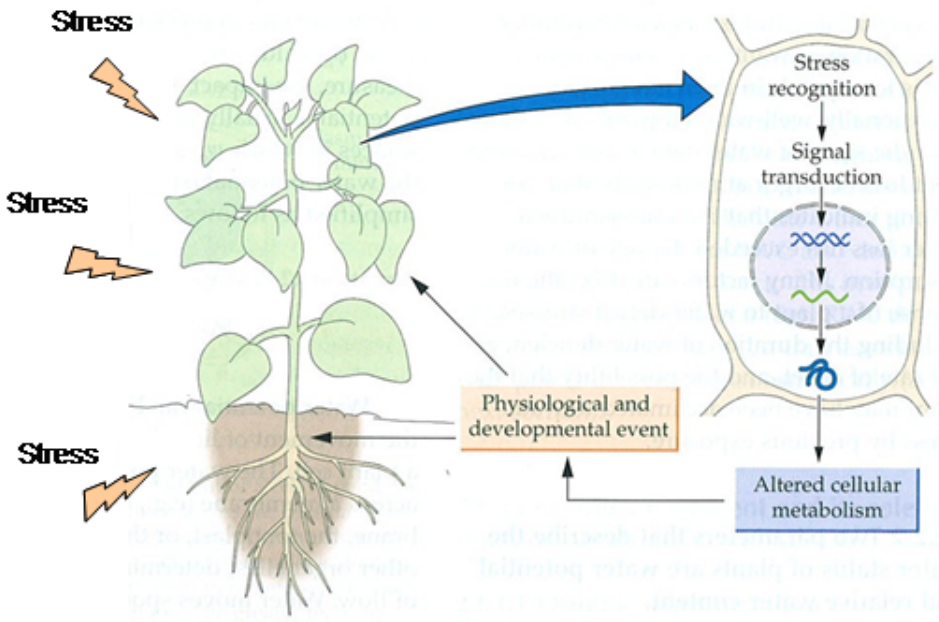


**Figure 1.2:** Plant response to stress from stress recognition via signal transduction and gene expression to altered cellular metabolism, finally resulting in physiological and developmental changes (Buchanan et al., 2000, modified).

#### 1.3.3.1 Signal perception and transduction

Until now little is known about how the plant senses stress. However, there must be a mechanism which enables the plant to sense water deficit stress. Effects of drought stress which the plant may sense include loss of turgor pressure, reduced water potential, changes in cell volume and conformational changes of cellular macromolecules (Bray, 1997). These changes may then initiate signal transduction cascades such as the histidine kinases which are most certainly involved in the initial sensing of dehydration stress. These osmosensors form a two-component system containing a histidine kinase as a sensor and a response regulator which transmits the phosphorylation signal, finally leading to gene activation. In *Arabidopsis*, the transmembrane histidine kinase AtHK1, which is up-regulated in response to drought stress, is thought to serve as osmosensor (Bray, 2002; Hirt and Shinozaki, 2004; Knight and Knight, 2001; Ramanjulu and Bartels, 2002). Two major forms of signalling in the plant are protein kinases which change the activity of target proteins via phosphorylation, and intracellular calcium ($Ca^{2+}$) which belongs to the so-called second messengers (Buchanan et al., 2000). One of the earliest responses of a plant to stress is the activation of MAPK (mitogen-activated protein kinase) cascades through an alteration of plasma membrane fluidity. Kinases such as the mitogen-activated

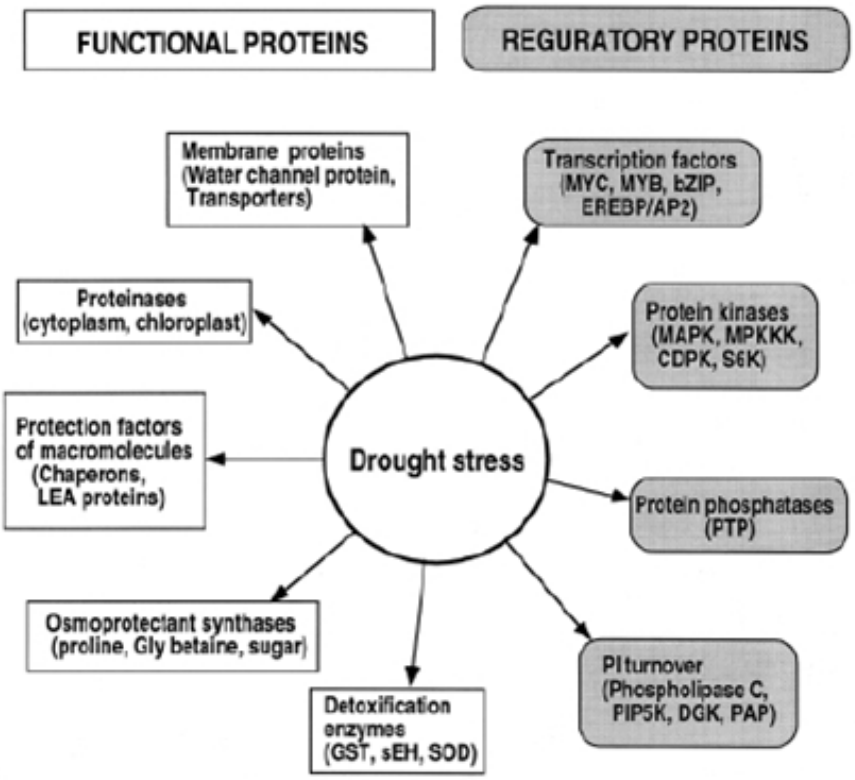


**Figure 1.3:** Drought stress inducible genes grouped into two categories - functional proteins and regulatory proteins - according to their presumed function (Yamaguchi-Shinozaki et al., 2002).

protein kinases (MAPKs) then regulate transcription by reversible protein phosphorylation. Activation upon dehydration has been shown for several kinases with sequence similarity to MAPKs. In Arabidiopsis, for example, AtMEKK1 is induced by drought, cold, and mechanical stress. In contrast, the levels of the MAPKs ATMPK4 and ATMPK6 do not change upon stress, instead the enzyme activities do increase.

Another early response to stress is an increase in cytosolic $Ca_2^+$ (See figure 1.4). Similar to the activation of MAPKs changes in the concentration of $Ca^{2+}$ can be induced by alterations in membrane fluidity and subsequent alteration of the $Ca^{2+}$ channels. This change in $Ca^{2+}$ concentration then can induce physiological changes such as the closure or opening of stomata. The precise effect of the calcium signal is supposed to depend not only on the magnitude of the induced $Ca^{2+}$-elevation but also on the $Ca^{2+}$-channel, the cellular location, and the availability of downstream signalling pathways, altogether defining the so-called “calcium-signature”. Furthermore, calcium can affect and interact with many different signalling pathways (“crosstalk”) which in turn involve many different proteins

and second messengers. Another aspect of calcium signalling are $Ca^{2+}$-regulated proteins including calmodulin, calcium-dependent protein kinases (CDPKs) and calcium-regulated phosphatases. CDPKs connect the two signalling pathways involving calcium and protein kinases, respectively. Phosphatases are generally thought to repress stress responses, examples being ABI1 and ABI2 proteins which are negative regulators of ABA signal transduction. MAP kinases (and other kinases), on the other hand, rapidly transmit signals which in turn induce/initiate the synthesis of protective compounds that reduce the negative effect of dehydration on the metabolism (Hirt and Shinozaki, 2004; Knight and Knight, 2001; Ramanjulu and Bartels, 2002). Other important signal molecules involved in the response to drought stress are abscisic acid (ABA) and sugars showing considerable interactions ("crosstalk") between their signalling pathways. Sugar and ABA play a role in many developmental processes (where they may act synergistically) and can alter gene expression upon drought stress. A detailed description of their action and role in signalling is given in reviews by Bray (2002); Finkelstein and Gibson (2001); Gibson (2004, 2005); Koch (2004); Rolland et al. (2006).

#### 1.3.3.2 Gene regulation

**Transcription factors** Transcription factors are proteins binding to promoter regulatory elements to facilitate transcription. They include basic-region leucine zipper (b-Zip) proteins, Myb-like proteins, Myc-like bHLH proteins and HD-ZIP proteins. Basic leucine zipper (b-Zip) proteins contain a basic region and a Leu-zipper motif. Several b-Zip factors bind ABA-responsive elements (ABREs) and induce gene expression by ABA and/or dehydration. Examples of these proteins are the wheat EmBP1, the tobacco TAF-1 and the OsZIP-1a (Shinozaki and Yamaguchi-Shinozaki, 1997; Ramanjulu and Bartels, 2002). Several Myb-type transcription factors are induced by dehydration. In *Arabidopsis* the gene AtMyb2 has been shown to be induced by salt, dehydration and ABA and positively regulates rd22 gene expression. The basic helix-loop-helix (bHLH) type transcription factor AtMYC2 which is induced by dehydration and ABA was also found to bind to the rd22 promoter. This suggests that both AtMYB2 and AtMYC2 play a role in gene expression control upon dehydration stress. In *C. plantagineum* three Myb transcription factors have been identified showing homology to AtMYB2. The Myb-like gene cpm10 in *C. plantagineum* is upregulated by ABA whereas the gene cpm7 is up-regulated upon dehydration in roots only (Hirt and Shinozaki, 2004; Ramanjulu and Bartels, 2002). Homeodomain-leucine zipper (HD-ZIP) genes are supposed to regulate development and are induced by many environmental stresses including dehydration.

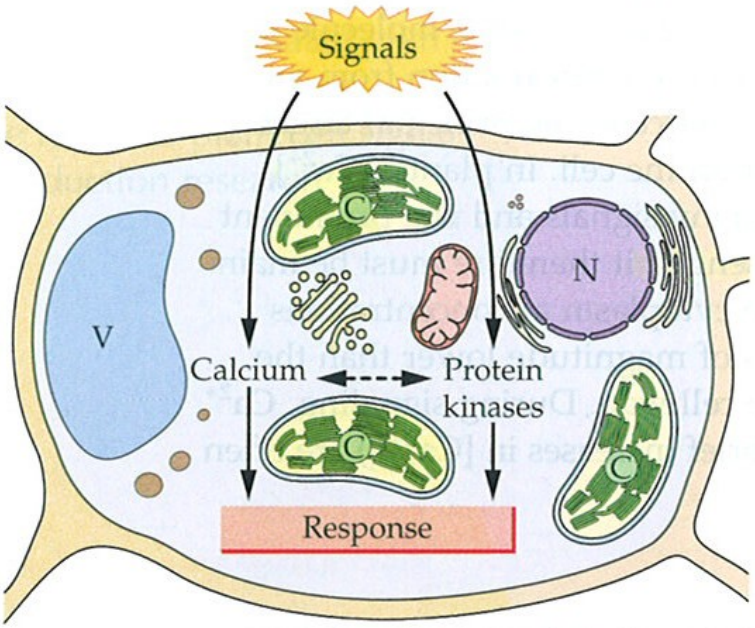


**Figure 1.4:** Calcium and protein kinase mediated signaling, respectively are two of the major signaling pathways in plants. The two pathways are connected via calcium-dependent protein kinases. V=vacuole, C=chloroplast, N=nucleus (figure from Buchanan et al. (2000))

In *Arabidopsis* two genes ATHB6 and ATHB7 are induced by dehydration in an ABA dependent manner. In *C. plantagineum* two HD-ZIP family II genes have been identified both being inducible by dehydration but only CpHB2 being inducible by ABA, too. This leads to the assumption of two pathways being induced by dehydration, one ABA-dependent and one ABA-independent. In addition to these positively reglulated genes identified in *C. plantagineum* other HD-ZIP genes are down-regulated by dehydration. This leads to the conclusion that some HD-ZIP proteins may function as repressors of dehydration-induced genes (Hirt and Shinozaki, 2004; Ramanjulu and Bartels, 2002).

Dehydration responsive element and ABA responsive element The dehydration responsive element (DRE) also referred to as C-repeat element (CRT) has been identified in many promoters of drought- and cold-regulated genes (e.g. RD29A). Two groups of transcription factors that bind to this cis-acting element have been identified: DREB1 (dehydration responsive element binding, also known as C-repeat binding factor (CBF)) which is induced by cold, and DREB2 being induced by dehydration. These binding factors are characterized by an AP2 domain which is a 60-amino acid DNA-binding motif. Members of the AP2-

domain subfamily play a role in pathogen defence, in jasmonate and ethylene signalling and are also known as ethylene responsive element-binding-factors (ERF proteins) (Hirt and Shinozaki, 2004; Knight and Knight, 2001). The ABA responsive promoter element (ABRE), representing a subgroup of the G-box elements and being important for ABA- and dehydration- responsive expression has first been identified in a LEA gene in rice (rab16A). Several bZIP-transcription factors have been shown to interact with this element and thus are termed ABA responsive element binding factors (ABF) or ABA responsive element binding (AREB) proteins. The gene RD29A (also known as LTI78 or COR78) contains both DRE and ABRE elements in its promoter and can be activated both by ABA-independent and ABA-dependent pathways (Hirt and Shinozaki, 2004; Knight and Knight, 2001).

#### 1.3.3.3 Functional changes

**Proteins with protective function** Late embryogenesis abundant (LEA) proteins were first identified in seeds where they are involved in maturation and desiccation. In addition, they have been shown to accumulate in vegetative tissues of plants in response to dehydration, low temperature, salinity, and exogenous ABA treatment. LEA proteins are very hydrophilic, glycine-rich, and remain soluble when boiled. LEA proteins are divided into five groups according to their properties. Group 1 is characterized by a 20-amino-acid motif, a representative being the Em (early methionine-labeled) protein from wheat. LEA proteins from Group 2 are also termed dehydrins, their characteristic feature being a lysine-rich 15-amino-acid motif which may form alpha-helices. In oak two dehydrins (QrDhn2 and QrDhn3) have been identified accumulating not only in somatic embryos but also in vegetative tissue in response to desiccation stress (Sunderlíková et al., 2009). Group 3 is characterized by a repeat motif of 11 amino acids, an example being HVA1 from barley which improved stress tolerance to drought and salinity in transgenic rice. Group 4 LEA proteins share a conserved N-terminal region predicted to form an alpha-helix. LEA proteins from Group 5 are rich in alanine, glycine, and threonine and are not soluble after boiling. These LEA proteins are thought to maintain structural integrity by binding proteins or membranes (Hirt and Shinozaki, 2004; Ramanjulu and Bartels, 2002; Buchanan et al., 2000). Another group of proteins involved in desiccation tolerance and being induced by dehydration stress are small heat shock proteins (HSP). They are thought to serve as chaperones maintaining protein function or refolding proteins which are already denatured (Hoekstra et al., 2001; Buchanan et al., 2000).

Aquaporins Aquaporins are channel proteins facilitating the transport of water across cell membranes. They have molecular weights of 26-34 kDa and display a conserved structure with six membrane spanning alpha helixes. In plants, four major groups of aquaporins have been identified. The most abundant aquaporins belong to the groups of tonoplast intrinsic proteins (TIP) and plasma membrane intrinsic proteins (PIP). TIPs are assumed to be involved in cell osmoregulation whereas PIPs play a major role in the control of transcellular water transport. The plasma membrane intrinsic proteins (PIPs) comprise two subgroups: PIP1 with low and PIP2 with high water channel activity. A third group comprises the nodulin-like membrane intrinsic proteins (NIPs) with unknown membrane location. Small basic membrane intrinsic proteins (SIPs), with unknown function and localization, constitute the fourth group of aquaporins. Due to significant sequence differences to the other groups of aquaporins it is assumed that SIPs differ in transport selectivity. It has been shown that water stress results in changed expression patterns of membrane intrinsic proteins (MIPs). A number of PIP genes are up-regulated in response to stress, including rd28 from *Arabidopsis*, the tomato-ripening-associated membrane protein TRAMP, and mipA in *Brassica oleracea*. Some PIP- and TIP genes, on the other hand, were down-regulated under drought stress including SunTIP18 in sunflower and Cp-TIP in the resurrection plant (Luu and Maurel, 2005; Tyerman et al., 2002; Ramanjulu and Bartels, 2002).

Detoxification Oxidative stress can be considered as a consequence of many environmental stresses such as dehydration leading to the accumulation of reactive oxygen species (ROS) including hydrogen peroxide, hydroxyl radicals, and superoxide anions. While a low concentration of reactive oxygen species is essential for the plant and serves as signalling molecules during stress, an accumulation of ROS causes damage to RNA, DNA, proteins, and membranes (Hirt and Shinozaki, 2004; Xiong and Zhu, 2002; Ramanjulu and Bartels, 2002). In order to prevent damaging effects of ROS plants have evolved complex protective mechanisms. They include antioxidant enzymes such as catalases, peroxidases and superoxide dismutase and free radical scavengers such as ascorbate, carotenoids, and oxidized (GSSG) and reduced glutathione (GSH) (Mundree et al., 2002). Furthermore, an aldehyde dehydrogenase seems to be involved in the detoxification of reactive aldehydes which form during the peroxidation of lipids by reactive oxygen species. Genes encoding an aldehyde dehydrogenase are expressed upon dehydration in *C. plantagineum* (Cp-ALDH) and *Arabidopsis* (Ath-ALDH3) (Hirt and Shinozaki, 2004).

Osmotic adjustment One important mechanism in plant tolerance to drought is osmotic adjustment which permits the plant to maintain osmotic pressure (turgor) under drought by actively increasing the concentration of solutes in the cytoplasm (Figure 1.5).

This allows the plant to extract water from the soil by keeping the water potential in the roots lower than in the soil. The solutes which accumulate during osmotic adjustment are termed osmolytes or compatible solutes. They are highly soluble molecules of low molecular weight, and due to their neutrality do not interfere with normal metabolic processes (Buchanan et al., 2000; Bray et al., 2000). Compatible solutes comprise betaines (e.g. glycine betaine), amino acids such as proline and sugars and polyols such as glucose, fructose, sucrose, trehalose, mannitol, sorbitol and quercitol. However, the distribution of these osmolytes varies with plant species. Sugars such as glucose and fructose can be released during starch degradation under stress but can quickly be re-utilized for starch synthesis upon stress relief. To this group of readily metabolizable osmolytes also the amino acid proline belongs, being synthesized upon stress and used as energy source upon re-watering of the plant (Buchanan et al., 2000). Glycine betaine and many polyols (e.g. quercitol), on the other hand, are irreversibly synthesized and thus are considered as stable osmolytes. In addition to their function in maintaining turgor many compatible solutes (e.g. glycine betaine, proline, and mannitol) are thought to stabilize proteins, protein complexes, and membranes. For example, they are able to prevent the dissociation of the oxygen evolving complex of photosystem II and can increase the thermal stability of

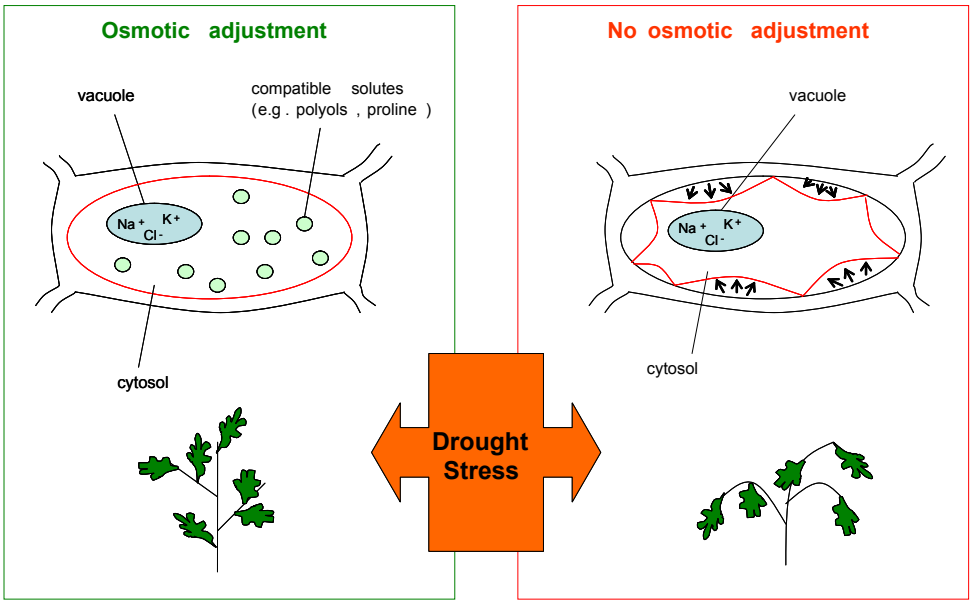


**Figure 1.5:** Osmotic Adjustment.

enzymes. Glycine-betaine, for example, can inhibit the inactivation of Rubisco under salt stress. Osmolytes protect proteins by preventing the disruption of their hydration shells through ions such as $Na^+$ or $Cl^-$. Unlike ions osmolytes do not penetrate the hydration shell, thus the hydration shell of the protein stays intact (Figure 1.6) (Bohnert and Shen, 1999; Buchanan et al., 2000). Another proposed function of compatible solutes is the scavenging of oxygen radicals whose production increases under stress. Among others, mannitol, sorbitol, proline and pinitol all were reported to have oxygen scavenging activity and thus may proctect proteins from oxidative damage (Bohnert and Shen, 1999). Much effort has already been put into transgenics trying to increase plant resistance to drought stress by increasing osmolyte production (Bohnert and Shen, 1999; Hare et al., 1998). However, not always the accumulation of compatible solutes resulted in more resistant phenotypes and further research needs to be conducted in this field.

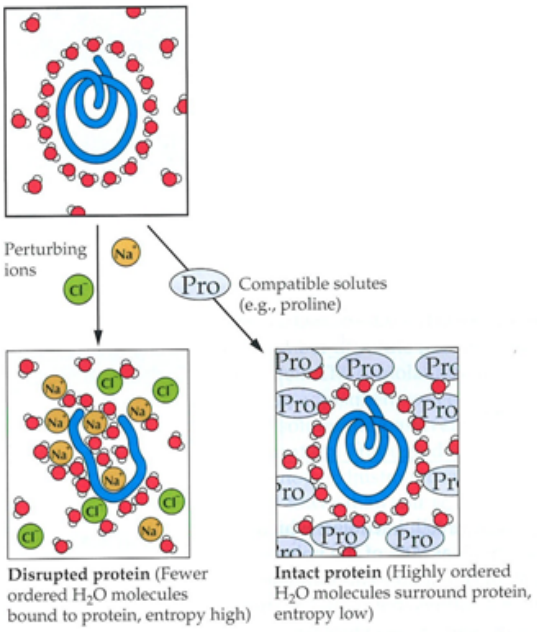


**Figure 1.6:** A hydration shell of a protein being disrupted by ions such as $Na^+$ and $Cl^-$ but staying intact in the presence of osmolytes such as proline. Figure from Buchanan et al. (2000).

## 1.4 Objectives

Drought and osmotic stress have been studied intensively in many plant species including monocotyledons (e.g. barley, maize, rice) and dicotyledons (e.g. *Arabidopsis*, sugarcane). Out of trees, poplar (as a model species) has been most extensively studied. Most of these studies, however, were focused on severe short term drought stress. Examples are given by Tuberosa and Salvi (2006) for *Arabidopsis*, rice, sorghum and maize. In addition, studies have been conducted on the monocotyledons barley and maize by Yu and Setter (2003); Talamè et al. (2006), respectively. *Arabidopsis* as a model plant in the dicotyledonous plants has been studied extensively, examples being represented by Huang et al. (2008); Kilian et al. (2007); Kreps et al. (2002). Another representative of the dicots, sugarcane has been examined by Rodrigues et al. (2009). In the group of trees studies on osmotic stress have been conducted by Gu et al. (2004) on *Populus euphratica*, for example, and Watkinson et al. (2003) on loblolly pine. All of these studies were restricted to a relatively short drought stress period ranging from several hours to a few weeks at a maximum. In addition, studies covering gene expression, such as the above mentioned, usually do not contain extensive data on the plants' morphological and physiological responses to stress. For this reason, the aim of this study was

1. to characterize the response of *Quercus robur* to drought stress and re-watering on the "whole-plant-level" including data on the
   a) morphological (shoot growth, stem diameter increment)
   b) phenological (flushing)
   c) physiological (carbohydrates, polyols, proline and related compounds) and
   d) gene expression level (using microarray technology) and
   e) proteome level (data not yet available).
2. to expose the oak plants to a long-term drought stress of one year and two years
3. to investigate the plant response during a re-watering period of several months after one year and two years of drought stress

This thesis constitutes the first of two parts of a project dealing with the monitoring of environmental influences on stress regulated genes in oak. The first part was conducted in cooperation with the "Centre de Recherche Public Gabriel Lippmann" in Luxembourg where physiological analyses and proteome analysis were/are carried out. In the second

part of the project the results of this study are used for the development of functional markers for the selection of oak plants with increased drought resistance potential.

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# CHAPTER 2

## Morphology & Physiology — The Effect of Long-term Drought Stress & Rewatering on Plant Growth & Stress Metabolites in *Quercus robur*


## Abstract

In a three-year glasshouse experiment five year old clonally propagated oak plants from tissue culture were subjected to controlled drought stress and subsequent rewatering. Phenotypic parameters were monitored and leaf material was sampled in regular intervals for the analysis of osmolytes including carbohydrates, polyols, and nitrogenous osmolytes (glycine betaine, trigonelline, and proline and analogues). The experiment comprised three treatments: 1) control, 2) one year drought, followed by one year rewatering, 3) two years drought, followed by one year rewatering. Drought stress led to significantly ($p < 0.05$) reduced shoot growth, diameter increment, and absence of a 2nd flush during the 2nd year of stress. Also, a significant increase ($p < 0.05$) in the concentrations of glucose, fructose, and galactose could be observed under drought stress in both years while for the nitrogenous compounds no clear/consistent correlation with the drought stress treatment could be found. During rewatering, oaks fully resumed growth and osmolyte concentrations went back to control levels. The growth pattern of rewatered oak plants, however, differed from that of the controls. Although plants were strongly impaired by drought stress and displayed a reduced first flush under rewatering they maintained the capability to fully recover and compensated for reduced growth during subsequent flushes.

## 2.1 Introduction

Oaks are estimated to comprise about 200-450 species (Krahl-Urban, 1959; Krüssmann, 1978; Neger and Münch, 1950) and are distributed over a very wide area from temperate regions to tropical zones (Kleinschmit, 1993). In Europe 24 species exist of which 15 are deciduous and nine evergreens (Kleinschmit, 1993). Pedunculate oak (*Quercus robur* L.) and sessile oak (*Q. petraea* [Matt.] Liebl.) are the most common deciduous forest tree species found in Central Europe (Thomas and Gausling, 2000). In addition, *Q. robur* has a distribution range from Europe to South West Asia and North Africa. This great natural range leads to a large variation within the species (Dissescu and Coca, 1973; Kleinschmit, 1993) and adaptations to a multitude of conditions. Depending on their natural distribution range from mesic to xeric sites, different oak species have developed a variety of responses and adaptations to various degrees of water stress on the morphological, phenological and physiological level. Under limited water supply trees usually show restricted growth and have leaves that are smaller, with less stomata and a thicker cuticula than trees on humid sites. Under severe drought stress the cyclic flushes of oak may even cease to occur and leaves might be shed early (Chaves et al., 2009; Dickson and Tomlinson, 1996). Plants have evolved a range of mechanisms to deal with water shortage. They may avoid drought by reducing shoot growth and/or deep rooting or tolerate drought through osmotic adjustment. Many oaks not only use drought avoidance strategies but show the ability for osmotic adjustment which is especially well developed in North American oak species (Abrams, 1990) but has also been described for European oaks (Kätzel and Löffler, 2004; Passarinho et al., 2006; Peltier et al., 1997; Épron and Dreyer, 1996). Osmotic adjustment is characterized through the active accumulation of compatible solutes such as carbohydrates, polyols or proline. These compatible solutes or osmolytes help the plant in maintaining turgor with increasing water deprivation. In addition, osmolytes have been proposed to play a role as osmoprotectants in scavenging free oxygen radicals (Hare et al., 1998), protecting protein structure, dry membranes and liposomes under stress (Crowe et al., 1992; Csonka, 1989; Ramanjulu and Bartels, 2002). Strategies to avoid or tolerate drought stress may occur individually or combined and are employed in different ways and intensities depending on time, intensity and duration of the drought stress at the respective site (Chaves et al., 2009). The aim of this study was to characterize the response of *Quercus robur* during a long-term drought stress (one year and two years) and re-watering. The morphological (plant growth), phenological (flushing) and physiological (carbohydrates, polyols) level was examined. To exclude additional variation (caused by

the plant material) five-year old oaks derived from somatic embryos were used.

## 2.2 Material & Methods

### 2.2.1 Plant Material and Growth Conditions

Plants from a 5-year-old oak clone P28 (*Quercus robur* L.), vegetatively propagated via somatic embryogenesis were used (Prewein et al., 2004). The plants were grown in peat moss (Einheitserde, Frux ED 63) in 20 L plastic containers in a greenhouse where air temperature (ambient) and humidity are regularly recorded. NPK long-term fertilizer was added after the end of the first growing season (see also Oufir et al. (2009)).

#### 2.2.1.1 Drought Stress Treatment

A preliminary experiment was carried out to estimate/determine the soil moisture level at which (first) stress symptoms occur (change in leaf angle) and to determine control conditions. Based on these results, soil water content for control plants (C) was kept at 30-50%. For the drought stress group (DS) soil moisture was set to 22-30% at the beginning of the first year. Later on, during summer (July, August) soil moisture was decreased to 13% in order to achieve similar visual symptoms (leaf angle). Usually, the difference in soil moisture between controls and drought stressed plants was 15-20% points. At the beginning of the growing season all plants were watered up to the saturation point of the soil (55-60%). The set of plants to be stressed were treated by withholding water. In the first year, water was applied manually, in the second year a combination of automatic and manual watering was used. The volumetric soil moisture content was measured with a ThetaProbe ML2x FD-Probe (Delta-T Devices, Cambridge, UK) usually three times a week. Soil moisture was measured at 10 and 15cm soil depth and at the soil surface.

#### 2.2.1.2 Re-watering

During re-watering (RW) plants were treated like controls with soil moisture content set to 30-50%.

### 2.2.2 Determining the Leaf Developmental Stages

The leaf development stages (DS) were determined on a scale of 1-10 for the first flush and six stages each for subsequent flushes (11-16, 17-22) based on characteristics given in

Figure 2.1. As a basis/reference for the development of the applied scheme the BBCH (Federal Biological Research Centre for Agriculture and Forestry) monograph on "Growth stages of mono-and dicotyledonous plants" was used (Meier, 2001).

### 2.2.3 Sampling

Four leaves from branch tips (first flush: development stage 8-9 and second flush: 14-15) were sampled from each plant, pooled, immediately frozen in liquid nitrogen and stored at -80 ℃(until further processing).

#### 2.2.3.1 First year (2006)

Sampling I after 3 days of drought exposure (average soil moisture for selected drought stressed trees was 20.5%, for selected controls 43.4%), and sampling II after 8 days of drought exposure (average soil moisture for selected drought stressed trees was 19.3%, for selected controls 45.7%), were conducted during the first flush (2006 May, table 2.1). Samples III (average soil moisture for selected drought stressed trees was 16.1%, for selected controls 49.4%) and IV (average soil moisture for selected drought stressed trees was 12.3%, for selected controls 40.3%) were taken during the second flush from newly developed shoots (June, July). Control plants were sampled after 35 and 41 days respectively, drought stressed plants 56 and 65 days respectively after start of the drought stress treatment. In order to keep the same development stage for both control and drought stressed plants, the latter were sampled 3 weeks later because their growth/second flush was delayed.

#### 2.2.3.2 Second year (2007)

For drought stressed plants and re-watered plants the first sampling during the first flush (April 2007) took place after four days (soil moisture for drought stressed plants was 14.5%, for re-watered plants 33.6%) the second sampling after 13 days (soil moisture for drought stressed plants was 8.8%, for re-watered plants 34.2%). The sampling times for the controls were delayed by one week because of later bud burst (soil moisture at sampling I was 37.7%, at sampling II 37.8%).
Sampling III and IV during the second flush (May/June) were conducted after 53 and 59 days for the re-watered plants (soil moisture at leaf sampling III 37.5% and 37.4% at sampling IV) while leaves of the controls were sampled after 80 and 87 days (soil moisture at sampling III 41.3%, at sampling IV 37.5%) because of the delayed plant development compared to the re-watered plants (second flush June/July).

**Leaf Development Stages Oak – first flush**

| | | | | | |
|---|---|---|---|---|---|
| 1 | | Brown (dormant) buds | 6 | | Leaf unfolding complete, leaves shining (reddish) |
| 2 | | Brown swollen buds | 7 | | Leaf expansion, leaves getting matt |
| 3 | | Green buds | 8 | | Darkening of leaves |
| 4 | | Bud break | 9 | | Leaves getting firm (and darker) |
| 5 | | Leaf unfolding | 10 | | Full size (leaves firm and dark) |

**Leaf Development Stages Oak – second flush**

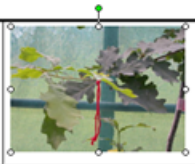
| | | | | | |
|---|---|---|---|---|---|
| 11 | | Leaf bud of 2nd flush | 14 |  | Shoot growth, leaf expansion, leaves light green |
| 12 | | Leaf unfolding | 15 | | Leaves getting firm and darker |
| 13 | | Leaf unfolding complete, leaves shining (reddish) | 16 | | Fully developed leaves |

**Figure 2.1:** Leaf Development Stages of Oak: for the first flush leaf development stages (DS) were determined on a scale of 1-10, for subsequent flushes (second and third) a six stage scale was applied.

Sampling V was conducted after 14 weeks in all treatments (soil moisture for selected plants of control: 33.5%, drought stress: 13.7%, re-watering: 33.2%).

#### 2.2.3.3 Third year (2008)

The same scheme as in the previous years was applied. Two samplings with one week distance were conducted during the first flush (April) and during the second flush (June). Because of a delayed second flush of the controls compared to the re-watered plants samplings III and IV were conducted two weeks later in the control group.

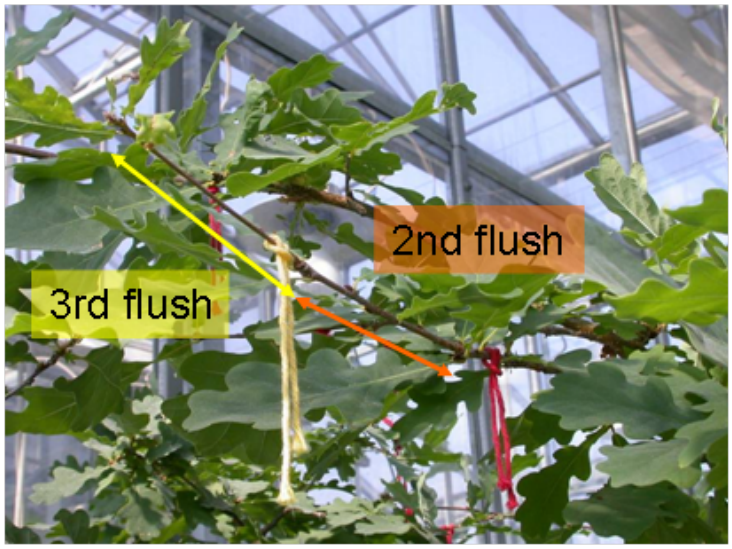



**Figure 2.2:** Shoot growth during 2nd and 3rd flush of *Quercus robur* plants. Individual flushes are indicated by arrows (red arrow = 2nd flush, yellow arrow = 3rd flush).

**Table 2.1:** Time schedule of leaf sampling (year, date, flush, days of stress) and volumetric soil moisture [%] for controls (C), drought stressed (DS) and re-watered (RW) plants at different sampling times in all experimental years.

| | | | | | volumetric soil moisture (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | all plants | | | plants selected for physiological analysis | | |
| Year | Date | Flush | Sampling | days (weeks) of stress | C | DS | RW | C | DS | RW |
| | 11.05. | I | I | 3 | 44,10 | 23,60 | x | 43,4 | 20,5 | x |
| | 16.05. | | II | 8 (1) | 43,90 | 20,60 | x | 45,7 | 19,3 | x |
| **2006** | 14.06. | II | III | 35 | 44,9 | x | x | 49,4 | | x |
| | 20.06. | | IV | 41 | 37,3 | x | x | 40,3 | | x |
| | 04.07. | | III | 56 (8) | x | 17,4 | x | x | 16,1 | x |
| | 13.07. | | IV | 65 ( 9) | x | 12,9 | x | x | 12,3 | x |
| | 11.04. | I | I | 4 | x | 15,5 | 33,9 | x | 14,5 | 33,6 |
| | 20.04. | | II | 13( 2) | x | 9,0 | 34,6 | x | 8,8 | 34,2 |
| | 22.04. | | I | 15( 2) | 38,7 | x | x | 37,7 | x | x |
| | 30.04. | | II | 23( 3) | 40,1 | x | x | 37,8 | x | x |
| **2007** | 30.05. | II | III | 53( 7,5) | x | x | 37,9 | x | x | 37,5 |
| | 05.06. | | IV | 59( 8,5) | x | x | 38,2 | x | x | 37,4 |
| | 26.06. | | III | 80( 11,5) | 41,7 | x | x | 41,3 | x | x |
| | 03.07. | | IV | 87( 12,5) | 37,9 | x | x | 37,5 | x | x |
| | 14.07. | | V | 98(14) | 33,4 | 13,6 | 34 | 33,5 | 13,7 | 33,2 |
| | 08.04. | I | I | | 32,5 | x | 36,4 | | | |
| | 16.04. | | II | | 39,5 | x | 48,8 | | | |
| **2008** | 10.06. | II | III | | x | x | 40,8 | | | |
| | 17.06. | | IV | | x | x | 35,0 | | | |
| | 24.06. | | III | | 31,1 | x | x | | | |
| | 01.07. | | IV | | 30,7 | x | x | | | |

### 2.2.4 Morphological Measurements

New shoots of each tree were counted and their length measured using a measuring tape in order to determine the mean total growth per tree (see Figure 2.2). Stem diameter was measured at 20cm stem height with a calliper gauge at the end of each growing season.

### 2.2.5 Experimental Design

In the first year (2006) 14 five-year-old oak plants for the control treatment (C) and 30 oak plants for the drought stress treatment (DS) were set up in a randomised block design. At the beginning of the growing season (April), all trees were watered up to the saturation point of the soil. The control group was then regularly watered up to the saturation point of the soil while the drought stress group was exposed to repeated cycles of water stress. At the end of the growing season the water regime was unified for the two groups (C and DS). At the beginning of the growing season 2007 (March) one subset of the previously stressed plants (30) was exposed to a second period of drought stress (15) while the other subset of plants was well watered (15). In a similar treatment cycle in the third year (2008) out of 15 plants stressed for two years 6 were re-watered, the rest was discarded. As a control six plants were kept (the rest was discarded). The experimental design is depicted in Figure 2.3.

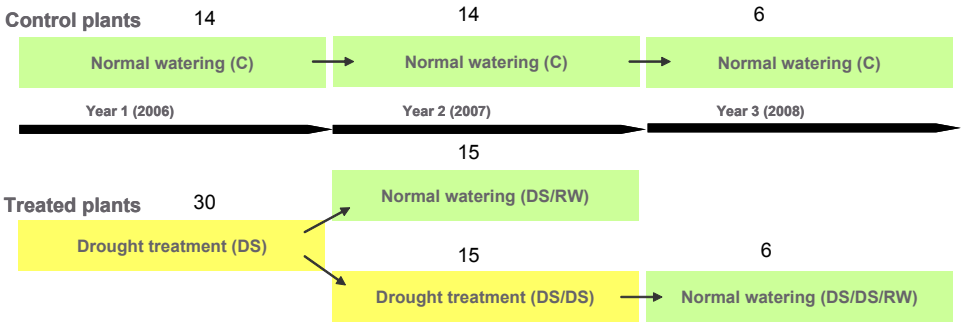



**Figure 2.3:** Experimental Design comprising a control group (C) of 14 plants in 2006 and 2007 which is reduced to 6 plants in 2008. The drought stress group (DS) with 30 plants in 2006 is split into two groups of 15 plants respectively in 2007. One of these groups is re-watered (DS/RW) the other group is kept under drought stress (DS/DS). In 2008 6 plants of the drought stress group (DS/DS) are kept for a re-watering treatment (DS/DS/RW).

## 2.2.6 Sample Preparation and Analysis of Stress Metabolites

Frozen, pooled plant material from four leaves per tree was ground in a Retsch MM 200 automatic grinder (Retsch GmbH & Co. KG, Haan, Germany) with a metallic bead for 2.5 min at 30 Hz. Ground plant material was aliquoted into 100 mg (±10 mg) samples, lyophilized over night and stored at room temperature (until further processing).

HPLC-analysis of stress metabolites was carried out by Mouhssin Oufir at the Public Research Center-Gabriel Lippmann, Department of Environment and Agrobiotechnologies (EVA), in Luxemburg.

### 2.2.6.1 Carbohydrates & Polyols

Carbohydrates and polyols were extracted according to Huylenbroeck and Debergh (1996) and analysed then using High Performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection HPAEC-PAD (Dionex ED 40, Dionex Corp., USA) according to Wilson et al. (1995). HPAEC-PAD analyses for carbohydrates were conducted on a Dionex HPLC ICS2500-BioLC (Sunnyvale, CA) with an AS50 autosampler and thermal compartment, a GS50 quaternary gradient pump, an EG50 eluent generator and an ED50 pulsed amperometric detector. The mobile phase was on-line generated KOH at 0.5 ml/min. The analytical column was a Dionex Carbopac PA-20 (3 mm ×150 mm) with a PA-20 guard column (3 mm ×50 mm) kept at 30°C. HPAEC-PAD analyses for polyols were conducted on Dionex DX-500 chromatograph (Sunnyvale, CA) constituted by a Spark Midas autosampler (Spark Holland B.V, Netherlands), a GP40 gradient pump and an ED40 pulsed amperometric detector. The analytical column was a Dionex Carbopac MA-1 (4 mm ×250 mm) with a MA-1 guard column (4 mm ×50 mm). Water and NaOH (500 mM) were used as eluents. The flow rate was 0.3 ml/min and the column was kept at 48 °C. Carbohydrates and polyols were quantified using calibration curves with standard solutions, ranging from 5 to 100 µmol/l and 10 to 1000 µmol/l respectively.

### 2.2.6.2 Proline and betaine analogues

Analysis of proline and betaine analogues was carried out according to OUFIR et al. (2009). See chapter 6 on page 85

## 2.2.7 Statistical Analyses

After verification of normal distribution and variance homogeneity the data from morphological measurements was analysed by t-test (comparison of two treatments) or oneway

analysis with subsequent student's t-test (comparison of three treatments), carbohydrate and polyol analyses were analysed by ANOVA and subsequent Tukey-test with $\alpha = 0.05$ using the software JMP IN 5.1.

## 2.3 Results

### 2.3.1 Plant Growth & Development

The drought stress treatment applied in this experiment consisted of a slowly decreasing soil moisture content down to 13% while the well-watered controls were kept at a soil moisture content of 30-50%. This kind of drought stress caused slight wilting and leaf curling symptoms as well as sporadic cases of early leaf colouring and leaf fall (end of July) in the drought stress group (Figure 2.4).

#### 2.3.1.1 Plant Development

In the first year of the experiment (2006) bud swelling of the oak plants started in the mid of April. With progression of the experiment the start of bud swelling shifted to mid of March in 2007 and finally to beginning of March in 2008. The reasons for this probably were the controlled greenhouse conditions during winter (while the plants were kept out of

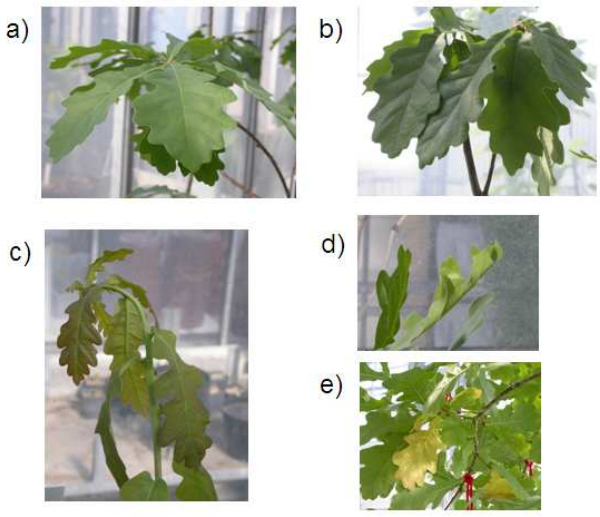



**Figure 2.4:** a) control and b-e) drought stress symptoms: b) change in leaf angle, c) symptoms on newly developed shoots, d), leaf curling, e) sporadic leaf colouring.

doors prior to the experiment). Additionally, shifts in time of flushing/plant development could be observed for all treated plants (drought stress and re-watered) when compared to the controls.

First year (2006): In the first year of the experiment (2006) the second flush of the drought stress treated plants (DS) occurred significantly later (3-4 weeks, $p<0.05$) than that of the controls (C) (Figure 2.5).

Second year (2007): In the second year (2007), both drought stress treated plants (DS-DS) and re-watered plants (DS-RW) showed a significantly earlier first flush ($p<0.05$) than the controls (C). For the re-watered plants (DS-RW) the same (trend) could be observed for the second flush whereas the drought stress treated plants (DS-DS) did not develop a further flush (Figure 2.5).

Third year (2008): In the third year of the experiment (2008) the re-watered plants (DS-DS-RW) again showed an earlier first flush and a significant earlier second flush ($p<0.05$) than the controls (C). Furthermore, the re-watered plants (DS-DS-RW) developed a third and even a fourth flush in 2008 which was not observed in the controls (C) (Figure 2.5).

#### 2.3.1.2 Shoot Growth

Growth cessation during drought stress The drought stress treatment of the oak plants resulted in a significantly reduced shoot growth (10-fold reduction, $p<0.05$) in the first year (DS). With continuing drought stress during the second year (DS-DS) growth came to a stop after the first flush already with flushes two and three being absent (See figure 2.5).

Compensation growth during re-watering The plants that were re-watered after one year of drought stress (DS-RW) and those that were re-watered after two years of drought stress (DS-DS-RW) showed a similar growth pattern. Compared to the controls (C) the growth of the re-watered plants was reduced significantly (2.5-fold reduction 2007, 4-fold reduction 2008, $p<0.05$) in the first flush, while the second (4.5-fold increase 2007, 9-fold increase 2008) and third flush (3-fold increase 2007, 20-fold increase 2008) were significantly stronger ($p<0.05$) in the re-watered plants (Figure 2.6). This compensation growth (second and third flush) under recovery/re-watering increased with increasing duration of the preceding drought stress. During the re-watering after the two-year drought stress period (DS-DS-RW) even a fourth flush could be observed.

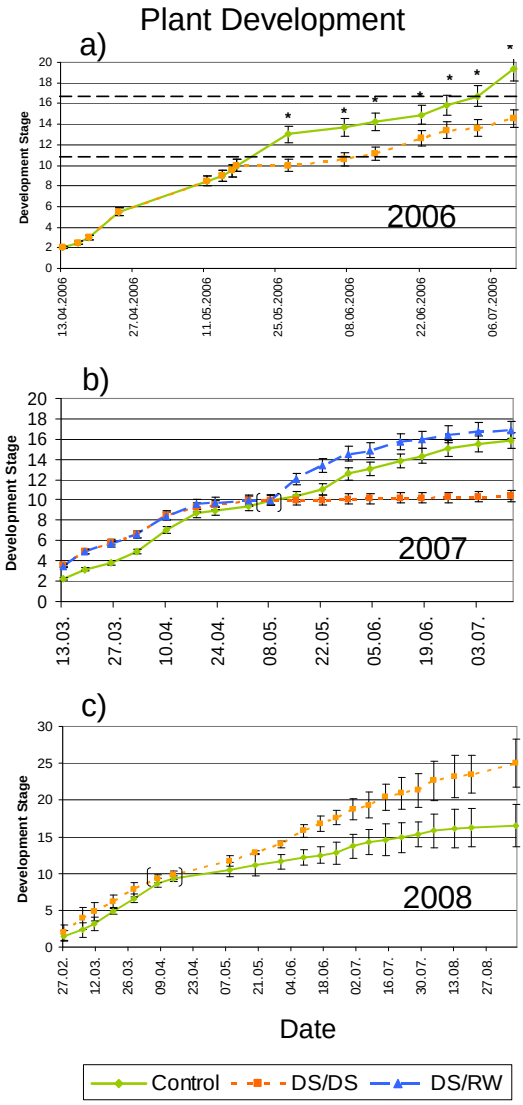



**Figure 2.5:** Plant development of a) control (C) and drought stressed plants (DS). b) control, drought stressed plants (DS/DS) and re-watered plants (DS/RW) and c) control and re-watered plants (DS/DS/RW). In a) points marked with an "*" are significantly different ($p<0.05$) from the controls, in b) and c) all points are significantly different except the ones marked with parentheses. The lower dashed line (Development Stage 11) indicates the start of the second flush, the upper one (Development Stage 17) the start of the third flush.

Surprisingly, during the first year the control plants (C) showed a similar growth pattern to that of the re-watered plants (DS-RW and DS-DS-RW). This could lead to the conclusion that the plants had been exposed to some stress (water, temp, mechanical stress) during the growing season preceding the start of the experiment.

#### 2.3.1.3 Stem Diameter

Stem diameter of drought stress treated plants was significantly smaller ($p<0.05$) compared to the controls when measured at the end of each growing season (2006: C, DS; 2007: C, DS-DS) (Figure 2.7). Re-watered plants (2007: DS-RW and 2008: DS-DS-RW) also had a smaller stem diameter than the controls although they had shown strong compensation growth during re-watering with significantly higher stem diameter increment ($p<0.05$) than the controls (Figure 2.8).

#### 2.3.1.4 Summary

Drought stress not only caused a delay in shoot growth (second flush 2006) but also a reduction in shoot and stem growth, followed by an earlier first flush in the following year (2007) and finally a growth cessation (2007). Re-watering led to an earlier first and second flush in the growing season following the drought stress as well as strong compensation growth in shoots and stem.

### 2.3.2 Analysis of Stress Metabolites

#### 2.3.2.1 Sugars

The major carbohydrates found in the oak leaves were glucose, fructose, and sucrose. In addition galactose, raffinose, arabinose, rhamnose and maltose could be detected.

Drought Stress During the first year of the experiment (2006) the drought stress treated plants showed a significant accumulation ($p<0.05$) of the monosaccharides glucose (5-fold), fructose (5-fold), and galactose (2-fold) compared to the controls (Figure 2.9). For the disaccharide sucrose, however, the increase under drought stress took place in the second flush only (1.6-fold concentration of controls in sampling III and IV, Figure 2.10). During the second year (2007) significantly higher concentrations ($p<0.05$) of the monosaccharides and sucrose could be detected for the drought stress treated plants at sampling times II (glucose and fructose 1.3-fold, galactose 3.3-fold, sucrose 2.5-fold) and V (glucose 1.5-fold, fructose 2.1-fold, galactose 3-fold, sucrose 1.2-fold) compared to the controls. (The drought

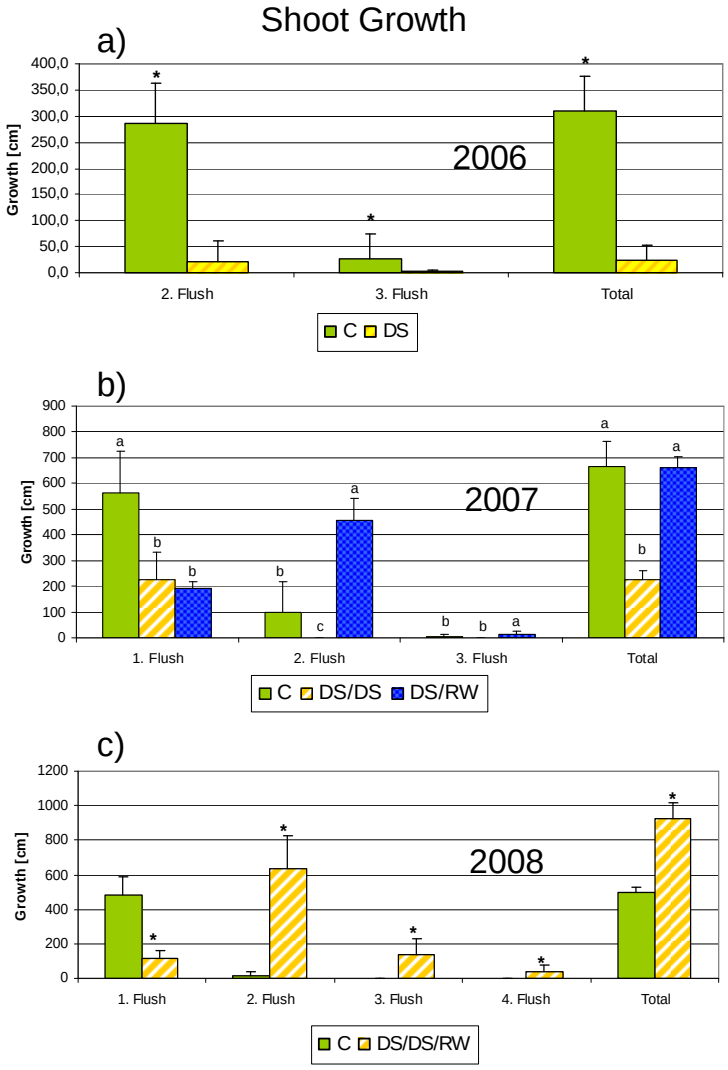



**Figure 2.6:** Shoot growth of a) control (C) and drought stress treated plants (DS) during the 1st year (2006), b) control, drought stress treated plants (DS-DS) and re-watered plants (DS-RW) during the 2nd year (2007) and c)control and re-watered plants (DS-DS-RW) after two years of drought stress in 2008. In a) and c) Columns marked with an * are significantly different ($p < 0.05$), in b) columns not marked with the same letter are significantly different.

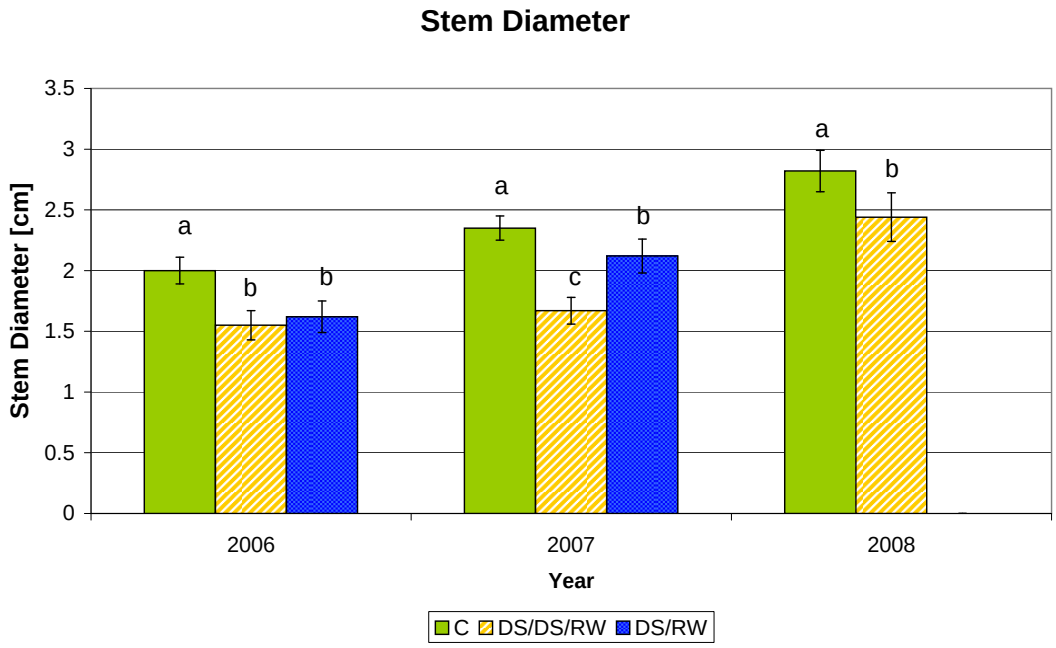



**Figure 2.7:** Stem diameter for all treatments from 2006 to 2008. Columns not marked with the same letter are significantly different. The columns with diagonal lines (DS/DS/RW) represent drought stress treatments in 2006 and 2007 but re-watering in 2008. The columns with cross hatch (DS/RW) represent drought stress in 2006 but re-watering in 2007.

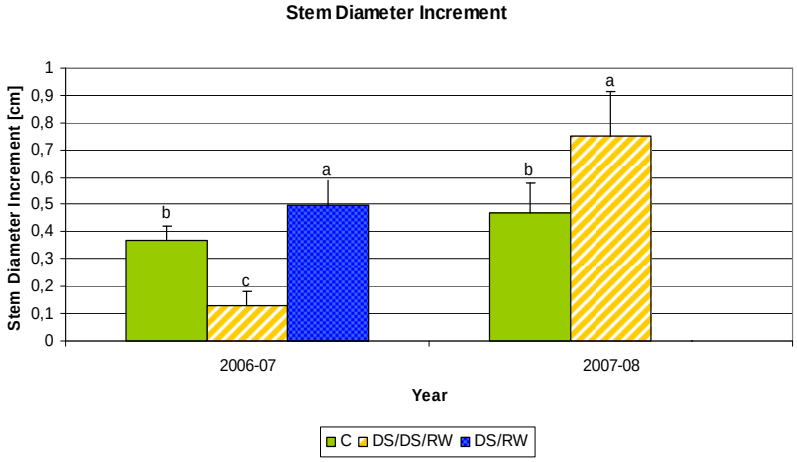



**Figure 2.8:** Stem diameter increment for all treatments from 2006 to 2008. Columns not marked with the same letter are significantly different. The columns with diagonal lines (DS/DS/RW) represent drought stress treatments in 2006 and 2007 but re-watering in 2008. The columns with cross hatch (DS/RW) represent drought stress in 2006 but re-watering in 2007.

stressed plants did not develop a second flush.) For the sugars arabinose, rhamnose, and maltose no notable changes could be detected in the drought stress treatment (supplemental material).

Re-watering The re-watered plants showed similar carbohydrate concentrations to the controls suggesting re-established osmotic conditions (at least regarding carbohydrates).

#### 2.3.2.2 Polyols

The major polyol detected in oak leaves was quercitol. Further polyols present were mannitol, trehalose, sorbitol and galactinol.

Drought Stress Quercitol was only analysed in the second year of the experiment where it showed significantly higher concentrations ($p<0.05$) in the drought stress treated plants at samplings II (1.3-fold increase) and V than in the controls (1.9-fold increase) (Figure 2.11). While for mannitol no significant changes could be observed between control and stress treatment in the first year of the experiment (2006, data not shown, see Appendix chapter A on page 115) a significantly higher concentration could be detected at all sampling times in 2007 for the drought stress treatment compared to the controls (sampling I 1.8-fold increase, II and V: 2.7-fold increase, Figure 2.11).

Re-watering The re-watered plants showed similar quercitol concentrations to the controls whereas the mannitol concentration in the re-watered plants was significantly increased (sampling I 1.7-fold increase, II 1.8, III 2.5, IV 2.3, $p<0.05$) at all sampling times except the last one (sampling V) (Figure 2.11).

The polyols trehalose, sorbitol and galactinol did not display notable changes in the drought stress treatment or the re-watering (supplemental material).

#### 2.3.2.3 Proline and Betaine Analogues

The nitrogenous osmoprotectants analysed comprise proline, hydroxyproline, methylproline, glycine betaine and trigonelline. For proline a significantly higher concentration could be measured in the drought stressed plants than the well-watered controls at sampling time II and V in 2007. Methylproline and trigonelline concentrations were significantly increased under stress at sampling time III in 2006 as well as I and II in 2007. Hydroxyproline concentration was significantly higher in both stressed and rewatered plants compared

to controls at sampling I 2007. Glycine betaine concentration in drought stressed plants was not significantly different from that of controls. Under rewatering glycine betaine concentration was significantly higher than in control plants at sampling time III in 2007 (figures in Chapter A starting on page 115).

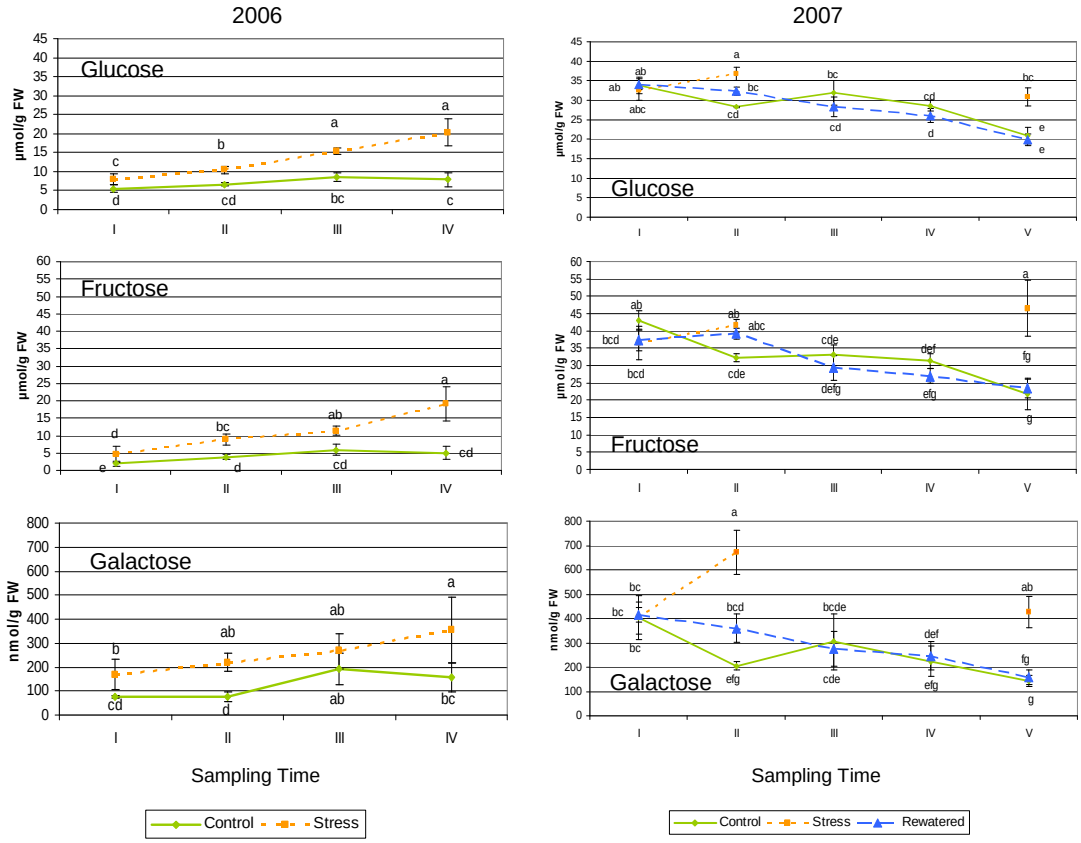



**Figure 2.9:** Monosaccharide concentrations in oak leaves of drought stress treated plants, re-watered plants, and controls. In 2007 samplings III and IV are missing in the drought stress group because the plants did not develop a second flush. Points not marked with the same letter are significantly different ($p < 0.05$).

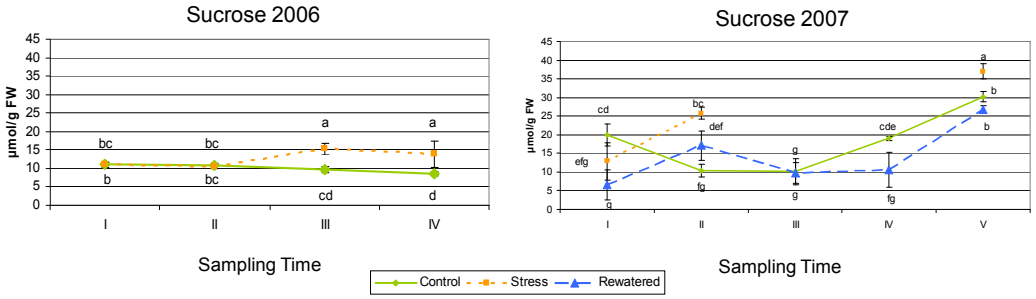



**Figure 2.10:** Sucrose concentrations in oak leaves of drought stress treated plants, re-watered plants, and controls. Samplings III and IV are missing in the drought stress group because the plants did not develop a second flush. Points not marked with the same letter are significantly different ($p < 0.05$).

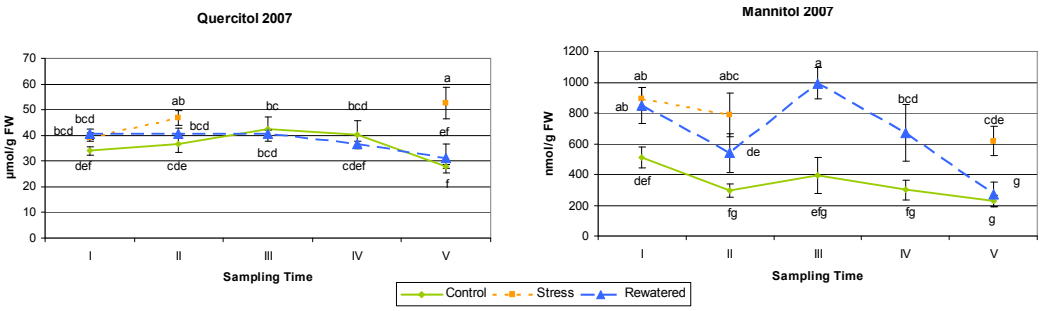



**Figure 2.11:** Quercitol and Mannitol concentrations in oak leaves of drought stress treated plants, re-watered plants, and controls. Samplings III and IV are missing in the drought stress group because the plants did not develop a second flush. Points not marked with the same letter are significantly different ($p < 0.05$).

## 2.4 Discussion

### 2.4.1 Plant Growth & Development

#### 2.4.1.1 Flushing Behaviour

For trees two types of growth are described: the *Quercus*- (oak-) type or type B according to Nitsch and the *Populus*- (poplar-) type or type A (Lyr and Hoffmann, 1992, and references within). Representatives of the *Quercus*-type (e.g. *Quercus*, *Fagus*, *Pinus*) stop shoot growth early in the season while root and radial growth continue. According to Lyr and Hoffmann (1992) a second flush (lammes shoots) can occur under good conditions. In the present study, plants growing under normal conditions showed three flushes during the first (2006), and two flushes during the second (2007) and third year (2008) of the experiment. Re-watered plants had two major flushes in 2007 (with a minor third flush) after one year of drought stress and four flushes in 2008 after two years of drought stress. These observations correspond to Bréda and Granier (1996) who observed three flushes on average in a 35-year-old *Quercus petraea* stand during five successive years. This growth pattern implies, that not only sufficient water supply during the current year determines the number of flushes and the intensity of shoot growth but also the soil moisture conditions during the preceding growing seasons. It seems that unfavourable conditions during the preceding years lead to a compensation growth in the second and third flush when water is available again. In contrast, good soil moisture conditions over several years lead to a constant and rather homogenous but less intense shoot growth mainly during the first flush (see b), c) in Figure 2.6). Three flushes in the controls during the first year only

consequently could be explained by the less controlled conditions the oak plants were exposed to prior to the start of the experiment (the plants were kept in containers on the field). In addition, the time of the flushing varied with the treatment. Drought stress not only led to a significantly delayed (4-5 weeks) second flush during the first year which can be attributed to the slower plant growth usually observed under drought stress. It further caused a significantly earlier first flush in the second year in both the re-watered plants and those that were kept under continuing drought stress. In the re-watered plants also the second flush occurred significantly earlier and a similar pattern could be observed for the third year. The re-watered plants (re-watering after two years of drought stress) again showed an earlier first flush compared to the controls and a significantly earlier second flush. This behaviour could be a strategy of the plant to avoid water deprivation during summer by shifting plant development to a period in the year when water supply is still sufficient. According to Kleinschmit (1993) provenances which flush late have their maximal water demand in July exceeding transpiration of early flushing provenances by 31%.

#### 2.4.1.2 Shoot Growth

Under drought stress the oak plants showed a significantly reduced shoot growth during the first year and a complete cessation of growth after the first flush in the second year. This corresponds to Dickson and Tomlinson (1996) who found that drought stress not only decreases the rate of leaf expansion, final leaf size and the number of leaves in a flush but that flushing of oak may be completely stopped by drought stress and photosynthate is redirected to root growth and storage instead (Dickson, 1991; Gordon et al., 1989). It is also known that a water shortage in June and July, as applied in this experiment, has a strong effect on tree growth (30-70% growth reduction) because in this time of the year the greatest biomass production takes place (Lyr and Hoffmann, 1992). Even if no visible symptoms develop, biomass production is reduced through the closure of stomata leading to restricted photosynthesis.

#### 2.4.1.3 Stem Diameter

Another parameter measured in this study was stem diameter increment/radial growth. Under drought stress radial growth was significantly reduced in both years (2006 and 2007) and though re-watered oak plants showed significantly increased radial growth compared to the controls they could not compensate for the low stem diameter caused during the previous drought stress year(s) (Figure 2.7 and Figure 2.8). This corresponds well with the

literature which states that in humid regions about 70-80% of the variation in radial growth can be attributed to drought stress while in arid regions it even reaches 90% (Kozlowski and Pallardy, 1997). In temperate zones radial growth of deciduous trees/broadleaved trees usually starts at the beginning of May and ceases at the end of August. Although the radial growth of oak and red oak in Europe starts already in April the maximal radial growth, however, is not reached before the end of July (Lyr and Hoffmann, 1992). While earlywood (April) seems to be influenced by temperature only, latewood growth seems to be restricted by water availability and was called "the soil moisture limited phase" by Hinckley et al. (1976). Similar findings were also reported by Becker et al. (1994) who mentioned fluctuations in precipitation in May, June, and August as limiting factor of stem wood growth. In contrast to shoot growth which is, at least partly, influenced by the previous year's soil moisture conditions radial growth is influenced only by climatic and nutritional conditions of the current year. For this reason, drought stress leads to a reduction and finally cessation of radial growth ahead of time (Lyr et al., 1992) as observed in the present study.

### 2.4.2 Stress Metabolites

The major compounds found in the leaves of *Quercus robur* in the present study were glucose, fructose, sucrose and quercitol as previously described for different oak species. (*Q. robur*, Lied (1994); *Q. suber*, Passarinho et al. (2006); *Quercus prinus*, Gebre and Tschaplinski (2002)).

#### 2.4.2.1 Sugars

Significantly higher concentrations of the monosaccharides glucose, fructose, and galactose could be detected under drought stress during both 2006 and 2007 compared to the control plants (Figure 2.9). These findings are supported by Épron and Dreyer (1996) who observed significant increases in glucose and fructose concentrations in 4-year old saplings of water-stressed *Quercus petraea*. These soluble carbohydrates are proposed to play a major role in osmotic adjustment, a mechanism being well documented for many North American oaks but also for *Q. robur* (Épron and Dreyer, 1996, and references within). For sucrose, the increase under drought stress took place in the second flush only (sampling III and IV) during the first year. Similar findings have been reported for chestnut oak (*Quercus prinus* L.) which under drought stress accumulated sucrose only later in the growing season (Gebre and Tschaplinski, 2002). During the second year sucrose concentration was significantly lower in stressed and re-watered plants at the

first sampling compared to the controls, but significantly higher at samplings II and V. These observations are likely to coincide with the varying growth patterns under different treatments. Sucrose concentration is significantly increased in the drought stressed plants during the second flush in the first year (2006) while the controls, which develop a third flush, show decreasing concentrations of sucrose. In the second year, the significantly higher concentration is present during sampling II (end of first flush) when controls already have to invest energy into the second flush while the growth of the drought stressed plants ceases. Furthermore, sucrose levels are highest in all treatments at the end of the growing season (sampling V, 2007).

#### 2.4.2.2 Polyols

During the second year (2007) of the drought stress treatment also significantly higher concentrations ($p<0.05$) of quercitol could be detected at sampling times II (first flush) and V (end of growing season) for the drought stress treated plants compared to the controls. For mannitol a significant increase at all sampling times of the drought stressed plants (I, II and V) was detectable.

#### 2.4.2.3 Possible function of carbohydrates and polyols in drought stress response

The observed carbohydrate/polyol pattern in the leaves supports the theory that during moderate drought stress or in temperate zones with sporadically occurring drought stress carbohydrates such as glucose, fructose and sucrose are accumulated which are readily metabolized as soon as sufficient water is available again. As opposed to this, plants in arid regions mainly accumulate cyclitols (e.g. quercitol), which are considered as stable osmolytes (Merchant et al., 2006). In this study, an intermediate situation seems to be present with significantly increased concentrations of glucose, fructose and sucrose under drought stress in both years (2006 and 2007) and an accumulation of quercitol under persisting drought stress during the second year of drought stress (2007). It has also been reported that hexose sugars, especially glucose, contribute directly to osmotic adjustment in oak (Épron and Dreyer, 1996) and other species (Clifford et al., 1998) while cyclitols (e.g. quercitol) are thought to have mainly a protective function during drought stress through the stabilization of the DNA structure (reviewed by Popp and Smirnoff (1995)). The polyol mannitol is also considered to have osmoprotective function, at low concentrations already, by scavenging hydroxyl radicals which are produced in higher amounts under drought stress (Bohnert and Shen, 1999; Smirnoff, 1993; Buchanan et al., 2000; Bray et al., 2000).

#### 2.4.2.4 Function of carbohydrates in drought stress relief (re-watering)

That carbohydrates such as glucose, fructose and sucrose are readily metabolized upon relief from drought stress could also explain why the re-watered plants show similar carbohydrate concentrations to the controls. It has been proposed before that osmolytes may serve as storage compounds for reducing power and function as a carbon or nitrogen source upon stress relief (Hare et al., 1998). This theory might also explain the strong compensation growth which occurs upon re-watering and would suggest a high adaptation potential of this *Quercus robur* clone to changing environmental conditions.

#### 2.4.2.5 Proline, Betaine and Analogues

The nitrogenous osmoprotectants proline, hydroxyproline, methylproline, glycine betaine and trigonelline did not show a clear response to drought stress in *Q. robur* leaves. Compared to quercitol and carbohydrates they only seem to play a minor role in the stress response of *Quercus robur*.

### 2.4.3 Conclusion

Although pedunculate oak (*Quercus robur* L.) is generally known to have a higher water requirement (Levy et al., 1992) and to be more prone to drought stress (Cochard et al., 1992; Vivin et al., 1993) than sessile oak (*Q. petraea*) it is not only found on sites well supplied with water but also on sites with low water supply such as sandy soils, on plateaus, and exposed slopes of well-drained limestone hills (Thomas and Gausling, 2000). The intermediate behaviour in the response to drought with an accumulation of carbohydrates that are quickly metabolized when water is present but also an accumulation of the more stable cyclitol quercitol which can serve as a protective strategy under longer-lasting drought periods enables *Q. robur* to grow in a broad range of habitats.

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# CHAPTER 3

## Gene Expression — Transcriptome-Analysis of *Quercus robur* Exposed to Long-term Drought Stress & Rewatering using cDNA-Microarrays


## Abstract

In a two-year glasshouse experiment, five year old oak clones (*Q. robur*) originating from tissue culture, were subjected to controlled drought stress. Three treatments were applied: 1) control, 2) one year drought followed by one year rewatering, and 3) two years drought. During the course of the experiment phenotypic parameters were monitored and plant material was sampled at regular intervals for analyses on the physiological level and the transcriptome level using a cDNA microarray with 18545 unique genes. The application of drought stress led to growth reduction and change in flushing time, osmotic adjustment and starch degradation, reinforcement of the cuticula, repression of photosynthesis, and induction of genes with protective function and senescence associated genes. Rewatering resulted in the (early) recovery of photosynthesis, down-regulation of genes with protective function and senescence-associated genes, slow but strong induction of growth processes, and upregulation of "rehydration-specific" genes. Suggested candidate genes for further selection of drought resistant oak trees are "WAX2" and lipid transfer protein (LTP), both being involved in cuticle biosynthesis. Other potential candidates for selection would be genes encoding for late embryogenesis abundant protein (LEA protein) and rare cold inducible 2B (RCI2B) with proposed function in maintaining membrane function and/or integrity under water stress. The possible function of these genes in drought tolerance is discussed.

## 3.1 Introduction

Environmental stress constitutes the major factor limiting plant productivity. Abiotic stresses such as drought, high temperature, and freezing which cause water deficit stress are responsible for the greatest losses and can reduce yield by up to 85% (Hare et al., 1998; Rodrigues et al., 2009). Water deprivation causes a complex set of responses in the plant. After perception of the stress at the cellular level (through changes in turgor pressure for example) signal cascades are activated triggering/causing alterations in gene expression which in turn lead to changes at the cellular, physiological and developmental level. On the molecular level, plant adaptation responses can be divided into three groups: a) osmotic homeostasis or osmotic adjustment, b) detoxification, damage control and repair, and c) growth control (Zhu, 2002). The aim of these adaptations is to maintain the functionality of the plant during increasing drought stress. The responses elicited not only vary with developmental stage, plant genotype, severity and duration of the stress, but also with the rate at which dehydration stress is applied (Blum, 2009; Bray, 1993; Buchanan et al., 2000). It is well known that fast application of stress may have a different effect on the plant than slowly increasing drought stress which gives the plant time to react and adapt to the stress by different mechanisms. Osmotic adjustment, for example, which is a powerful mechanism of the plant to withstand stress, may take one to three weeks to be fully expressed (Blum, 2009). Very rapid and severe stresses as they are applied in many laboratory and greenhouse studies (Huang et al., 2008; Kilian et al., 2007; Yu and Setter, 2003; Kreps et al., 2002; Talamè et al., 2006) therefore cannot be transferred easily to the field situation where drought stress usually develops much slower. Only few studies have been conducted in tree species on the response to drought stress on the molecular level so far (pine: Watkinson et al. (2003), poplar: Bogeat-Triboulot et al. (2007)). Hence, in the current study the response of oak trees (*Quercus robur*) to a long-term drought stress over a period of one and two years, respectively was analysed on the gene expression level using microarray technology. In order to investigate potential long term effects of water deficit stress the plants were monitored for an additional year (after one year of stress) under well watered conditions.

## 3.2 Materials and Methods

### 3.2.1 Plant Material and Growth Conditions

Five year old oak plants of clone P28 (*Quercus robur* L.) stemming from somatic embryogenesis were used (Prewein et al., 2004). The plants were transferred into fresh peat moss in 20 L plastic containers prior to the experiment and kept in a greenhouse where air temperature (ambient) and humidity are regularly recorded. After the end of the first growing season NPK long-term fertilizer was applied.

#### 3.2.1.1 Water regime

Prior to the experiments, we determined soil moisture conditions for control and drought stress treatment based on stress symptoms observed on oak plants (change in leaf angle). At the start of the growing season all plants were watered up to the saturation point of the soil (55-60%). In the first year, water was applied manually and soil moisture conditions for the control plants were set to 35-50%. For drought stressed plants, treated by withholding water, volumetric soil moisture was set to 15-25%. In the second year a combination of automatic and manual watering was used and the soil moisture for controls was kept at 30-50% and at 10-15% volumetric soil moisture for the drought stressed plants. The volumetric soil moisture content was measured with a ThetaProbe ML2x FD-Probe (Delta-T Devices, Cambridge, UK) usually three times a week during the course of the experiment. Soil moisture was measured at 10 and 15 cm soil depth and at the soil surface.

#### 3.2.1.2 Sampling

Four fully grown leaves from the tips of branches were sampled after three and eight days of stress in 2006 and after four and 13 days in 2007 during the first flush. During the second flush samples were taken after eight (56 days) and approximately 9 weeks (65 days) of stress in 2006 and after 7.5 weeks (53 days) for rewatered plants only in 2007 (Table 3.1). In the second year (2007) continued drought stress caused a cessation of growth after the first flush. For this reason, an additional leaf sampling was carried out on old leaves of the first flush after 14 weeks (98 days) in all plants. Sampled leaves from each plant were pooled, frozen in liquid nitrogen and stored at -80 °C until further processing.

**Table 3.1:** Time schedule of leaf sampling (year, date, flush, days of stress) and volumetric soil moisture (%) for controls (C), drought stressed (DS) and re-watered (RW) plants at different sampling times in 2006 and 2007. Samples in brackets were not used for microarray-analysis

| | | | | | volumetric soil moisture (%) | | |
|---|---|---|---|---|---|---|---|
| **Year** | **Date** | **Flush** | **Sampling** | **days (weeks) of stress** | **C** | **DS** | **RW** |
| | 11.05. | I | I | 3 | 43,9 | 21,7 | x |
| | 16.05. | | II | 8 (1) | 45,0 | 20,1 | x |
| **2006** | 14.06. | II | III | 35 | 46,7 | x | x |
| | 20.06. | | IV | 41 | 39,2 | x | x |
| | 04.07. | | III | 56 (8) | x | 17,2 | x |
| | 13.07. | | IV | 65 ( 9) | x | 12,6 | x |
| | 11.04. | I | I | 4 | x | 14,1 | 34,0 |
| | 20.04. | | II | 13( 2) | x | 9,0 | 33,7 |
| | 22.04. | | I | 15( 2) | 37,1 | x | x |
| | 30.04. | | II | 23( 3) | 38,1 | x | x |
| **2007** | 30.05. | II | III | 53( 7.5) | x | x | 38,6 |
| | [ 05.06. | | IV | 59( 8.5) | x | x | 38.8 ] |
| | 26.06. | | III | 80( 11.5) | 40,2 | x | x |
| | [ 03.07. | | IV | 87( 12.5) | 37,2 | x | x ] |
| | 14.07. | | V | 98(14) | 32,5 | 13,3 | 34,3 |

### 3.2.2 Experimental Design

In the first year (2006) 14 five-year-old oak plants for the control treatment (C) and 30 oak plants for the drought stress treatment (DS) were set up in a randomised block design. At the beginning of the growing season (April), all trees were watered up to the saturation point of the soil. The control group was then regularly watered up to a soil moisture content of 35-55% while the drought stress group was exposed to repeated cycles of water stress. At the end of the growing season the water regime was unified for the two groups (C and DS) at control level. At the beginning of the growing season 2007 (March) one subset of the previously stressed plants was exposed to a second period of drought stress (15) while the rest was well watered (15). The experimental design is depicted in Figure 3.1. For microarray analyses four trees per treatment were selected.

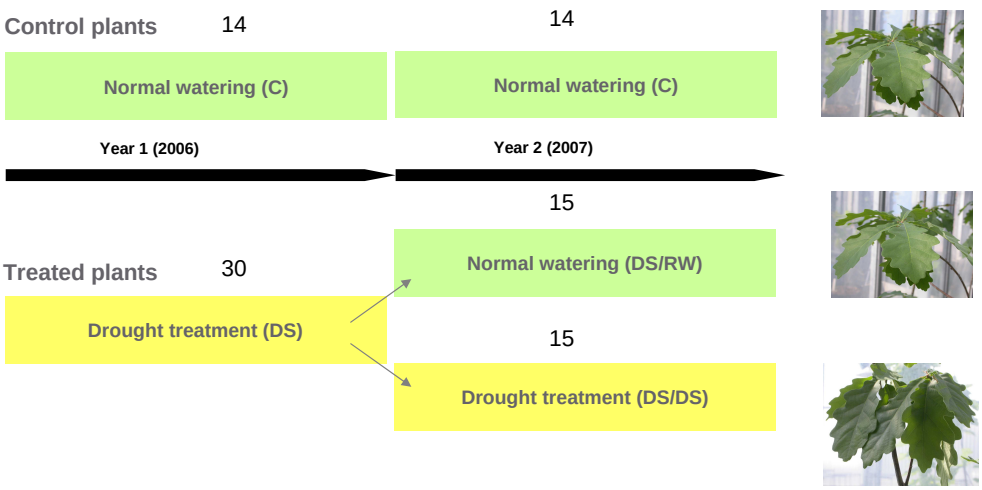


**Figure 3.1:** Experimental design comprising a control group of 14 plants in 2006 and 2007 and a drought stress group of 30 plants in 2006 which is split up in two groups in 2007. One group (15 plants) is kept under drought stress, the other group (15 plants) is irregated regularly till the end of the experiment. Pictures show oak leaves during normal watering (control, rewatering) and during drought stress (change in leaf angle).

### 3.2.3 Sample Preparation and RNA-Extraction

Total RNA was extracted using a modified method of Chang et al. (1993). Frozen, pooled plant material from four leaves per tree was ground in a Retsch MM 200 automatic grinder (Retsch GmbH & Co. KG, Haan, Germany) with a metallic bead for 2.5 min at 30 Hz. Approximately 200 mg of ground plant material was thoroughly mixed with 5 ml of extraction buffer (3% CTAB, 3% PVP K 40, 100 mM Tris-HCl pH 8.0, 25 mM EDTA, 2 M NaCl, 2.67% $\beta$-mercaptoethanol) pre-warmed to 65°C and incubated for 10 min. The homogenate was extracted twice with 5 ml of chloroform:isoamyl alcohol (24:1) by vortexing and centrifugation at 5000 rpm for 20 min. The aqueous phase was kept and one-quarter volume of LiCL was added. The RNA was precipitated at 4°C over night and centrifuged for 30 min at 4°C and 10000 rpm. The pellet was dissolved in 700 µl SSTE (1 M NaCl, 0.5% SDS, 10mM Tris-HCl pH 8.0, 1mM EDTA) and extracted twice with 700 µl chloroform:isoamyl alcohol (24:1) by centrifugation at 10000 rpm for 10 min. A precipitation of the RNA at -80°C for 1 h with two volumes of ethanol (99.8%) and centrifugation at 13000 rpm for 30 min followed. The pellet was washed with 70% ethanol twice and then allowed to dry. The RNA was resuspended in DEPC-treated water and the concentration determined using a NanoDrop spectrophotometer (NanoDrop Technologies,

Delaware, USA). RNA quality was assessed by standard agarose gel electrophoresis.

### 3.2.4 Construction of cDNA microarrays containing oak EST clones

For printing of the PICME (Platform for Integrated Clone Management; `http://www.picme.at`) oak microarray 11,305 expressed sequences tags (ESTs) of *Quercus petraea* and 6,940 ESTs of *Quercus robur* from INRA-Bordeaux and 300 ESTs of *Q. robur* from PICME were used. Probes were printed onto glass slides coated with $\gamma$-amino-propyl-silane (GAPS II; Corning Inc., Corning, NY, USA) using an Omnigrid 100 contact spotter (Gene Machines, San Carlos, CA, USA) with 48 SMP3 stealth pins (Telechem International Inc.). cDNA was fixed by 300 mJ UV irradiation. The slide blocking was performed in freshly prepared solution of 1.79 g succinic anhydride in 100 ml of 1-methyl 2-pyrrolidone, to which 4.48 ml 1 M sodium borate (pH 8) was added after succinic anhydride had dissolved. Slides were agitated in blocking solution for 15 min in the dark and then washed five times with millipore water. After denaturation in a water bath at 95 °C for 1 min the slides were transferred into ice-cold ethanol and dried with compressed air.

### 3.2.5 Preparation of fluorescent probes

2 µg of RNA were amplified using the Amino Allyl MessageAmp™II aRNA Amplification Kit (Applied Biosystems Inc, USA) according to manufacturer instructions. An aliquote of 20 µg of amino allyl modified RNA (aRNA) was then coupled to Cy3 or Cy5 reactive dye (GE Healthcare). Concentration and dye incorporation rate were determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Delaware, USA). Following purification, the probes (0.5 µg) were mixed with hybridization buffer (Amersham Biosciences) and formamide at the ratio of 1:1:2 (by vol.), respectively.

### 3.2.6 Hybridizations and microarray analyses

For each treatment (and sampling time) slides were co-hybridized with labelled RNA from the reference (Cy5) and one biological replicate consisting of an individual tree (Cy3) of either control, drought stressed plants, or re-watered plants. Depending of the availability of material 2-6 biological replicates were used. As a reference sample a pool of shoot and root material of young *Quercus robur* and *Quercus rubra* seedlings as well as leaves of old oaks (Prater), drought stressed oaks and controls from the experiment were used. Hybridizations were carried out using a Tecan HS 400 Hybridization Station (Tecan Austria GmbH, Austria) at 42 °C for 15 h. The arrays were washed with 1×SSC

for 30 s at RT, 1x SSC, 0.2% SDS twice for 10 min at 50°C, 0.1×SSC, 0.2% SDS for 10 min at RT and 0.2×SSC twice for 5 min at RT. Microarrays were scanned on a Tecan LS 200 Scanner (Tecan Austria GmbH). Image analysis was carried out in the software Spot version 3.1 (Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia). Data analysis was done using bioconductor in the R environment (`http://www.bioconductor.org/`, `http://www.r-project.org/`). Gene expression data was normalized within and between arrays using variance stabilization normalization (vsn). After normalization, controls and empty spots were excluded to increase power and the package "limma" was used to fit a linear model for each gene. Genes were considered as differentially expressed when $p<0.05$ after multiple testing correction using the method of Benjamini & Hochberg (BH). Genes were annotated using four different databases (est2uni, nr, swissprot, tair7) and assigned to functional groups according to Gene Ontology.

## 3.3 Results

### 3.3.1 Differentially expressed genes

Gene expression analysis was carried out using a cDNA microarray containing 18,545 ESTs from *Quercus petraea* and *Quercus robur*. Transcriptome analysis of leaves of stressed (one and two years) and rewatered plants (after one year of drought) was carried out at several time points (Table 3.1 on page 50) doing pairwise comparisons to the control. A total of 588 genes (3.1%) were differentially expressed (at an adjusted p-value <0.05) at least once during the experiment. Of these genes, 518 (88%) showed a very specific response pattern being differentially expressed at only one time point and treatment. The largest group of differentially expressed genes comprised genes of unknown function in both treatments (drought stress and rewatering) followed by genes involved in the response to stress. Although most functional groups were represented in both treatments (DS and RW), (Figure 3.2) distribution and size of groups varied strongly between treatments (Figure 3.3 and Figure 3.4). More genes involved in growth and cell wall modification, for instance, were induced under rewatering than under drought stress. On the other hand, a higher number of genes involved in signalling and metabolism were down-regulated under rewatering than under drought stress. Between the two years of stress differences could be observed, too. A higher number of stress responsive genes, for example, was induced in the second year of stress compared to the first while more genes involved in signalling were activated in the first year.

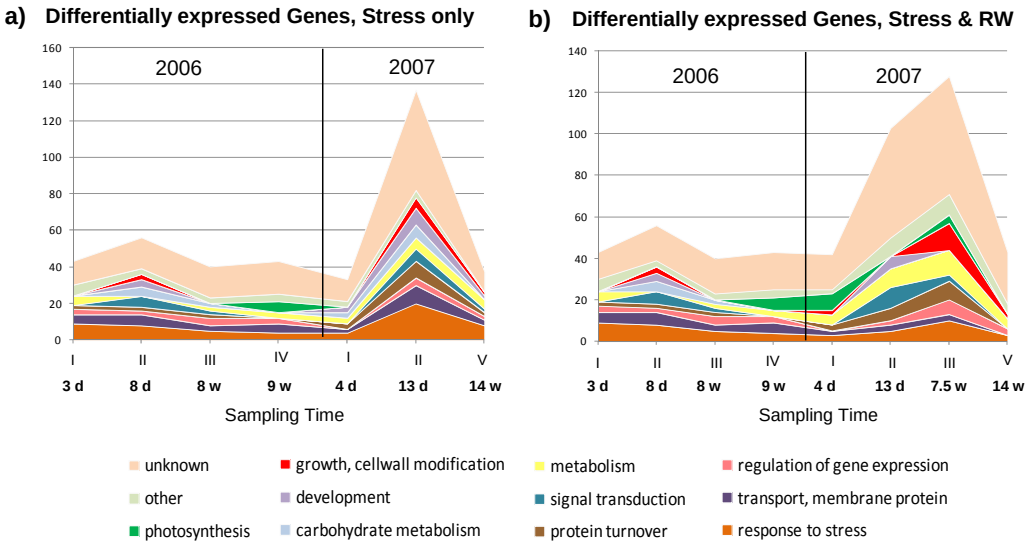


**Figure 3.2:** Differentially expressed genes during a) drought stress at sampling times I-IV in 2006 and samplings I, II and V in 2007. b) Drought stress in 2006 and rewatering in 2007 with samplings I, II, III, and V for rewatered plants. Times of sampling are given in days (d) and weeks (w) of stress exposure.

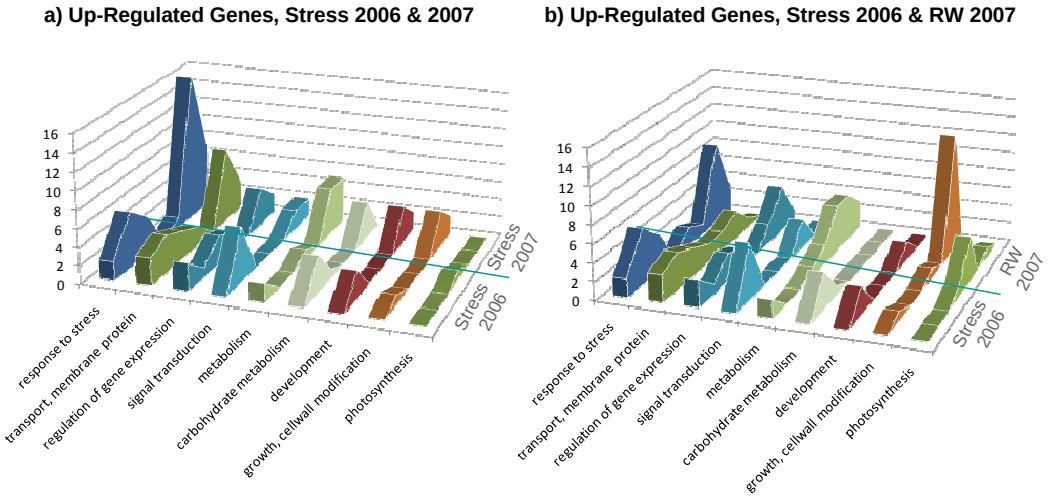


**Figure 3.3:** Number of up-regulated genes for selected functional groups during a) stress in 2006 and 2007 and b) stress in 2006, rewatering (RW) in 2007.

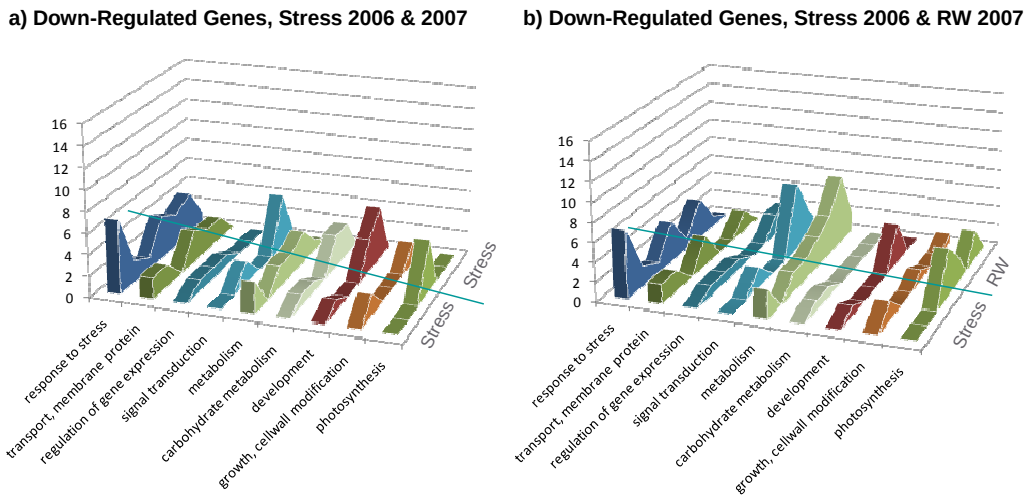


**Figure 3.4:** Number of down-regulated genes for selected functional groups during a) stress in 2006 and 2007 and b) stress in 2006, rewatering (RW) in 2007.

#### 3.3.1.1 Drought Stress

Genes being up regulated under drought stress include some with proposed protective function such as LEA (late embryogenesis abundant), RCI2B (rare-cold-inducible gene 2b), and ELIP1 (early light inducible protein 1) and many other stress responsive genes such as PP2CA (protein phosphatase 2CA), ALDH7B4 (aldehyde deyhdrogenase 7B4), drought-induced gene 21, metallothionein-like protein, responsive to desiccation 22, and LOS1 (low expression of osmotically responsive genes 1). Also, three genes being involved in senescence (CLPR1, SAG21, and putative senescence-associated protein) as well as transcription factor WRKY which is involved in defence, wounding, and senescence were induced. In addition, concurrent with the increase in glucose concentration in leaves (see Chapter 2) several amylases (alpha- and beta-amylase) functioning in starch breakdown were up-regulated (Figure 3.5). Also up-regulated by drought stress were transport related genes such as ATINT1 (inositol transporter), transporter SEC22, and lipid transfer proteins (LTP1 & LTP3). On the other hand, genes encoding HSP70 (heat shock protein 70) and GLP3 (germin-like protein) as well as several photosynthesis-related genes and one gene being involved in growth (Expansin A8), were down regulated.

#### 3.3.1.2 Rewatering

In the rewatered plants, stress responsive genes encoding GLP3 and MLP423 Bet v allergen family proteins which had been down regulated under drought stress were up regulated. In

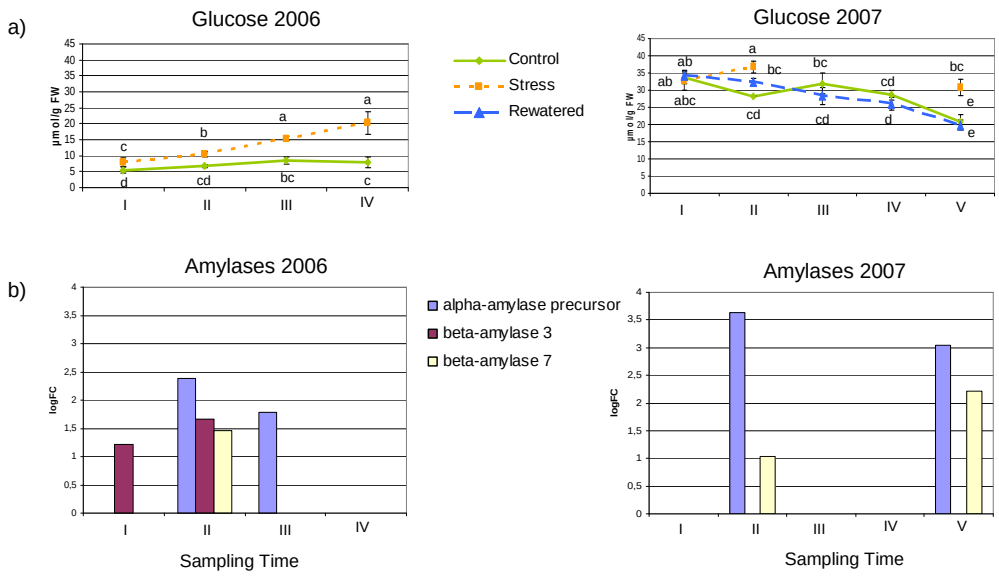


**Figure 3.5:** a) Concentration of glucose in leaves of drought stressed plants at different sampling times in 2006 and 2007. Points not marked with the same letter are significantly different. At certain time points carbohydrate breakdown-related genes (encoding for amylases) were detected as being differentially expressed. b) Graphs indicate the log fold change of amylase encoding genes at different samplings of drought stressed plants in 2006 and 2007.

addition, a large number of genes being involved in growth and cell wall modification were up regulated (Table 3.2 and 3.3). These include several genes involved in lignin biosynthesis such as laccase 10, caffeoyl-CoA 3-O-methyltransferase, FERULATE-5-HYDROXYLASE 1, and cellulose synthase. Expansin A8, which is supposed to be involved in growth was down regulated during the first days of rewatering when shoot growth was still reduced compared to the control plants but up regulated after several weeks when rewatered plants showed strong compensation growth (Figure 3.6). Two genes involved in senescence, CLPR1 and SAG21, as well a thioredoxin which is involved in detoxification processes were also down-regulated as opposed to the drought stress treatment. Photosynthesis-related genes were up regulated with the exception of genes encoding ELIP1 (early light inducible protein).

**Table 3.2:** Differentially expressed genes (adjusted p-values <0.05) of selected functional groups at sampling times I-V in 2006 and I, II and V in 2007 for drought stressed plants (DS) and sampling times I, II, III, and V in 2007 for rewatered plants (RW). For each gene accession number, annotation and log-fold change are given. Continued in table 3.3.

| | | | 2006 DS | | | | 2007 DS | | | 2007 RW | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FG | Name | Annotation | I | II | III | IV | I | II | V | I | II | III | V |
| carbohydrate metabolism | LG0AAA19M15 | acidic endochitinase (CHIB1) | | -1,57 | | -2,18 | | -1,92 | | | | | |
| | LG0AAD28G23 | ATPGMP, PGM1, STF1, PGM PGM (PHOSPHOGLUCOMUTASE) | | 1,31 | | | | | | | | | |
| | LG0AAD2O3 | ATPHS2, PHS2 ATPHS2/PHS2 (ALPHA-GLUCAN PHOSPHORYLASE 2); phosphorylase/ transferase, transferring glycosyl groups | | | | | -1,38 | | | -1,27 | | | |
| | LG0AAD22O3 | ATTPPA ATTPPA (Arabidopsis thaliana trehalose-6-phosphate phosphatase); trehalose-phosphatase | | | | | | -1,45 | | | | | |
| | LG0AAD50L11 | Alpha-amylase precursor - Streptomyces thermoviolaceus | | 2,38 | 1,79 | | | 3,64 | 3,05 | | | | |
| | LG0AAB25G15 | BMY3 BMY3 (beta-amylase 3); beta-amylase | 1,22 | 1,66 | | | | | | | | | |
| | LG0AAD7M4 | BMY7, TR-BAMY BMY7/TR-BAMY (beta-amylase 7); beta-amylase | | | | | | 1,04 | | | | | |
| | LG0AAD36D20 | BMY7, TR-BAMY BMY7/TR-BAMY (beta-amylase 7); beta-amylase | | 1,46 | | | | | 2,21 | | | | |
| | LG0AAA20D4 | BMY7, TR-BAMY BMY7/TR-BAMY (beta-amylase 7); beta-amylase | | | | | | | | | | -2,19 | |
| | LG0AAD7O22 | glycosyl hydrolase family 1 protein | | | -1,02 | | | | | | | | |
| | LG0AAA4E21 | ISA1, ATISA1, ARA1 ARA1 (ARABINOSE SENSITIVE 1); ATP binding / galactokinase | | | | | | -1,23 | | | | | |
| | LG0AAA20P10 | MDH MDH (malate dehydrogenase); malate dehydrogenase | | | | | -1,16 | | | | | | |
| | LG0AAC6J14 | ribulose-phosphate 3-epimerase, cytosolic, putative / pentose-5-phosphate 3-epimerase, putative | | | | | | | | | 0,97 | | |
| | LG0AAD27N24 | starch synthase, putative | | | | | -1,39 | | | | | | |
| | LG0AAD21J11 | starch synthase, putative | | | | | | | | -1,29 | | | |
| | LG0AAB19I13 | SUS4 SUS4; UDP-glycosyltransferase/ sucrose synthase/ transferase, transferring glycosyl groups | | | | | | | | | | 2,77 | |
| growth, cellwall modification | LG0AAB16A4 | XTH9 XTH9 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9); hydrolase, acting on glycosyl bonds | | | | | | 1,16 | | | | | |
| | LG0AAB29P15 | XTH9 XTH9 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9); hydrolase, acting on glycosyl bonds | | | | | | 1,85 | | | 1,48 | | |
| | LG0AAC22P21 | XTH9 XTH9 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9); hydrolase, acting on glycosyl bonds | -0,87 | | | | | | | | | | |
| | LG0AAD9N21 | arabinogalactan-protein | | | | | | 1,95 | | | | | |
| | LG0AAD33K16 | putative arabinogalactan protein [Lotus japonicus] | | | | | | | 2,13 | | | | |
| | LG0AAC9G7 | fasciclin-like arabinogalactan protein 10 [Gossypium hirsutum] | | | | | 1,12 | | | | | | |
| | LG0AAD7A22 | ATPME1 ATPME1 (Arabidopsis thaliana pectin methylesterase 1); pectinesterase | | | | | | 1,33 | | | | | |
| | LG0AAA16C1 | ATPSK3, PSK1 PSK1 (PHYTOSULFOKINE 3 PRECURSOR); growth factor | | | | | | 1,51 | | | | | |
| | LG0AAC20N18 | BURP domain-containing protein / polygalacturonase, putative | | | | | | | | | | | -2,09 |
| | CU656890 | BURP domain-containing protein / polygalacturonase, putative | | -1,90 | | | | | | | -1,82 | | 1,46 |
| | LG0AAA3C11 | caffeoyl-CoA 3-O-methyltransferase, putative | | | | | | | | | | 2,92 | |
| | LG0AAB22L24 | CESA8, IRX1, ATCESA8, LEW2 CESA8 (CELLULASE SYNTHASE 8); cellulose synthase/ transferase, transferring glycosyl groups | | | | | | | | | | 3,77 | |
| | LG0AAB14O2 | chitinase | | | | | | | | | | 3,41 | |
| | LG0AAB6L15 | chitinase | | | | | | | | | | 3,23 | |
| | LG0AAC14E10 | cinnamoyl-CoA reductase-related | | | | | | | 1,69 | | | | |
| | LG0AAD29B22 | cinnamyl-alcohol dehydrogenase, putative | | | | | | | | 1,25 | | | |
| | LG0AAD30N15 | CYP84A1, FAH1 FAH1 (FERULATE-5-HYDROXYLASE 1); ferulate 5-hydroxylase | | | | | | | | | | 3,29 | |
| | LG0AAB19K10 | CYP84A1, FAH1 FAH1 (FERULATE-5-HYDROXYLASE 1); ferulate 5-hydroxylase | | | | | | | | | | 2,19 | |
| | LG0AAD9C10 | EXP8, ATEXP8, ATHEXP ALPHA 1.11, ATEXPA8 ATEXPA8 (ARABIDOPSIS THALIANA EXPANSIN A8) | | -2,78 | | | | -1,89 | | -1,69 | | 2,79 | |
| | LG0AAC14O14 | FLP1, YRE, WAX2 WAX2; catalytic | | 0,92 | | | | 1,37 | | | | | |
| | LG0AAB5I12 | LAC10 LAC10 (laccase 10); copper ion binding / oxidoreductase | | | | | | | | | | 2,90 | |
| | LG0AAD25G23 | Ligninase H2 precursor - Phanerochaete chrysosporium (White-rot fungus) (Sporotrichum pruinosum) | | | | | | -1,52 | | | | | |
| | CR628186 | Ndr family protein | | | | | | | | | | 1,70 | |
| | LG0AAD27O5 | OMT1, ATOMT1 ATOMT1 (O-METHYLTRANSFERASE 1) | | | | | | | | | | 2,51 | |
| | LG0AAD37D10 | pectinesterase family protein | | | | | | | | | | 1,84 | |
| | LG0AAD22F1 | plant cell wall protein SITFR8B [Lycopersicon esculentum] | | | | | | | | | | 2,10 | |
| | LG0AAD37H24 | proline-rich protein PRP1 [Castanea sativa] | | | | | | | | | -0,95 | | |
| development | LG0AAC23J10 | ATCOP1, DET340, FUS1, EMB168, COP1 COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1) | | | | | | | | | | -2,44 | |
| | LG0AAD2I3 | ATLEA5, SAG21 SAG21 (SENESCENCE-ASSOCIATED GENE 21) | | | | | | | | | -1,48 | | |
| | LG0AAC4H12 | CCL CCL (CCR-LIKE) | | | | | | -2,90 | | | -1,95 | | |
| | LG0AAB13H3 | EAT1, EFE EFE (ethylene forming enzyme) | | | | | | -1,32 | | | | | |
| | LG0AAD44H15 | EAT1, EFE EFE (ethylene forming enzyme) | | -1,12 | | | | -1,07 | | | -0,87 | | |
| | LG0AAB17G13 | EAT1, EFE EFE (ethylene forming enzyme) | | | | | | -1,34 | | | | | |
| | LG0AAD13L6 | GASA1 GASA1 (GAST1 PROTEIN HOMOLOG 1) | | 1,75 | | | | 1,53 | | | 1,36 | | |
| | LG0AAD13L23 | GASA1 GASA1 (GAST1 PROTEIN HOMOLOG 1) | | 2,11 | | 1,61 | | | | | | | |
| | LG0AAC20O13 | GASA1 GASA1 (GAST1 PROTEIN HOMOLOG 1) | | | | | | 1,80 | 3,48 | | | | |
| | CU657317 | GASA1 GASA1 (GAST1 PROTEIN HOMOLOG 1) | | 2,57 | | | | 1,87 | | | | | |
| | LG0AAD50O15 | gibberellin-regulated family protein | | | | | | 1,11 | | | | | |
| | LG0AAD3M7 | GR-RBP7, CCR2, ATGRP7 ATGRP7 (COLD, CIRCADIAN RHYTHM, AND RNA BINDING 2); RNA binding | | | | | -1,59 | | | | -1,35 | | |
| | LG0AAA20H19 | GR-RBP7, CCR2, ATGRP7 (COLD, CIRCADIAN RHYTHM, AND RNA BINDING 2); RNA binding / DNA binding | | | | | -1,14 | | | | | | |
| | LG0AAD35I5 | GR-RBP7, CCR2, ATGRP7 (COLD, CIRCADIAN RHYTHM, AND RNA BINDING 2); RNA binding / DNA binding | | | | | -2,43 | | | | | | |
| | LG0AAD3M7 | GR-RBP7, CCR2, ATGRP7 (COLD, CIRCADIAN RHYTHM, AND RNA BINDING 2); RNA binding | | | | | -1,59 | | | | -1,35 | | |
| | LG0AAB25I24 | LSH1 LSH1 (LIGHT-DEPENDENT SHORT HYPOCOTYLS 1) | | | | | | -0,95 | | | | | |
| | LG0AAD48O4 | oxidoreductase, acting on the CH-CH group of donors | | | | | | | | | | | 1,36 |
| | LG0AAC26K7 | putative senescence-associated protein [Cupressus sempervirens] | | | | | | | 2,97 | | | | |
| | CU657048 | UNE3, PGA2, TATC, APG2 APG2 (ALBINO AND PALE GREEN 2) | -1,58 | | | | | | | | | | |
| oxidation, aduct | LG0AAC14E13 | ACD1-LIKE ACD1-LIKE; electron carrier | | | | | | | | | -1,20 | | |
| | LG0AAD45D13 | ATH9 ATH9 (thioredoxin H-type 9) | | | | | | | | | | | -2,33 |
| | LG0AAD46L5 | ATM4, TRX-M4, ATHM4 ATHM4 (Arabidopsis thioredoxin M-type 4); thiol-disulfide exchange intermediate | | | | | | | | | | -2,14 | |
| | LG0AAB24C1 | thioredoxin family protein | | 1,93 | | 1,66 | | | 3,12 | | | | |
| | LG0AAC7D2 | thioredoxin family protein | 1,29 | | | | | | | | | | |
| | LG0AAD1N6 | ATNTRB, NTRB NTRB (NADPH-dependent thioredoxin reductase B) | 1,02 | | | | | | | | | | |
| | LG0AAD49A16 | FQR1 FQR1 (FLAVODOXIN-LIKE QUINONE REDUCTASE 1) | | | | | | | | | | 1,88 | |
| | LG0AAD41H20 | oxidoreductase, 2OG-Fe(II) oxygenase family protein | | | 0,98 | | | 1,78 | | | | | |
| | LG0AAA9K18 | oxidoreductase/ zinc ion binding | | | | | | | | | | | -1,44 |
| | LG0AAB25E8 | UDP-glucose 6-dehydrogenase, putative | | | | | | | | | | 1,95 | |
| photosynthesis | LG0AAA13I12 | AB140, CAB140, LHCB1.3, CAB1 CAB1 (CHLOROPHYLL A/B BINDING PROTEIN 1); chlorophyll binding | | | | | | | | 1,45 | | | |
| | LG0AAD45L18 | ATPPC1 ATPPC1 (PHOSPHOENOLPYRUVATE CARBOXYLASE 1); phosphoenolpyruvate carboxylase | | | | | | 2,30 | | | | | |
| | LG0AAC31J6 | ELIP, ELIP1 ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN); chlorophyll binding | | | | 2,39 | | | | | -1,51 | | |
| | LG0AAA8H2 | ELIP, ELIP1 ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN); chlorophyll binding | | | | | | | | -1,64 | | | |
| | LG0AAA18E23 | ELIP, ELIP1 ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN); chlorophyll binding | | | | | | | | | | -2,37 | |
| | LG0AAA7N24 | ELIP, ELIP1 ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN); chlorophyll binding | | | | | | | | | | -2,38 | |
| | LG0AAA10J24 | ELIP, ELIP1 ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN); chlorophyll binding | | | | | | | | | | -3,24 | |
| | LG0AAA2I22 | ELIP, ELIP1 ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN); chlorophyll binding | | | | | | | | -2,16 | | | |
| | LG0AAA19E24 | ELIP, ELIP1 ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN); chlorophyll binding | | | | | | | | -1,69 | | | |
| | LG0AAC28K8 | LHCB1.4, LHB1B1 LHB1B1 (Photosystem II light harvesting complex gene 1.4); chlorophyll binding | | | | | 1,37 | | | 1,54 | | | |
| | LG0AAA14O9 | LHCB2.2 LHCB2.2 (Photosystem II light harvesting complex gene 2.2); chlorophyll binding | | | | -1,46 | | | | | | | |
| | LG0AAA20L8 | LHCB3*1, LHCB3 LHCB3 (LIGHT-HARVESTING CHLOROPHYLL BINDING PROTEIN 3) | | | | | | | | 2,14 | | | |
| | LG0AAA15H8 | OEE2, PSBP-1 PSBP-1 (OXYGEN-EVOLVING ENHANCER PROTEIN 2); calcium ion binding | | | | | | | | 1,17 | | | |
| | LG0AAA4O9 | PSAF PSAF (photosystem I subunit F) | | | | -1,05 | | | | | | | |
| | LG0AAA8E4 | PSAO PSAO (photosystem I subunit O) | | | | | | | | 1,23 | | | |
| | LG0AAA11B16 | PSBQ, PSBQ-2 PSBQ/PSBQ-2 (photosystem II subunit Q-2); calcium ion binding | | | | -1,50 | | | | | | | |
| | LG0AAC14I16 | PSBX PSBX (photosystem II subunit X) | | | | -1,51 | | | | | | | |
| | CR628050 | PSBX PSBX (photosystem II subunit X) | | | | -1,43 | | | | | | | |
| regulation of gene expression | LG0AAA5E12 | AGL14 (AGAMOUS-LIKE 14); DNA binding / transcription factor | | | | | | | | | | -2,33 | |
| | LG0AAC14D8 | ARAC7, ATROP9, RAC7, ROP9 ARAC7/ATROP9/RAC7/ROP9 (rho-related protein from plants 9); GTP binding | | | | 1,31 | | | | | | | |
| | LG0AAB17D11 | AtMYB85, MYB85 MYB85 (myb domain protein 85); DNA binding / transcription factor | | | | | | | | | | | -2,14 |
| | LG0AAD24N12 | ATRDRP1, RDR1 RDR1 (RNA-DEPENDENT RNA POLYMERASE 1); RNA-directed RNA polymerase/ nucleic acid binding | | | 1,39 | | | | | | | | |
| | LG0AAD22E19 | ATWRKY53, WRKY53 WRKY53 (WRKY DNA-binding protein 53); transcription factor | | 2,68 | 1,81 | | | 3,99 | 3,26 | | | | |
| | LG0AAD41M10 | basic helix-loop-helix (bHLH) family protein | | | | | | 1,31 | | | | | |
| | LG0AAD29N12 | cleavage and polyadenylation specificity factor (CPSF) A subunit C-terminal domain-containing protein | | | | | | | | 1,38 | | | |
| | LG0AAB19G13 | CPL4 (C-TERMINAL DOMAIN PHOSPHATASE-LIKE 4) | | | -1,63 | | | | | | | | -2,20 |
| | CR627819 | DNA-binding protein-related | | | | | | | 2,52 | | | | |
| | LG0AAD18N22 | glycine-rich RNA binding protein [Cryptococcus neoformans var. neoformans JEC21] | | | | -2,31 | | | | | | | |
| | CU657114 | GTE6 GTE6 (GENERAL TRANSCRIPTION FACTOR GROUP E6); DNA binding | | | | | | | | | | -1,82 | |
| | LG0AAB28M3 | HAP5C HAP5C (HEME ACTIVATED PROTEIN 5C); DNA binding / transcription factor | | | | | | | | | 1,24 | | |
| | LG0AAD5A4 | harpin-induced protein-related / HIN1-related / harpin-responsive protein-related | | | | | | | | | | 1,64 | |
| | LG0AAA13I11 | HIT-type zinc finger-containing protein C1orf181 homolog - Mus musculus (Mouse) | | | | | | | | | | 2,27 | |
| | LG0AAD31A16 | hydroxyproline-rich glycoprotein DZ-HRGP [Volvox carteri f. nagariensis] | | | | | | | | | -1,10 | | |
| | LG0AAD37E11 | LHY1, LHY LHY (LATE ELONGATED HYPOCOTYL) | | | | | | | | | | -1,99 | |
| | CR627988 | Light-inducible protein CPRF-2 - Petroselinum crispum (Parsley) (Petroselinum hortense) | 1,19 | | | | | 2,56 | | | | | |
| | LG0AAD42M1 | myb family transcription factor | | | | | | 1,61 | | | | | |
| | LG0AAB25A16 | MYB transcription factor MYB177 [Glycine max] | | | | 1,77 | | | | | | | |
| | LG0AAD37N7 | transcription factor | | | | | | | | | | 2,39 | |
| | LG0AAA3A14 | transcriptional factor B3 family protein | | | 1,04 | | | | | | | | |
| | LG0AAC10N4 | VIP2 VIP2 (VIRE2 INTERACTING PROTEIN2); transcription regulator | | | | | | | | | 1,36 | | |
| | CU656661 | zinc finger (C3HC4-type RING finger) family protein | | -0,76 | | | | | | | | | |
| | LG0AAC30E6 | zinc finger (C3HC4-type RING finger) family protein | | | | | | | | | | 3,20 | |
| | LG0AAA14K19 | zinc finger (ZPR1-type) family protein | | | | | | | | | | 2,11 | |
| | LG0AAD35I13 | zinc finger protein-related | | | | | | | | | | | 2,01 |

**Table 3.3:** Continued from table 3.2.

| | | | 2006 DS | | | | 2007 DS | | | 2007 RW | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FG | Name | Annotation | I | II | III | IV | I | II | V | I | II | III | V |
| signaling | LG0AAB3N12 | ARF16 ARF16 (AUXIN RESPONSE FACTOR 16); mRNA binding / transcription factor | | | | -1,13 | | | | | | | |
| | LG0AAB6E2 | ARR12 ARR12 (ARABIDOPSIS RESPONSE REGULATOR 12); transcription factor/ two-component response regulator | | | | | | | | | 1,27 | | |
| | LG0AAA10K11 | ASK1, SNRK2-4, SNRK2.4, SRK2A ASK1 (ARABIDOPSIS SERINE/THREONINE KINASE 1); kinase | | | -1,60 | | | | | | | | |
| | LG0AAD43C12 | ATMKK9 ATMKK9 (Arabidopsis thaliana MAP kinase kinase 9); kinase | | | | | -1,39 | | | | -1,33 | | |
| | LG0AAB71I1 | ATPI4K ALPHA ATPI4K ALPHA (Arabidopsis thaliana phosphatidylinositol 4-kinase alpha); inositol or phosphatidylinositol kinase | | 2,54 | | 3,47 | | | | | | -2,99 | |
| | LG0AAB24D5 | ATPIN1, PIN1 PIN1 (PIN-FORMED 1); transporter | | | | | | -1,28 | | | | | |
| | LG0AAD6L14 | CML24, TCH2 TCH2 (TOUCH 2); calcium ion binding | | | | | | | | | -0,90 | | |
| | LG0AAC22K9 | DC1 domain-containing protein | | 1,67 | | | | 2,20 | | | | | |
| | CU639821 | FBL6, EBF1 EBF1 (EIN3-BINDING F BOX PROTEIN 1); ubiquitin-protein ligase | | | | | | | 2,39 | | | | |
| | LG0AAB18M24 | GTP-binding protein-related | | | | | | | | | 1,09 | | |
| | LG0AAD48E14 | leucine-rich repeat transmembrane protein kinase, putative | | | | | | -1,81 | | | -2,14 | | |
| | LG0AAD15C24 | leucine-rich repeat transmembrane protein kinase, putative | | | | | | -1,44 | | | -1,42 | | |
| | CU656459 | leucine-rich repeat transmembrane protein kinase, putative | | | -1,07 | | | | | | | | |
| | LG0AAA6D23 | NTF2A NTF2A (NUCLEAR TRANSPORT FACTOR 2A); Ran GTPase binding / protein transporter | | | | | | | | | | -1,86 | |
| | LG0AAB24E22 | OST1, SNRK2-6, SNRK2.6, SRK2E, P44 OST1 (OPEN STOMATA 1); kinase/ protein kinase | | 1,64 | | | | 1,25 | | | | | |
| | CU639700 | phosphatidic acid phosphatase-related / PAP2-related | | | | | | | | 1,91 | | | |
| | LG0AAB8E2 | phosphatidic acid phosphatase-related / PAP2-related | | | | | | | | | 1,17 | | |
| | LG0AAD43L16 | protein kinase family protein | | | | | | -1,26 | | | -1,26 | | |
| | LG0AAB27F17 | protein kinase family protein | | | | | | | | | 1,04 | | |
| | LG0AAD15H15 | protein kinase family protein | | | | | | 1,06 | | | | | |
| | LG0AAD43L16 | protein kinase family protein | | | | | | -1,26 | | | -1,26 | | |
| | CU640516 | protein kinase family protein | | | | | -1,74 | | | -1,34 | -1,12 | | |
| | LG0AAD12P23 | protein kinase, putative | | | | | | | | | -1,49 | | |
| | LG0AAC21F15 | protein phosphatase 2C, putative / PP2C, putative | | | | | | -1,26 | | | | | |
| | LG0AAB13J11 | CPK8, CDPK19 CDPK19 (CALCIUM-DEPENDENT PROTEIN KINASE 19); calcium- and calmodulin-dependent protein kinase/ kinase | | | | | | | | | 1,73 | | |
| | CU640322 | RLK4, CRK10 CRK10 (CYSTEINE-RICH RLK10); kinase | | | | | | | | | | -1,91 | |
| | LG0AAB19J18 | SIP3, SnRK3.14, CIPK6 CIPK6 (CBL-INTERACTING PROTEIN KINASE 6); kinase | 1,13 | | | | | | | | | | |
| | LG0AAA3K5 | SIP3, SnRK3.14, CIPK6 CIPK6 (CBL-INTERACTING PROTEIN KINASE 6); kinase | | 1,15 | | | | | | | | | |
| | LG0AAD3J10 | SIP3, SnRK3.14, CIPK6 CIPK6 (CBL-INTERACTING PROTEIN KINASE 6); kinase | | | | | | | 2,45 | | | | |
| | LG0AAA10G19 | XLG2 XLG2 (extra-large GTP-binding protein 2); signal transducer | | | | | | -1,20 | | | | | |
| stress | LG0AAA11N5 | AGP17 (ARABINOGALACTAN PROTEIN 17) [Arabidopsis thaliana]; Lysine-rich arabinogalactan protein 17 precursor (Lys-rich AGP 17) | | | | | | 1,05 | | | | | |
| | CU656278 | ALDH7B4 ALDH7B4 (ALDEHYDE DEHYDROGENASE 7B4); 3-chloroallyl aldehyde dehydrogenase | | | | | | 1,08 | | | | | |
| | CR627971 | ATCHITIV, CHIV, ATEP3 ATEP3 (Arabidopsis thaliana chitinase class IV); chitinase | | | | | | | | | | | 3,03 |
| | LG0AAB10E20 | ATCOR413-PM2 COR413-PM2 (cold regulated 413 plasma membrane 2) | | | | | | 1,23 | | | | | |
| | LG0AAD9O16 | ATLOX2, LOX2 LOX2 (LIPOXYGENASE 2) | | | | | | | | | | | 1,80 |
| | LG0AAD16O9 | ATPER1 ATPER1 (Arabidopsis thaliana 1-cysteine peroxiredoxin 1); antioxidant | | | | | | | | | | 2,02 | |
| | LG0AAB15C11 | ATPP2CA, AHG3 AHG3/ATPP2CA (ARABIDOPSIS THALIANA PROTEIN PHOSPHATASE 2CA); protein binding / protein phosphatase type 2C | | 1,57 | | | | 1,67 | | | | | |
| | LG0AAD6A20 | ATPP2CA, AHG3 AHG3/ATPP2CA (ARABIDOPSIS THALIANA PROTEIN PHOSPHATASE 2CA); protein binding / protein phosphatase type 2C | | 1,73 | | | | | | | | | |
| | LG0AAA7C15 | CYP77A4 CYP77A4 (cytochrome P450, family 77, subfamily A, polypeptide 4); oxygen binding | -1,27 | | | | | | | | | 3,29 | |
| | CR627776 | DI21, ATDI21 ATDI21 (Arabidopsis thaliana drought-induced 21) | | 3,17 | | | | | 3,17 | | | | |
| | LG0AAC20B18 | disease resistance-responsive family protein / dirigent family protein | | | | | | | 2,87 | | | | |
| | CU657271 | DNAJ heat shock N-terminal domain-containing protein | | | | | -2,97 | 2,06 | | | | | |
| | LG0AAD11D18 | EDS1 EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1); signal transducer/ triacylglycerol lipase | | | | | | -1,77 | | | -1,74 | | |
| | LG0AAA16L11 | GAPC-2 GAPC-2; glyceraldehyde-3-phosphate dehydrogenase | | | | | | | | | -0,85 | | |
| | LG0AAA12B13 | GLP1 GLP1 (GERMIN-LIKE PROTEIN 1); manganese ion binding / metal ion binding / nutrient reservoir | | | | | | | | | | 2,17 | |
| | LG0AAA16M9 | GLP3A, GLP3B, GLP3 GLP3 (GERMIN-LIKE PROTEIN 3); manganese ion binding / metal ion binding / nutrient reservoir | | | -3,24 | -2,17 | -2,08 | | | | | 4,24 | |
| | LG0AAA1E14 | GLP3A, GLP3B, GLP3 GLP3 (GERMIN-LIKE PROTEIN 3); manganese ion binding / metal ion binding / nutrient reservoir | | -1,53 | -2,30 | -1,69 | | | | | | | |
| | LG0AAA2B20 | GLP3A, GLP3B, GLP3 GLP3 (GERMIN-LIKE PROTEIN 3); manganese ion binding / metal ion binding / nutrient reservoir | -1,70 | | | | | | | | | 1,92 | |
| | LG0AAA16M9 | GLP3A, GLP3B, GLP3 GLP3 (GERMIN-LIKE PROTEIN 3); manganese ion binding / metal ion binding / nutrient reservoir | | | -3,24 | -2,17 | -2,08 | | | | | 4,24 | |
| | LG0AAA1E14 | GLP3A, GLP3B, GLP3 GLP3 (GERMIN-LIKE PROTEIN 3); manganese ion binding / metal ion binding / nutrient reservoir | | -1,53 | -2,30 | -1,69 | | | | | | | |
| | CU656866 | 17.6 kDa class I small heat shock protein (HSP17.6B-CI) | | | | | | | | | | | 3,21 |
| | LG0AAA15B17 | HSP70 HSP70 (heat shock protein 70); ATP binding | -1,43 | | | | | | | | | | |
| | LG0AAA5K19 | HSP70 HSP70 (heat shock protein 70); ATP binding | -1,40 | | | | | | | | | | |
| | CU639824 | HSP70-1, AT-HSC70-1, HSC70, HSC70-1 HSC70-1 (heat shock cognate 70 kDa protein 1); ATP binding | -1,12 | | | | | | | | | | |
| | LG0AAC22H21 | HSP70-1, AT-HSC70-1, HSC70, HSC70-1 HSC70-1 (heat shock cognate 70 kDa protein 1); ATP binding | -0,95 | | | | | | | | | | |
| | LG0AAA11F23 | inositol-3-phosphate synthase isozyme 2 / myo-inositol-1-phosphate synthase 2 / MI-1-P synthase 2 / IPS 2 | | | | | | | | 1,01 | | | |
| | LG0AAD26H5 | isoflavone reductase, putative | -0,98 | | | | | | | | | | |
| | LG0AAD21G11 | isoflavone reductase, putative | | | | | -1,90 | | | | | | |
| | CU657720 | lactoylglutathione lyase | | -1,51 | | -1,39 | | | | | | | |
| | LG0AAC18B20 | late embryogenesis abundant protein [Catharanthus roseus] | | | | | | 4,50 | 3,28 | -1,58 | | | |
| | CR627993 | Late embryogenesis abundant protein Lea5 - Citrus sinensis (Sweet orange) | | 2,67 | 2,59 | | | 5,39 | 3,14 | | 2,64 | | |
| | LG0AAC15L2 | late embryogenesis abundant protein, putative / LEA protein, putative | | 1,38 | | | | | | | | | |
| | LG0AAD45D14 | lipocalin, putative | | | | | | 1,20 | | | | | |
| | LG0AAB14E16 | maturation-associated SRC1-like protein [Carica papaya] | | | | | | 1,07 | 1,82 | | | | |
| | LG0AAD46K14 | maturation-associated SRC1-like protein [Carica papaya] | | | 1,59 | | | 2,59 | | | | | |
| | LG0AAC19F10 | maturation-associated SRC1-like protein [Carica papaya] | | | | | | 1,76 | | | | | |
| | LG0AAD21M21 | Metallothionein-like protein 1 - Pisum sativum (Garden pea) | | | | | | 1,09 | | | | | |
| | LG0AAA5F10 | Metallothionein-like protein 1 - Pisum sativum (Garden pea) | | | | | | 0,78 | | | | | |
| | LG0AAA10A6 | MLP423 Bet v I allergen family protein | | | | | | | | 1,90 | | 4,54 | |
| | LG0AAA2I21 | MLP423 Bet v I allergen family protein | | | | | | | | | | 1,99 | |
| | LG0AAD18B20 | MLP423 Bet v I allergen family protein | | | | | | -1,64 | | | | | |
| | CR628213 | MT3 MT3 (METALLOTHIONEIN 3) | 1,90 | | | | | | | | | | |
| | LG0AAA20P6 | PAG1 PAG1 (20S proteasome alpha subunit G1); peptidase | | | | | | | | | -1,62 | -2,18 | |
| | LG0AAA7F9 | peroxidase, putative | | | | | | | | | | 2,22 | |
| | LG0AAD25D17 | PR3, PR-3, CHI-B, B-CHI, ATHCHIB ATHCHIB (BASIC CHITINASE); chitinase | | | | | | | | | | 1,90 | |
| | LG0AAD49A3 | PR3, PR-3, CHI-B, B-CHI, ATHCHIB ATHCHIB (BASIC CHITINASE); chitinase | | | | | -1,41 | | | | | | |
| | LG0AAD48P10 | pyruvate decarboxylase PDC2 [Laccaria bicolor S238N-H82] | | | 1,57 | | | | | | | | |
| | LG0AAB10A20 | RCI2B RCI2B (RARE-COLD-INDUCIBLE 2B) | | 1,42 | | | | 1,34 | 2,43 | | | | |
| | LG0AAA10C8 | RCI2B RCI2B (RARE-COLD-INDUCIBLE 2B) | | 2,27 | | | | 2,57 | 3,04 | | | | |
| | LG0AAC24C5 | RCI2B RCI2B (RARE-COLD-INDUCIBLE 2B) | 1,09 | 1,50 | | | | 1,40 | | | | | |
| | LG0AAA10C8 | RCI2B RCI2B (RARE-COLD-INDUCIBLE 2B) | | | | | | | | | -0,83 | | |
| | LG0AAD10L19 | RD19A, EMB3005, RD19 RD19 (RESPONSIVE TO DEHYDRATION 19); cysteine-type peptidase | | | | | | -0,89 | | | | | |
| | LG0AAA6M12 | RD22 RD22 (RESPONSIVE TO DESSICATION 22) | | | | | | 1,41 | | | | | |
| | LG0AAD37E3 | AT1G56075.1, LOS1 (Low expression of osmotically responsive genes 1); translation elongation factor/ translation factor, nucleic acid binding | | | | | | 0,89 | | | | | |
| | LG0AAD37M8 | SEN2, CAT3 CAT3 (CATALASE 3); catalase | | | | | 4,48 | | | | | | |
| | LG0AAA15D7 | SH1, FAD8 FAD8 (FATTY ACID DESATURASE 8); omega-3 fatty acid desaturase | | | | | | | -1,72 | | | | |
| | LG0AAD51A12 | SRC2, (AT)SRC2 (AT)SRC2/SRC2 (SOYBEAN GENE REGULATED BY COLD-2); protein binding | | | | | | | | | | 3,08 | |
| | LG0AAB16D23 | universal stress protein (USP) family protein | | | | | | | | | -1,04 | | |
| | LG0AAD9B5 | peroxidase 64 (PER64) (P64) (PRXR4) | | | -1,39 | | | | | | | | |
| | LG0AAA1O17 | SH1, FAD8 FAD8 (FATTY ACID DESATURASE 8); omega-3 fatty acid desaturase | | | | -1,83 | | | | | | | |
| transport | LG0AAD31O19 | (VACUOLAR ATP SYNTHASE SUBUNIT B2); hydrogen ion transporting ATP synthase, rotational mechanism | | | | | | 1,00 | | | | | |
| | LG0AAB25J6 | AAC1 AAC1 (ADP/ATP CARRIER 1); binding | | | | | | 1,04 | | | | | |
| | LG0AAD36J11 | AAC2 AAC2 (ADP/ATP CARRIER 2); binding | -1,09 | | | | | | | | | | |
| | LG0AAA20I17 | AHA8 AHA8 (ARABIDOPSIS H(+)-ATPASE 8); ATPase | | | | | | -0,90 | | | | | |
| | LG0AAB5G2 | anion exchange family protein | | | | | | -1,13 | | | | | |
| | LG0AAA4P24 | ATCNGC2, CNGC2, DND1 DND1 (DEFENSE NO DEATH 1); cation channel/ cyclic nucleotide binding / inward rectifier potassium channel | | -1,08 | | | | | | | | | |
| | LG0AAA2D3 | ATG8D ATG8D (autophagy gene 8-related); microtubule binding | | | | | | 1,55 | | | | | |
| | LG0AAA4I2 | ATP synthase gamma chain, mitochondrial (ATPC) | | | | -1,04 | | | | | | | |
| | LG0AAB5M4 | AVPL1, AVP2 AVP2 (ARABIDOPSIS VACUOLAR H+-PYROPHOSPHATASE 2) | | | | | -2,09 | | | | | | |
| | LG0AAD44I22 | BOU BOU (A BOUT DE SOUFFLE); binding | | 1,12 | | | | | | | | | |
| | LG0AAD24P1 | cytochrome c oxidase family protein | | | | | | | | | 1,62 | | |
| | LG0AAA9O18 | ELD1, ABI8, KOB1 KOB1 (KOBITO) | | | | | | | | | 1,07 | | |
| | CU657855 | fortisome [Medicago truncatula] | | | | | | | 1,90 | | | | |
| | LG0AAD7P10 | haloacid dehalogenase-like hydrolase family protein | | | 1,22 | | | | | | | | |
| | LG0AAD14N3 | heavy-metal-associated domain-containing protein | | | 1,34 | | | | | | | | |
| | LG0AAC21E21 | Encodes a Protease inhibitor/seed storage/LTP family protein | | | | -1,99 | | | | | | | |
| | LG0AAA14O8 | LTP1, ATLTP1, LP1 LP1 (nonspecific lipid transfer protein 1) | | | | | | 0,95 | | | | | |
| | CU657255 | LTP1, ATLTP1, LP1 LP1 (nonspecific lipid transfer protein 1) | | | | | | 1,25 | | | | | |
| | LG0AAA7B19 | LTP1, ATLTP1, LP1 LP1 (nonspecific lipid transfer protein 1) | | | | | | 1,66 | | | | | |
| | LG0AAC4C24 | LTP3 LTP3 (LIPID TRANSFER PROTEIN 3); lipid binding | | | | | | 1,98 | | | | | |
| | LG0AAC27F5 | protease inhibitor/seed storage/lipid transfer protein (LTP) family protein | -2,38 | -1,35 | | | | | | 1,72 | | | |
| | LG0AAD50M24 | protease inhibitor/seed storage/lipid transfer protein (LTP) family protein | | | 2,26 | | | | | | | | |
| | LG0AAD27N10 | nascent polypeptide-associated complex (NAC) domain-containing protein / BTF3b-like transcription factor, putative | | | | | | | 2,75 | | | | |
| | LG0AAC16P20 | mechanosensitive ion channel domain-containing protein / MS ion channel domain-containing protein | | | | | | | | | | 3,11 | |
| | LG0AAD43D15 | NIP1;2, NLM2 NIP1;2/NLM2 (NOD26-like intrinsic protein 1;2); water channel | | | | | | -1,14 | | | | | |
| | LG0AAA18F13 | PIP2D, PIP2;5 PIP2;5/PIP2D (plasma membrane intrinsic protein 2;5); water channel | | | | -2,51 | | | | | | | |
| | CU640246 | TIP2;1, DELTA-TIP1, AQP1, ATTIP2;1, DELTA-TIP DELTA-TIP (delta tonoplast integral protein); water channel | | | | | | | | 1,11 | | | |
| | LG0AAC2B21 | TOM6 TOM6 (TRANSLOCASE OF THE OUTER MITOCHONDRIAL MEMBRANE 6) | | | | | -1,53 | | | | | | |
| | LG0AAC11H12 | integral membrane protein, putative | | | | | | | | | | | -2,00 |
| | LG0AAB26H1 | sugar transporter, putative | | | | | | | | | 1,41 | | |
| | LG0AAD45E20 | ATINT1 ATINT1 (INOSITOL TRANSPORTER 1); carbohydrate transporter/ sugar porter | 1,74 | 2,83 | | 2,51 | | 2,11 | | | | | |
| | LG0AAA14C3 | ATSEC22, SEC22 SEC22 (secretion 22); transporter | 2,18 | 2,24 | | | | 3,11 | | | | | |
| | LG0AAD45N16 | ATKUP3, ATKT4, KUP3 KUP3 (K+ uptake permease 3); potassium ion transporter | | | | | | | | | | -2,10 | |
| | LG0AAA13N22 | transporter-related | | | | | | | | | | -3,31 | |

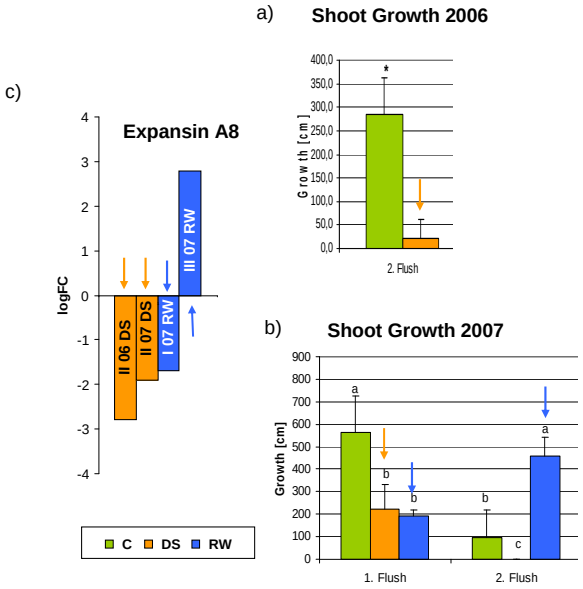


**Figure 3.6:** a) Shoot growth of control (C) and drought stressed plants (DS) during the second flush in 2006. Columns marked with an * are significantly different ($p<0.05$). b) Shoot growth of control, drought stressed, and rewatered plants (RW) during first and second flush in 2007. Columns not marked with the same letter are significantly different c) Log fold change of gene Expansin A8 at sampling time II (first flush) in 2006 and 2007 for drought stressed plants (DS) and at samplings I (first flush) and III (second flush) 2007 for rewatered plants (RW).

## 3.4 Discussion

### 3.4.1 Drought Stress

It is known that, under drought stress, the plant activates different response pathways depending on time and intensity of the stress. Genes involved in signal transduction and gene regulation, for instance, are induced fast within a few hours of stress while genes encoding functional proteins are induced more slowly (Yamaguchi-Shinozaki and Shinozaki, 2006). In *Quercus robur* the long-term drought stress treatment (two growing seasons) activated a very specific response at each sampling time depending on intensity and duration of the water deficit with the majority of the genes (88%) being induced at only one time point. In loblolly pine Watkinson et al. (2003) also found a very specific plant response depending on intensity and duration of the drought stress treatment. In barley, Talamè et al. (2006) reported that a high number of genes were exclusively differentially expressed at one treatment type (drought stress, dehydration shock and, rehydration).

This behaviour suggests that the plant is able to sense the intensity of the stress and accordingly activates specific response pathways.

Only a few days after the start of the mild drought stress several genes encoding Hsp 70, which are known to have chaperone function and usually are reported to be induced upon stress (Buchanan et al., 2000; Bukau and Horwich, 1998; Li et al., 1994), were down regulated in *Quercus robur*. A similar response was found by Bogeat-Triboulot et al. (2007) in another tree species, *Populus euphratica*, exposed to mild drought stress. These authors suggested that poplar might either not be able to activate protective processes or that an acclimation process had already taken place in poplar while in most studies rapid stress responses were analysed. In the case of *Quercus robur*, this contradictory behaviour was only shown in the case of Hsp70 which, apart from its proposed function as chaperone, can also play an important role in normal metabolism assisting in the folding of newly translated proteins and in disassembling of oligomeric protein structures, guide the translocation of proteins across membranes, facilitate the proteolytic degradation of unstable proteins, and may control the biological activity of transcription factors (Bukau and Horwich, 1998). Other genes with proposed protective function such as LEA (late embryogenesis abundant) and RCI2B (rare cold inducible 2B) (Buchanan et al., 2000; Capel et al., 1997; Medina et al., 2001) displayed increased transcript levels under drought stress. It is suggested that this response does not indicate a failure of activation of protective processes in trees but rather a different response based on the physiology of trees and the nature of the stress applied. Another indication for the activation of protective processes is that genes involved in photosynthesis are not down-regulated until the last sampling during the first year of stress. Besides, a gene encoding ELIP1 (early light inducible protein 1) which has a putative function in the protection of photosynthesis against environmental stress (Adamska et al., 1992a,b; Hutin et al., 2003) is up-regulated at the same time. Interestingly, no photosynthesis-related genes are significantly down-regulated at any of the time points analysed in the second year of the drought treatment despite a slightly increased stress level. In loblolly pine exposed to cycles of mild drought stress Watkinson et al. (2003) found that trees showed a recovery of photosynthetic rate after the first cycle of stress suggesting photosynthetic acclimation. Another explanation for the maintenance of photosynthesis could be a more efficient "defense" of the plants against water deprivation through increased transcript levels of genes involved in protective processes. While, during the first year, genes encoding LEA were induced 1.4 to 2.6 fold under stress compared to the controls their induction increased to levels/ratios between 3.1 and 5.4 in the second year of drought stress. LEA-proteins are known to stabilize proteins and to prevent protein

degradation. Studies with rice and yeast, for instance, resulted in increased drought stress resistance when LEA-proteins were overexpressed (Buchanan et al., 2000). Another gene, encoding ALDH7B4 which is turgor responsive and postulated to be involved in an unknown adaptive metabolic pathway (Kirch et al., 2004) was induced in the second year only. Also several genes encoding LTPs (lipid transfer proteins ) which are supposed to be involved in cuticle biosynthesis and deposition were up-regulated during the second year of water deficit stress indicating again the enforced stress defence in the second year (Cameron et al., 2006; Jung et al., 2003). Furthermore, several circadian controlled genes with a putative role in stress regulation (Kreps et al., 2002) were down-regulated during the second year of drought stress.

Besides (these) specific responses being initiated at a certain stress level and/or duration only *Q. robur* generally seems to apply both tolerance and avoidance mechanisms to deal with the long-term drought stress. An example for the application of drought avoidance mechanisms in *Quercus robur* is the reduction and, with persisting and increasing water deficit stress, inhibition of growth (see chapter 2) accompanied by the down-regulation of Expansin A8, a gene being involved in growth processes (Cosgrove, 1997; Cosgrove et al., 2002). Another drought avoidance mechanism is wax deposition on the leaves to reduce water loss under drought stress. Genes involved in this process are WAX2 which is induced under drought stress in both years and LTPs. WAX2 and LTPs function in cuticle membrane and cuticle wax production (Cameron et al., 2006; Chen et al., 2003; Jung et al., 2003) and an up-regulation of these genes could consequently lead to a decreased water loss during drought stress through reduced transpiration rates. Alternative functions ascribed to LTPs are the repair of stress-induced damage in membranes and/or changing the lipid composition of membranes which in turn might change the fluidity of the membrane and thus the permeability to toxic ions (Oono et al., 2003, and references within). These functions, however, would allocate LTPs to the tolerance mechanisms applied by the plant. Other tolerance mechanisms expressed by *Q. robur* comprise the induction of genes with protective function such as LEA, RCI2B, and ALDH7B4 as well as osmotic adjustment. In *Q. robur* osmotic adjustment is mainly achieved by the accumulation of the monosaccharides glucose and fructose and the cyclitol quercitol. In accordance with these findings starch degradation through amylases was up-regulated in both years resulting in the breakdown product glucose (via maltose). The induction of amylases by abiotic stress together with an increase in the compatible solutes maltose and glucose serving in stress tolerance has been observed in previous studies (Jacobsen et al., 1986; Kaplan et al., 2006).

### 3.4.2 Rewatering

A rewatering treatment after one year of drought stress was applied for one growing season to examine the long-term effects of drought on *Quercus robur*.

The physiological data showed that both rewatered plants and those under continued drought stress developed an earlier first flush than the controls. Furthermore, growth during the first flush was strongly reduced in both treatments compared to the controls accompanied by a down-regulation of Expansin A8. The similarities in the phenotype of drought stressed and rewatered plants during the first flush are reflected in the gene expression pattern. During the first flush of the second year many genes belonging to the functional groups of development (CCR-like, EFE, GASA1, CCR2), signalling (MAP kinase kinase 9, putative leucine-rich repeat transmembrane protein kinase, protein kinase family protein) and unknown genes showed a similar trend (up-/down-regulated) in rewatered plants and those kept under continued drought stress. In the second flush, however, the behaviour of the two groups, rewatered and drought stressed, strongly diverges. In the rewatered plants a strong compensation growth takes place accompanied by the induction of many genes involved in growth and cell wall modification, many of them participating in lignin biosynthesis, while the plants kept under continued drought stress cease to grow. However, in order to achieve this strong compensation growth energy from photosynthesis and a reallocation of energy to growth processes is necessary. Photosynthesis-related genes which were repressed under drought stress at the end of the first year were induced (sampling I) under rewatering. A similar behaviour of photosynthesis-related genes was observed in rice under drought stress and rehydration (Zhou et al., 2007) and in poplar under salt stress and recovery (Gu et al., 2004). In contrast, now unnecessary processes which use up energy for the protection against drought such as increased production of protective molecules and detoxifying systems were down-regulated. Early light inducible protein 1 (ELIP1), for example, which is supposed to protect photosynthesis against damage caused by environmental stress (Adamska et al., 1992a,b; Hutin et al., 2003) was down-regulated in several cases under rewatering but up-regulated under drought stress. Thioredoxin, which is thought to have many different functions from photosynthesis regulation, prevention of oxidative stress, and repair of oxidatively damaged proteins to the control of apoptosis (Arnér and Holmgren, 2000; Grant, 2001) also was induced under drought stress but repressed under rewatering. A similar behaviour could be observed for two genes with proposed function in senescence, CLPR1 and SAG21 which were down-regulated under rewatering as opposed to the drought stress treatment. Apart from

its proteolytic function CLP also seems to have chaperone activity (Squires and Squires, 1992) similar to SAG21 which shows strong sequence similarity to late embryogenesis abundant (LEA) proteins known to have chaperone function (Weaver et al., 1998). In accordance with this, one late embryogenesis abundant protein (LEA) and one RCI2B were down-regulated during rewatering while they had been induced under drought stress. In *Arabidopsis* Oono et al. (2003) also identified several LEA genes as being induced under stress but repressed under rehydration. These findings indicate a very effective and flexible protection machinery of the plant against drought induced damage only activated under stress. Upon rewatering, when protection against dehydration damage is not needed anymore energy can be reallocated to recovery processes such as photosynthesis and growth and genes which were repressed under drought stress are activated. Genes encoding MLP423 Bet v allergen family protein, for example, which had been down-regulated under drought stress were induced under rewatering corresponding to the findings of Oono et al. (2003) who identified pollen allergen homolgs to be rehydration-inducible in *Arabidopsis*. The function of these genes in the rehydration process is at present not fully understood. Additionally, more than 10 unknown genes were detected to be induced under rewatering which could be interesting candidates for further research.

In summary, drought stress elicited the induction/repression of genes involved in

- growth and time of flushing,
- osmotic adjustment and starch degradation,
- reinforcement of the cuticula,
- photosynthesis,
- protective processes, and
- senescence.

Rewatering resulted in the induction/repression of genes involved in

- the recovery of photosynthesis,
- protective processes,
- senescence,
- growth and cell wall related processes, and
- rehydration.

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# CHAPTER 4

## Conclusion & Perspectives

*Quercus robur* seems to apply both drought avoidance and tolerance mechanisms when exposed to water deficit stress: the former includes a change in flushing time, growth reduction, and transpiration reduction through increased wax production and cuticle biosynthesis. The latter comprises osmotic adjustment by means of an accumulation of quercitol, fructose, and glucose and the concurrent breakdown of starch to glucose through the induction of genes encoding amylases. While many of these responses occur in both years of drought stress some are specific to a certain degree and/or duration of stress. In general, the response to water deprivation seems to be stronger in the second year leading to a better defence and consequently protection of metabolic processes (e.g. photosynthesis). This suggests an adaptation process to persisting water deficit. Genes being involved in this adaptation process may thus serve for the selection of drought tolerant trees. Potential candidates for further selection might be the gene "WAX2" and lipid transfer protein, both being involved in cuticle biosynthesis. The induction of these genes could lead to reduced water loss under drought stress through a reinforcement of the cuticula resulting in decreased transpiration rates. Furthermore, lipid transfer proteins have a proposed function in the repair of stress-induced damage in membranes. Other potential candidates for selection would be genes encoding for late embryogenesis abundant protein (LEA protein) and rare cold inducible 2B (RCI2B) with proposed function in maintaining membrane function and/or integrity under water stress. Increased synthesis of these proteins might prevent damage and maintain metabolic processes even at low water potential. In addition, these genes only seem to be produced under drought stress. When water is available again the production of these genes seems to cease, indicating a very effective protection mechanism which is only activated when needed. Thus, when water supply is sufficient again energy can be reallocated to other processes. In general, slow evolving drought stress even when persisting for two years, as applied in this experiment,

does not seem to cause long-term damage in the oak plants since a strong compensation growth was observed under rewatering. Also, the production of osmolytes goes back to control levels under rewatering and genes involved in protective and detoxification processes as well as senescence-associated genes are down-regulated. From this behaviour it can be concluded that mild long-term drought stress over two years as it may occur in nature leads to the activation of many adaptation related processes during the stress period. This ensures the survival of the plants while (irreversible) damage is kept low and facilitates the full recovery of *Quercus robur* upon rewatering. Most studies conducted so far applied a short but severe dehydration stress resulting in stronger gene expression changes and a higher number of differentially expressed genes (Kreps et al., 2002; Talamè et al., 2006). When comparing the plants' responses to mild and strong dehydration/drought stress, however, only a small overlap can be observed suggesting that different response pathways are activated in relation to the degree of damage caused by the stress (Talamè et al., 2006; Watkinson et al., 2003). This indicates the need of studies under more naturally induced drought conditions in order to understand gene regulation enabling the selection of candidate genes involved in adaptation processes for further selection of drought tolerant plants.

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# Part II

# Technical Aspects

# CHAPTER 5

# Technical Aspects of Microarray Analysis

## 5.1 From Raw Material to Microarray Images

### 5.1.1 Extraction of RNA, labeling, hybridization, and scanning

For Microarray Analysis RNA was extracted from leaves of four individual trees of *Quercus robur* clone P28 H9 for each treatment (4 biological replicates). RNA for the reference sample RNA was extracted from different tissues and treatments. RNA-samples were amplified to have a sufficient amount of RNA available for subsequent steps (AminoAllyl-Amplification, for details see Chapter 3 on Gene Expression on page 47) and labelled with fluorescent dyes. To each microarray slide an experimental sample labelled with Cy3 and an aliquote of the reference sample labelled with Cy5 were co-hybridized. After over-night hybridization the slide was scanned at fixed laser intensities for the green (Cy3) and red (Cy5) channel as determined in a preliminary study (Figure 5.1).

### 5.1.2 Selection of hybridization scheme

The microarray experimental design chosen for this study was a reference design, meaning that all comparisons were made indirectly/in a transitive fashion via a reference. As opposed to this, in a direct design samples (treatments) are compared directly. The advantages of using a reference design are the reduction of technical artefacts by always using the same reference on each slide, its robustness to the loss of arrays (e.g. through poor quality), the possibility to compare each sample with all others and the extendibility of the experiment (Simon et al., 2002). A disadvantage is lower sensitivity (greater experimental noise) compared to a direct design. However, for large experiments direct comparison between all pairs ("all-pairs design") is hardly feasible (Yang and Speed, 2002). Another

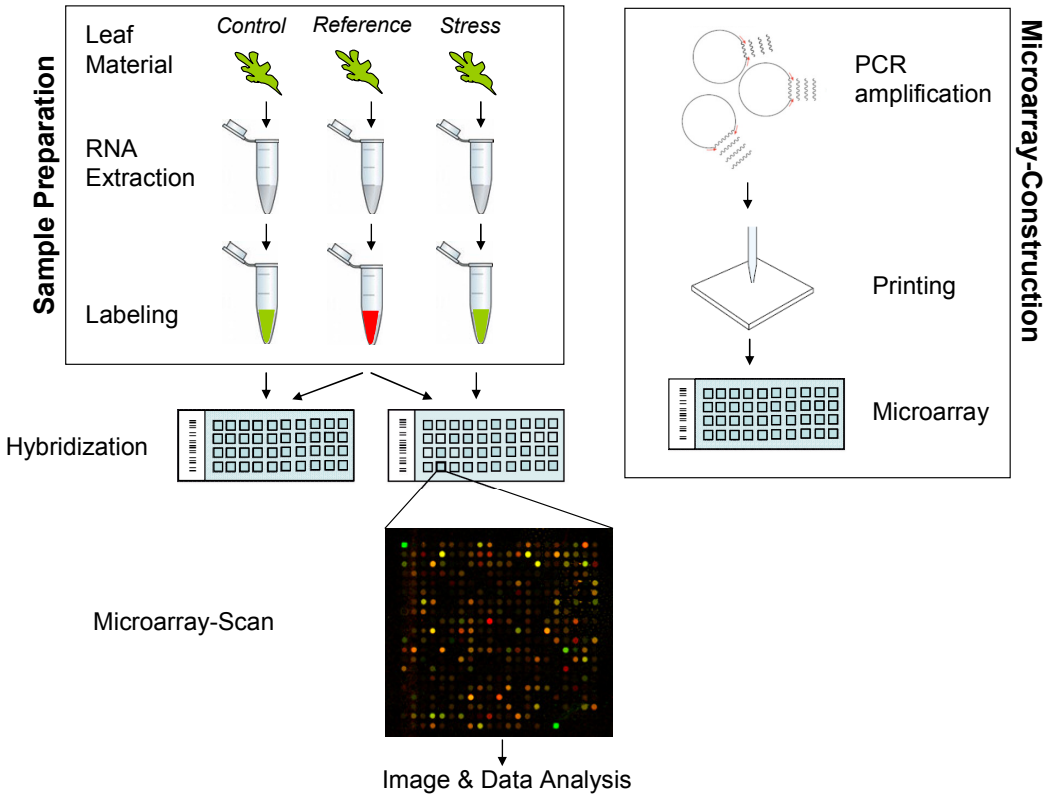


**Figure 5.1:** Microarray construction and sample preparation for microarray hybridization.

drawback of the reference design is the difficulty of estimating gene expression for genes with low expression values in the reference sample (Townsend, 2003). In order to avoid this problem the reference sample for this experiment was created by extracting and pooling RNA from different tissues and treatments (see chapter 2 on Morphology & Physiology on page 21 for details). Through this strategy every gene which is expressed at a high level in one treatment/tissue will still be present at a reasonable concentration in the pooled reference sample (Townsend, 2003). Considering these arguments a preliminary experiment was conducted to compare a direct dye swap design with a reference design. The results of this preliminary experiment suggested sufficient sensitivity of the reference design and was thus used in the main experiment.

## 5.2 From Image to Raw Data

### 5.2.1 Image Analysis

After scanning, image analysis was carried out with the software "Spot" (Beare et al., 2005). Although the commercial software "GenePix" was available the freeware "Spot" was selected because of several advantages as listed below.

Advantages of Spot compared to GenePix:

- Automated image analysis →much faster
- Detection of weak spots/no spots are lost →in GenePix a high percentage of spots is flagged "not found"
- Better (default) background substraction than in GenePix (see example diagrams below)

During Image Analysis background is (usually) subtracted in order to eliminate effects such as non-specific hybridization (Yang et al., 2001). Below (Figure 5.2 and Figure 5.3), an example from the data of a cDNA array which was self-self hybridized is shown to illustrate differences between different background correction methods (Smyth et al., 2008). Self-self hybridization means that the same RNA was hybridized to both channels and no actual differential expression is present for this array. The data is shown in an MA-plot which is a plot of log-intensity ratios (M-values) versus log-intensity averages (A-values). In Genepix, (as a default) the median local background is subtracted resulting in a fanning of the M-values at low intensities (Figure 5.2).

In the following plot (Figure 5.3), background subtracted spot intensities for the same array are shown after "morph" background subtraction with the software Spot. By using this background subtraction method the variability of the M-values is stabilized as a function of intensity. Through this reduction of dependence of variability on intensity the subsequent statistical analysis with the empirical Bayes methods (limma) for assessment of differential expression will be more powerful, especially at low intensities (Smyth et al., 2008).

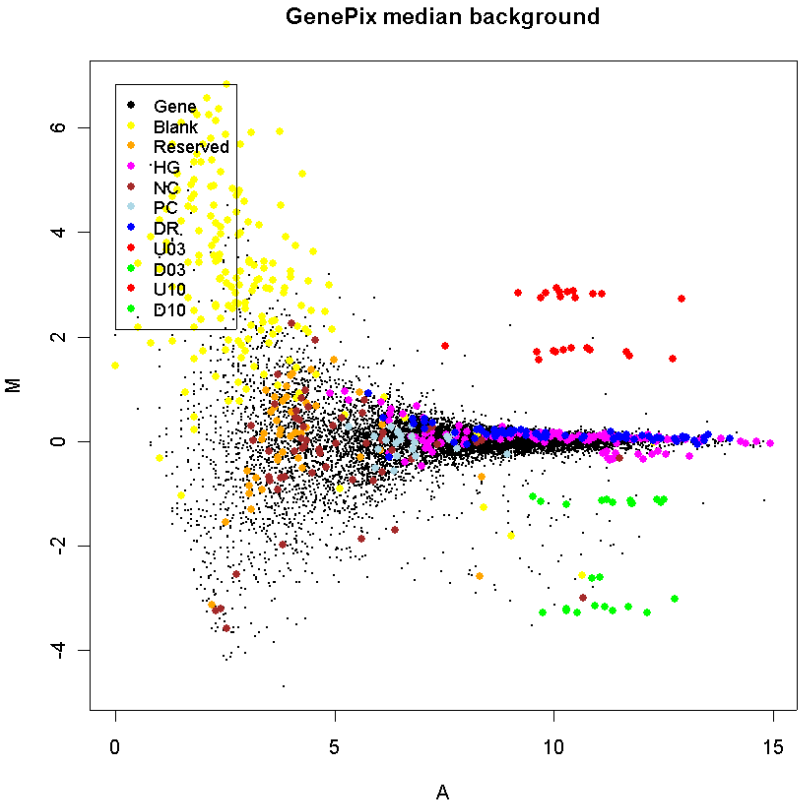


**Figure 5.2:** M/A-plot of background corrected spot intensities in Genepix using the default local median background subtraction. Graph taken from Smyth et al. (2008).

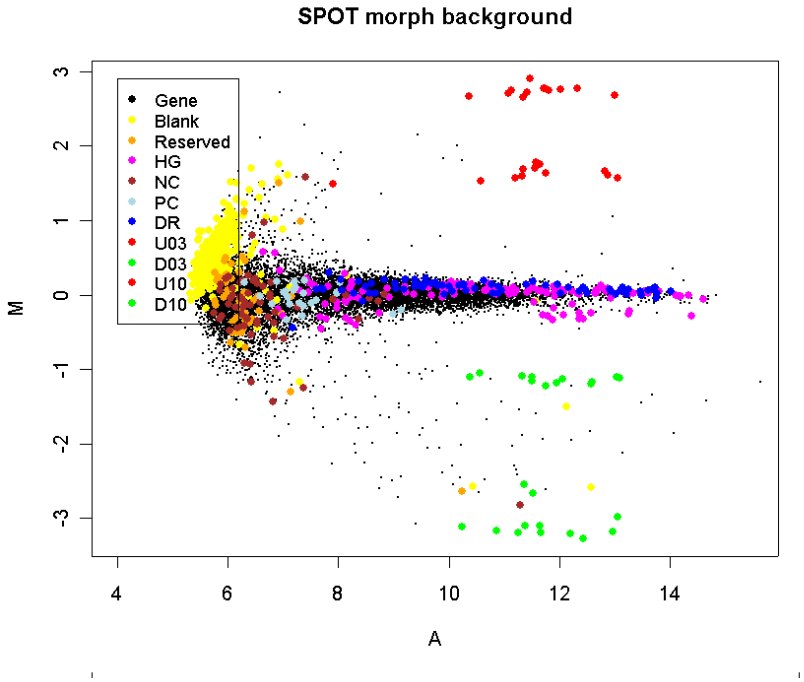


**Figure 5.3:** M/A-plot of background subtracted spot intensities in Spot with method "morph". Graph taken from Smyth et al. (2008).

### 5.2.2 Quality Check

After Image Analysis in Spot data was read into "R" and Quality Control was conducted using the package arrayQuality (Paquet et al., 2004) which displays several diagnostic plots (Figure 5.4):

1. MA-plot of raw M with coloured lines representing the loess curves for each print-tip group. The trend of the loess curves is an indicator of the amount of normalization required.

2. MA-plot of data density normalized with the method "print-tip loess". Yellow colour indicates a high number of spots, blue a lower density.

3. Spatial plot of rank of raw M values without background subtraction. Each spot is ranked according to its M value with blue representing the higher rank and yellow the lower one. This plot facilitates the detection of uneven hybridization.

4. Spatial plot of M values ranks normalized with the method "print-tip loess". Each spot is ranked according to its M value with blue representing the higher rank and yellow the lower one. This plot helps verifying if normalization removed any spatial effects.

5. Spatial plot of raw A values with signal intensity indicated by colour. Dark colour indicates a strong signal, white colour indicates missing spots.

6. Histogram of the signal-to-noise log-ratio (SNR) for channels Cy3 and Cy5. On top of the histogram mean and variance are indicated. The signal-to-noise log-ratio is a good indicator for dye problems.

Figure 5.5 shows an example of a slide with uneven hybridization.

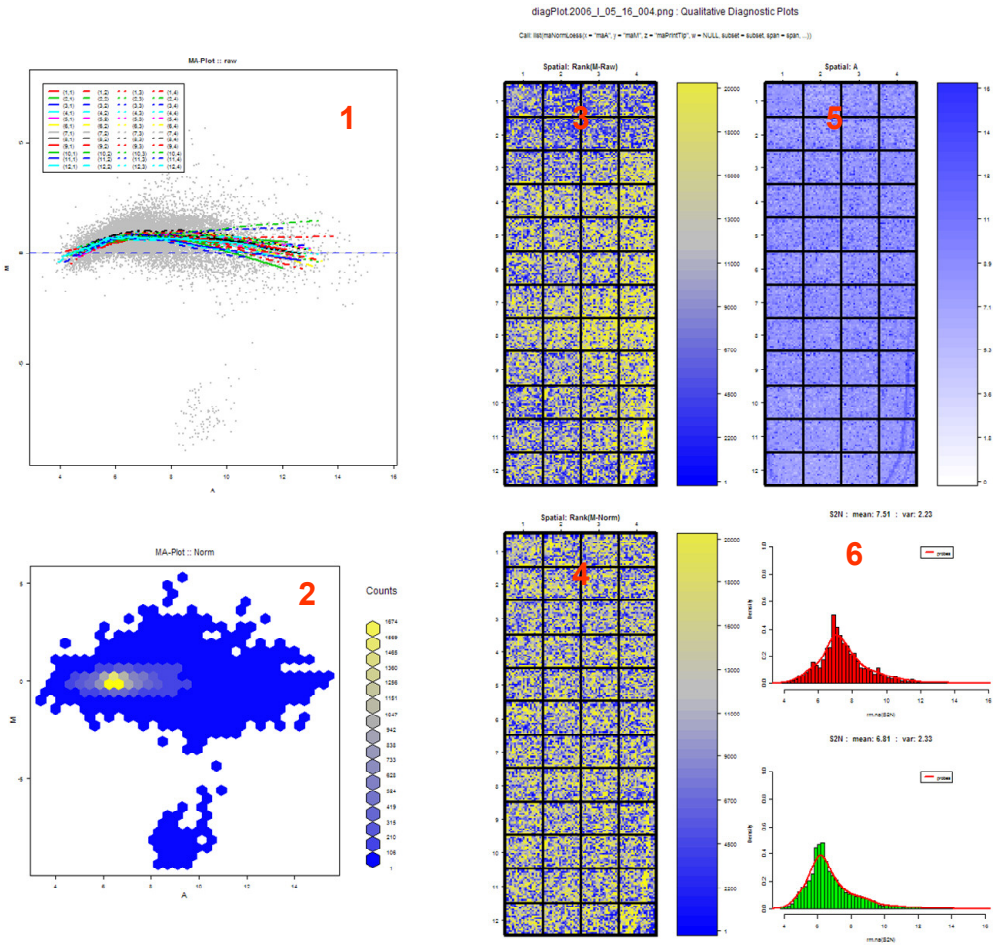


**Figure 5.4:** Diagnostic Plots for microarray quality control representing a microarray of good quality.

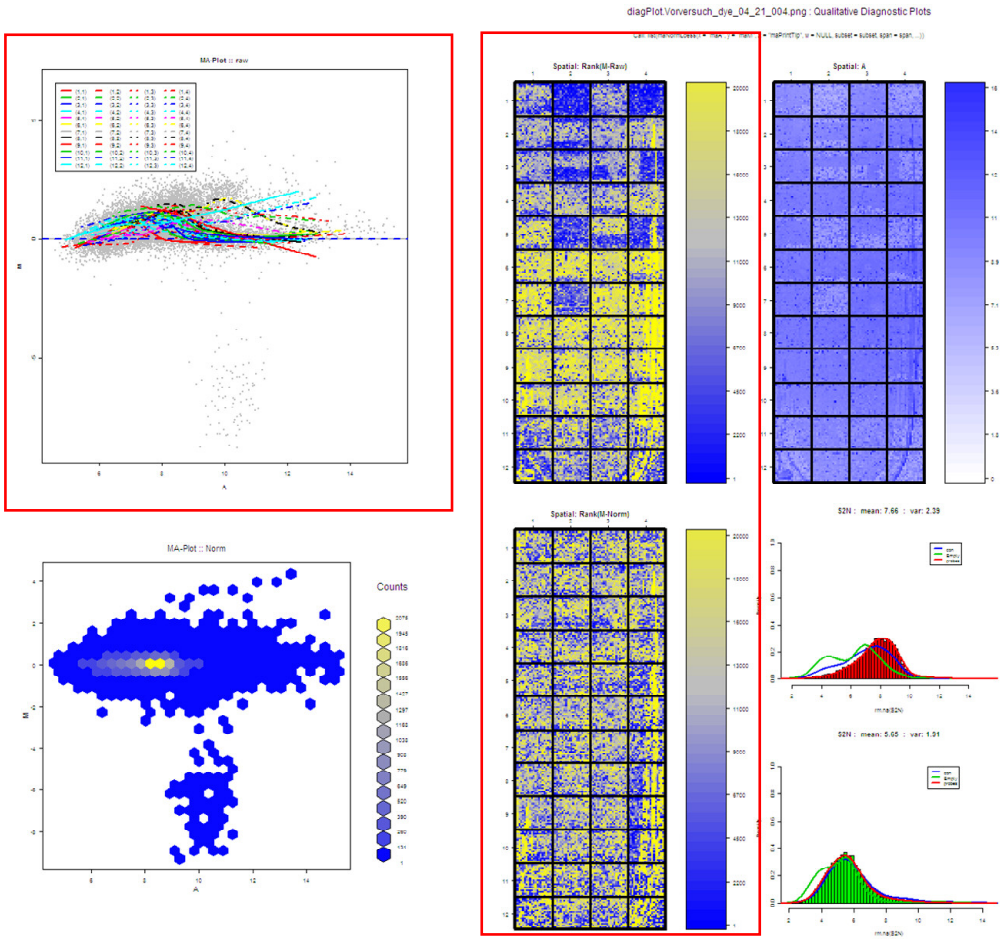


**Figure 5.5:** Diagnostic Plots for microarray quality control representing a microarray with uneven hybridization (3, 4, 1) which cannot be fully removed by normalization.

## 5.3 From Raw Data to Results

### 5.3.1 Data Preprocessing

After quality control, normalization is usually conducted to remove spatial effects within a slide and adjust/balance possible differences in signal intensity between individual arrays (Figure 5.6).

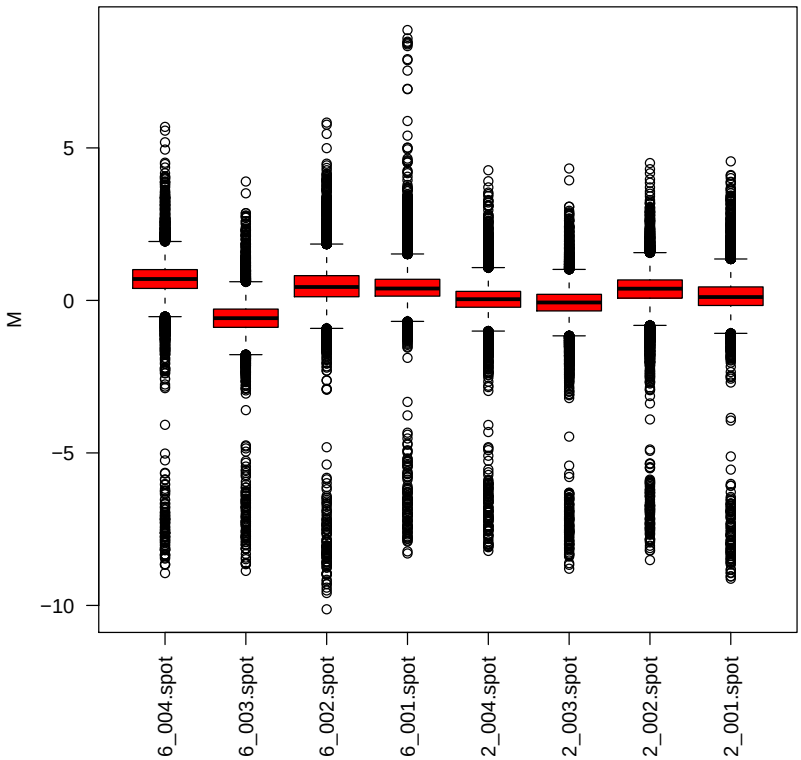


**Figure 5.6:** Boxplot of log-ratios (M) across eight arrays. Using this boxplot the need for between-array normalization can be assessed by displaying scale differences among arrays.

This can be done by applying separate normalization methods such as "print-tip loess" normalization for within-array normalization and, for example quantile normalization for between-array normalization, if necessary. However, there is a "bias-variance trade-off". The more complex the normalization procedure the more technical variation can be removed but, on the other hand, the more biological signal might get lost (Yang and Paquet, 2005). Therefore, in this study the normalization method "variance stabilization normalization" (vsn) was applied which is a mild normalization procedure, also referred to as "separate-channel normalization", that covers both within- and between-array normalization (Huber,

2008; Yang and Paquet, 2005). After normalization, genes which were not expected to be differentially expressed such as controls, blanks, and empty spots were filtered out as recommended/described by Hahne et al. (2008) to increase the power of the subsequent statistical analysis.

### 5.3.2 Statistical Analysis and Annotation of Results

Statistical analysis of the data was carried out using the "robust fit" function of Limma (Smyth, 2005) which fits multiple linear models and is, as the name indicates, more robust against outliers than the default function "lmFit". Then contrasts were made and moderated t-statistics computed using the empirical Bayes (eBayes) function. The data was then corrected for multiple testing using the method of Benjamini and Hochberg (1995). This method controls the false discovery rate, i.e. the expected proportion of false discoveries among the rejected hypotheses. As an output, differentially expressed genes with an adjusted p-value $<0.05$ were selected (see table A.6 on page 125 for a sample results table as obtained from R). In the case of an array containing 18545 unigenes, as it was used here, an "un-adjusted" p-value $<0.05$ would mean that by chance alone about 927 genes would be identified as differentially expressed. Using a method for multiple testing correction this number can be strongly reduced by lowering the actual p-value for each gene to about 0.05/18545 in the respective case (Sachs, 1999). Furthermore, the B-statistic was considered as a means to evaluate the reliability of the data. The B-statistic is the log-odds that a gene is differentially expressed. In the case of B=2, for example, the odds of differential expression is $e^2 = 7.38$. This means, the probability that the gene is differentially expressed is $7.38/(1 + 7.38) = 0.88$ or 88%. A B-statistic of 0 would mean that there is a 50-50 chance of the gene to be differentially expressed (Smyth et al., 2008).

Finally, all genes differentially expressed at adj. p $<0.05$ were annotated using four databases (est2uni, nr, swissprot, and tair) and assigned to functional groups according to Gene Ontology. For an overview of microarray image and data analysis see Figure 5.7.

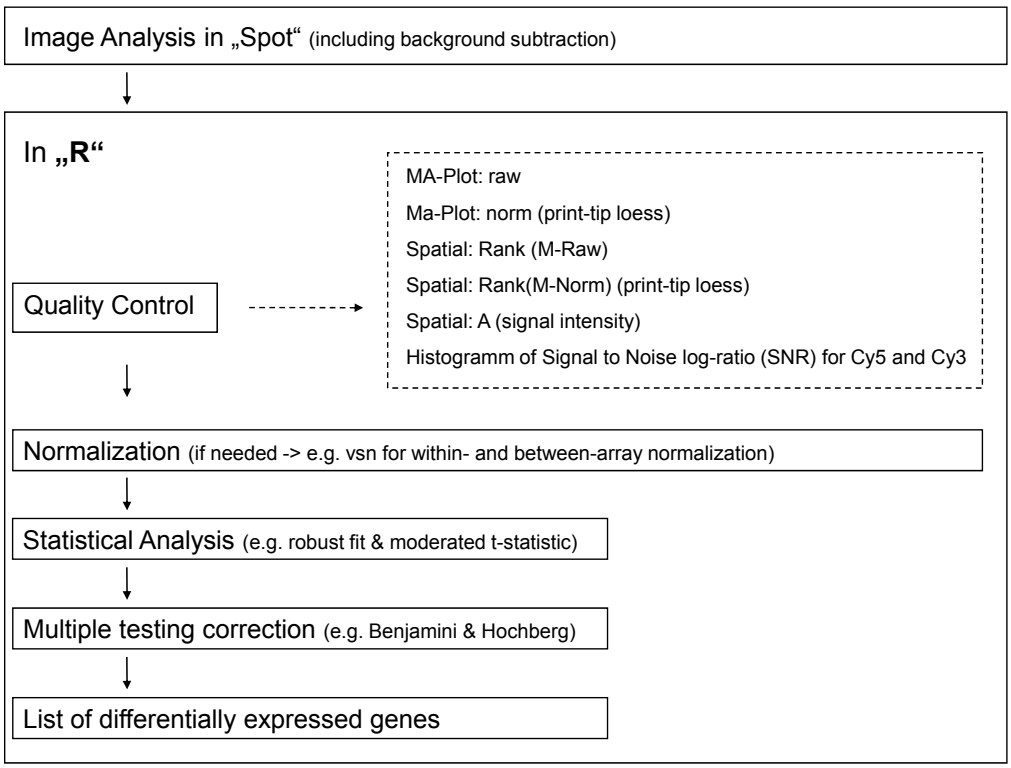


**Figure 5.7:** Overview of microarray analysis starting with image analysis followed by quality control, normalization, statistical analysis and multiple testing correction resulting in a list of differentially expressed genes.

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# CHAPTER 6

## Simultaneous measurement of proline and related compounds in oak leaves by high-performance ligand-exchange chromatography and electrospray ionization mass spectrometry for environmental stress studies

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## Abstract

A mass spectrometer was coupled to high-performance-ligand-exchange liquid chromatography (HPLEC) for simultaneous analysis of stress associated solutes such as proline, hydroxyproline, methylproline, glycine betaine and trigonelline extracted from leaves of drought stressed oaks and an internal standard namely N-acetylproline. Methanol/chloroform/water extracts were analyzed using an Aminex HPX-87C column and specifically quantified by the positive ion mode of an electrospray ionisation-mass spectrometry (ESI-MS) in single ion monitoring (SIM) mode. The recovery of N-acetyl proline added to oak leaf extracts ranged from 85.2% to 122.% for an intra-day study. Standard calibration curves showed good linearity in the measured range from 0.3125 to 10 µmol/l with the lowest correlation coefficient of 0.99961 for trigonelline. The advantages of this alternative procedure, compared to previously published methods using fluorescence or amperometric detections, are the simultaneous and direct detection of osmoprotectants in a single chromatographic run, a minimal sample preparation, a good specificity and reduced limits of quantification, ranging from 0.1 to 0.6 µmol/l. Fifty-six days of water deficit exposure resulted in increased foliar free proline levels (2.4-fold, $P < 0.001$, 155 µmol/g FW) and glycine betaine contents (2.5-fold, $P < 0.05$, 175 µmol/g FW) of drought stressed oak compared to control.

## 6.1 Introduction

Unfavourable environmental conditions for growth and development of plants are defined as environmental stresses, which are divided into biotic stress and abiotic stress. Living organisms such as insects, pathogenic fungi, bacteria, and viruses can be responsible for biotic stress that can harm plants. Abiotic stress is caused by nonliving environmental factors such as, for instance, unfavourable conditions of water, temperature, salt, light, nutrition, that can have disastrous effects on plants. Abiotic stress such as drought poses serious threat to global agriculture production and food security. Drought stress can directly or indirectly affect physiological status of plants by altering metabolism, growth and development (Bray, 2001). Active or passive accumulation of osmoprotectants, like proline (P) (Paleg and Aspinall, 1981), hydroxyproline (HP), methylproline (MP) (Naidu et al., 1987), trigonelline (T) and glycine betaine (GB) (Ashraf and Foolad, 2007; Jones and Storey, 1981; Rhodes and Hanson, 1993; Verbruggen and Hermans, 2008), is an important adaptation mechanism for plants in response to osmotic stresses including water deficit and high salinity levels. Thus, many organisms synthesize solutes that help in retaining

water within cells or protect organelles from dehydration. Glycine betaine presents both properties (Incharoensakdi et al., 1986; Robinson and Jones, 1986). L-proline, proline analogues such as N-methyl-L-proline and L-4-hydroxyproline and glycine betaine, betaine analogues such as trigonelline (nicotinic acid N-methyl betaine) act as osmolytes in higher plants. Some of the corresponding biosynthetic pathways are shown in Fig. 6.1 (Brouquisse et al., 1989; Essery et al., 1962; Kuttan and Radhakrishnan, 1973; Weretilnyk and Hanson, 1990). The role of osmoprotectants is to be accumulated in response to water or saline stress (Hanson et al., 1991; Rathinasabapathi et al., 2001) in order to keep an osmotic balance with the environment (Bray et al., 2000; Robinson and Jones, 1986) and to preserve the quaternary structure of complex proteins (Papageorgiou and Murata, 1995). Thus, the monitoring of these osmoprotectants is of prime importance to study the response of plant metabolism during abiotic stress events.

Historically, glycine betaine content was carried out according to previously published method (Grieve and Grattan, 1983) and proline content was quantified by the ninhydrin-colorimetric method (Bates et al., 1973; Singh et al., 1973), which is not selective since ninhydrin reacts with a free alpha-amino group, the latter group being contained in all amino acids, peptides, or proteins. Moreover, accuracy of quantification by ninhydrin can be distorted by plant extracts with high carbohydrate content (Magné and Larher, 1992).

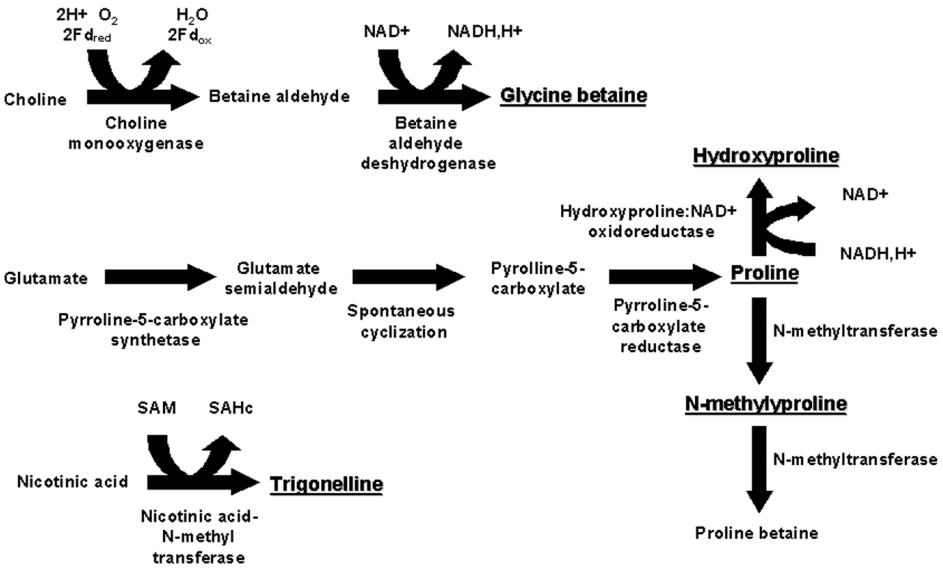


**Figure 6.1:** Scheme of biosynthetic pathways of nitrogenous osmoprotectants (proline, hydroxyproline, N-methylproline, glycine betaine and trigonelline) in plants (SAM, S-adenosylmethionine; SAHc, S-adenosylhomocysteine; underlined compounds are analysed in this study).

In addition to this colorimetric method, several chromatographic methods are frequently used. However, none of these methods has the advantage to quantify all compounds of interest in a single run (quantification of both proline and hydroxyproline (Bouchereau et al., 1999; DeMeglio and Svanberg, 1996; Hanko and Rohrer, 2004; Inoue et al., 1999; Mayadunne et al., 2005; Sormiachi et al., 1995; Woo and Ahan, 1996), glycine betaine (Larher et al., 2003; Rhodes et al., 1987) and glycine betaine and trigonelline (de Zwart et al., 2003)). Moreover, sample preparation is not always straightforward in many cases with steps of chemical conversion (Wu, 1993) or precolumn derivatization (Bouchereau et al., 1999; Rhodes et al., 1987; Sormiachi et al., 1995; Woo and Ahan, 1996; de Zwart et al., 2003) coupled to an additional SPE step (Inoue et al., 1999; Mayadunne et al., 2005). Due to the absence of natural chromo- or fluorophore groups, only a limited number of detectors can be suitable for these osmoprotectants. After derivatization, Diode Array Detector (DAD) (Bouchereau et al., 1999; Larher et al., 2003; Sormiachi et al., 1995; Woo and Ahan, 1996; de Zwart et al., 2003) or Fluorescence Detector (FLD) can be used, the latter allowing a limit of detection down to 1 fmol (Inoue et al., 1999; Sultana et al., 2001; Wu, 1993). Pulsed Amperometric Detection (PAD) can provide an effective detection without derivatization, but interferences can be noticed when samples have a high sugar content (Hanko and Rohrer, 2004). Mass spectrometry (MS) (Mayadunne et al., 2005; Rhodes et al., 1987) and autoradiography (DeMeglio and Svanberg, 1996) can also be used to detect underivatized osmoprotectants. The chromatographic separation of these osmoprotectants can be performed by reverse phase chromatography (Bouchereau et al., 1999; Inoue et al., 1999; Larher et al., 2003; Sormiachi et al., 1995; Woo and Ahan, 1996; Wu, 1993; de Zwart et al., 2003), anion exchange chromatography (Hanko and Rohrer, 2004), by two-dimensional GC (GC X GC) (Mayadunne et al., 2005), thin layer chromatography (DeMeglio and Svanberg, 1996), by capillary electrophoresis (Zhang et al., 2002) or ligand exchange chromatography (Rosset et al., 1991). However, because of the low sensitivity (50 to 1000 pmol) obtained by UV detection at 195 nm (Naidu, 1998), and the extensive preparation needed (derivatization and SPE) for RP-HPLC-FLD or DAD, other new alternative methods were developed namely a high performance anion exchange chromatography (HPAEC) with PAD method and an isocratic high-performance ligand exchange chromatography (HPLEC) with MS method, which was finally selected. This paper describes this new one-step, reliable, specific, selective and sensitive method for the separation and simultaneous quantification of proline, hydroxyproline, methylproline, glycine betaine, trigonelline and N-acetylproline as internal standard (IS).

## 6.2 Experimental

### 6.2.1 Chemicals and reagents

Milli-Q water, 18.2 MΩ-cm resistivity, was used for all eluent and standard preparations. Analytical reagent grade methanol and chloroform from VWR International (Leuven, Belgium) were used. 50% (w/w) sodium hydroxide, sodium acetate, anhydrous and disodium calcium salt-EDTA ($CaNa_2EDTA$) were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA) for preparation of HPLC mobile phases. All compounds of interest: L-Proline, Betaine Hydrochloride, Trigonelline Hydrochloride, N-Methyl-L-Proline, L-4-Hydroxyproline and N-acetyl-DL-proline were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA) for preparation of working standard solutions.

### 6.2.2 Preparation of plant material

For this experiment, plants from a five-year-old oak clone P28 (*Quercus robur* L.), vegetatively propagated via somatic embryogenesis were used (Prewein et al., 2004). The plants were grown in peat moss (Einheitserde, Frux ED 63) in 20 l plastic containers in a greenhouse where air temperature (ambient) and humidity are regularly recorded. NPK long-term fertilizer was added after the end of the first growing season. The volumetric soil moisture content was measured with a ThetaProbe ML2x FD-Probe (Delta-T Devices, Cambridge, UK) usually three times a week. Soil moisture was measured at 10 cm and 15 cm soil depth. For the experiment, a control (14 five-year-old plants) and a drought stress group of oak plants (30 five-year-old plants) were set up in a randomised block design. At time 0 (April), all trees were watered up to the saturation point of the soil. The control group was then regularly watered up to the saturation point of the soil while the drought stress group was exposed to repeated cycles of water stress (about 30-15% soil moisture). Leaf samples were taken three times from all trees. For proline analysis five plants per treatment were selected. Control leaves were sampled when the soil moisture was higher than 40% whereas sampling of drought stress exposed leaves was carried out when the soil moisture was lower than 24% (Average soil moisture for selected trees was 20.5%). Sampling times were further scheduled by leaf development stage. At time I (after 3 days of drought exposure, average soil moisture for selected drought stressed trees was 20.5%) and at time II (after 8 days of drought exposure, average soil moisture for selected drought stressed trees was 19.3%), fully developed leaves of the first flush

(May) were sampled. At time III (average soil moisture for selected drought stressed trees was 16.1%), fully developed leaves of the second flush were taken (from newly developed shoots). Control plants were sampled after 35 days, drought stressed plants 56 days after start of the drought stress treatment. In order to keep the same development stage for both control and drought stressed plants, the latter were sampled three weeks later because their growth/second flush was delayed by three to four weeks. From each tree, four leaves were sampled and immediately put into liquid nitrogen and then stored at -80 ℃prior to HPLC analysis.

### 6.2.3 Sample preparation

Pooled plant material from four leaves per tree was ground in a Retsch MM 200 automatic grinder (Retsch GmbH & Co. KG, Haan, Germany) with two metallic beads for 2.5 min at 30 Hz. Ground plant material was aliquoted into 100 mg (±10 mg) samples, lyophilised over night and stored at room temperature (until further processing). One mL of ice-cold methanol/chloroform/water (60:25:15, v/v/v) was immediately added to the powder. After vortexing and shaking for 30 min at 4 ℃with an Eppendorf Thermomixer Comfort (Eppendorf, Hamburg, Germany) at 1400 rpm, samples were centrifuged at 10000 g for 10 min at 4 °C. The clear upper methanol-water phase was collected and stored below 4 °C for the estimation of osmoprotectants and the remaining residue was extracted against 0.5 mL of ice-cold methanol/chloroform/water (60:25:15, v/v/v) (Naidu, 1998). The resulting supernatant was pooled with the first one and dried in a SpeedVac concentrator (Heto, Thermo Electron Corporation, Waltham, MA, USA). The final dried extract was re-suspended in 1 mL of milli-Q water, filtered through 0.45 µm Acrodisc PVDF syringe filter and diluted 2 X prior to HPLC analysis. In order to assess the recovery of the method, N-acetyl DL-proline was added as internal standard (IS) to the extraction mixture at two different final concentrations of 0.01 mM and 1 mM.

### 6.2.4 Sample analysis by HPAEC-PAD

HPAEC-PAD analyses were conducted on a Dionex HPLC ICS-2500/BioLC (Dionex Corp., Sunnyvale, CA, USA) with an AS50 autosampler, a GS50 gradient pump and an ED50 electrochemical detector. The analytical column was a Dionex Carbopac PA-100 (4 x 250 mm) kept at 30 ℃. After optimization, selected eluents were pure water (A), 500 mM NaOH (B) and NaOH 100 mM + NaOAc 500 mM (C). The gradient program was as follows: 50% B (15 min), 50% B to 100% C (2.5 min), 100% C (5 min) 100% C to 50% B

(2.5 min) and column equilibration at 50% B during 5 min. Total run time was 30 min. The injection volume for all samples was 25 µL.
The PAD detection was achieved with a gold working electrode and an Ag/AgCl reference electrode, with a data collection rate of 1 Hz. The potential was set to 0.13V during 0.04 s, 0.33V during 0.17 s, 0.55V during 0.25 s, 0.33V during 0.1 s (integration between 0.21 and 0.56 s), -1.67V during 20 ms, 0.93V during 10 ms and 0.13V during 10 ms.
Proline, glycine betaine and analogues were quantified using eight-points calibration curves with in-house standard solutions, ranging from 5 to 200 µmol/l; every 10 injections, a check standard solution was used to confirm the calibration of the system.

### 6.2.5 Sample analysis by HPLEC-MS

Isocratic HPLEC-MS experiments were performed on a Dionex BioLC chromatograph, with an AS50 autosampler, a GS50 gradient pump (Dionex Corp., Sunnyvale, CA, USA), an external column heater (Bio-Rad, Hercules, CA, USA) and a Finnigan MSQ quadrupole spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The mobile phase was degassed (vacuum filtration through a Millipore HA 0.45 mm filter) Milli-Q water containing 5 mg/l disodium calcium salt-EDTA. Prior to initial use and after running about fifty samples, the column was regenerated by passing 500 mg/l disodium calcium salt-EDTA solution at a flow rate of 0.1 ml(/min overnight. The column was then washed with the mobile phase until baseline stabilisation. The analytical column was an Aminex HPX-87C column (300 ×7.8 mm i.d., 9-µm particle size) with a micro guard Carbo C (4.6 ×3 mm) cartridge (Bio-Rad, Hercules, CA, USA). The flow rate was 0.6 ml/min and the column was heated at 80 ℃. Total run time was 55 min. The injection volume for all samples was 20 µl. MS data were acquired on a quadrupole spectrometer with an electrospray interface (ESI). The probe temperature was fixed at 500 ℃with a needle voltage of 4.0 kV (Guignard et al., 2005). All chromatographic and MS data were interpreted using the softwares Chromeleon 6.5 and Xcalibur 1.3. All compounds of interest (Fig. 6.2) were quantified using seven-points calibration curves with in-house standard solutions, ranging from 0.625 to 40 µmol/l; every 10 injections, a check standard solution was used to confirm the calibration of the system. Biological replicates (five per time point) were injected several times at different days in order to check that the freeze and thaw process (-20 ℃) of samples and the storage in the autosampler (10 ℃) did not have an impact on the results (data not shown).

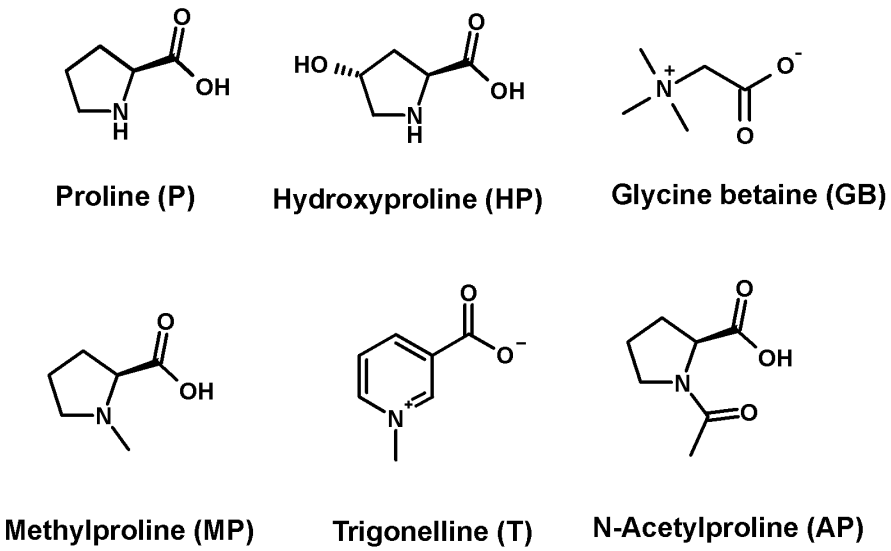


**Figure 6.2:** Chemical structures of proline, glycine betaine and analogues analysed in this study. N-acetylproline (AP) was used as internal standard (IS).

## 6.3 Results and discussion

### 6.3.1 Analysis of osmolytes by HPAEC-PAD

The aim of the present study was to develop an analytical method allowing the direct quantification (without derivatization) of the investigated nitrogenous osmoprotectants, with better detection limits than those obtained with UV detection (between 50 to 1000 pmol according to compounds) (Naidu, 1998). For that reason, we have first evaluated HPAEC-PAD as an alternative method. This technique is known for its high sensitivity and selectivity for carbohydrates and polyols (Guignard et al., 2005), but it may also be used for the analysis of nitrogen-containing compounds (Hanko and Rohrer, 2004) by simply adapting the anion-exchange stationary phase and the detection waveform. In our case, a Dionex CarboPac PA-100 was selected, and the oxidizing waveform was tuned to obtain the highest sensitivity for amino acids. This method allowed a significant decrease of the detection limits (20 µM for MP, 2 µM for P and HP) and a satisfactory chromatographic separation was obtained by gradient elution as shown in Fig. 6.3. However, the obtained sensitivity remained insufficient to detect all solutes in our samples. Especially, MP can hardly be quantified below 40 µM (data not shown). Moreover, the pulsed amperometric

cell was unable to detect QACs (T, GB), which do not appear to have a free electron pair on nitrogen (Fig. 6.2) and therefore cannot be further oxidized on the electrode surface. As these compounds may be present in plant extracts, this method was not selected for further development.
Nevertheless, HPAEC-PAD can be used as a routine method to quantify others osmoprotectants like carbohydrates and polyols (Guignard et al., 2005).

## 6.3.2 Analysis of osmolytes by HPLEC-MS

### 6.3.2.1 Chromatographic separation

Due to its high separation capacity for small and polar natural compounds like carbohydrates or amino acids, an Aminex HPX-87C analytical column was chosen. Usually, deionized water is the advised eluent for this stationary phase. However, as its ionic strength cannot be stable for long periods, the use of pure water as mobile phase for compounds of interest may result in poor reproducibility or unstable retention times. Furthermore, due to the high complexity of the sample matrix, a wide array of natural compounds (carbohydrates, polyols, pigments,...) may potentially be co-extracted with

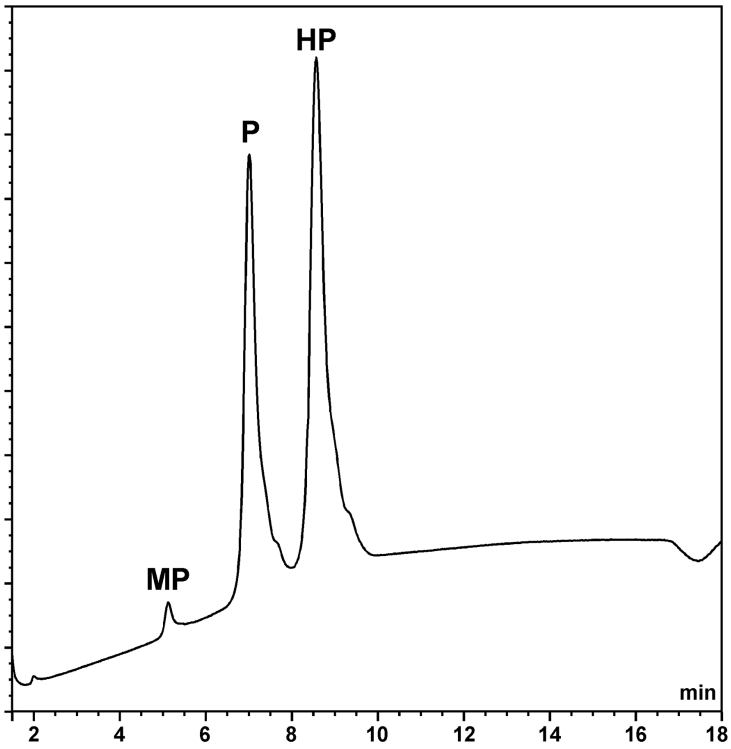


**Figure 6.3:** HPLEC-PAD chromatogram of nitrogenous osmoprotectants standard solution at 100 µM (MP: N-Methylproline; P: Proline; HP: Hydroxyproline).

proline, glycine betaine and analogues, affecting progressively the separation capacity of the column. Therefore, a mobile phase with a higher eluting strength ($CaNa_2EDTA$ aqueous solution) was preferred (Naidu, 1998). The molarity of the EDTA solution was chosen as a compromise between the ionic strength and the MS physical limits. Indeed, a high concentration in EDTA shortens the total run time and improves the peak shape, but it may also cause electrical shortcuts or salt deposits in the MS interface, which can induce a rapid pollution of the MS source and a drastic loss of sensitivity. Considering these different limiting factors, a 5 mg/L concentration, which can be considered as a maximal value to avoid MS troubles, was used for this analysis (Naidu, 1998). Even with this eluent, it has been noticed that, after about fifty runs, the adsorption of matrix compounds on the stationary phase led to a progressive degradation of its chromatographic properties. To avoid this, the column was regenerated each time after 30-50 sample injections with 500 mg/L $CaNa_2EDTA$, then stabilized with 5 mg/L $CaNa_2EDTA$ prior to quantitative analysis.

#### 6.3.2.2 MS parameters

Proline, glycine betaine and analogues were analysed in positive ESI mode. Each compound was detected and quantified on a dedicated SIM (Selected Ion Monitoring) channel corresponding to its $[M^+Na]^+$ adduct, which ensures a highly specific detection. The entrance cone voltage of each channel was optimized between 10 and 100V (10V steps) to obtain the highest sensitivity and the dwell times were adjusted to maximize the signal/noise ratios, without losing chromatographic resolution (>20 points per peak). The main MS parameters are reported in table 6.1, and resulting chromatograms for a standard mixture and sample extracts can be shown in Fig. 6.4

**Table 6.1:** Quantification parameters of proline, glycine betaine and analogues.

| Target compound | Retention time (min) | m/z[1] (amu) | Dwell time (s) |
|---|---|---|---|
| N-Acetylproline (AP) | 6.0 | 180 | 2.0 |
| Hydroxyproline (HP) | 25.0 | 154 | 1.0 |
| Glycine & betaine (GB) | 30.8 | 140 | 0.5 |
| Methylproline (MP) | 35.2 | 152 | 1.0 |
| Proline (P) | 40.6 | 138 | 1.0 |
| Trigonelline (T) | 49.8 | 160 | 2.0 |

[1] m/z ratio of the sodium adduct

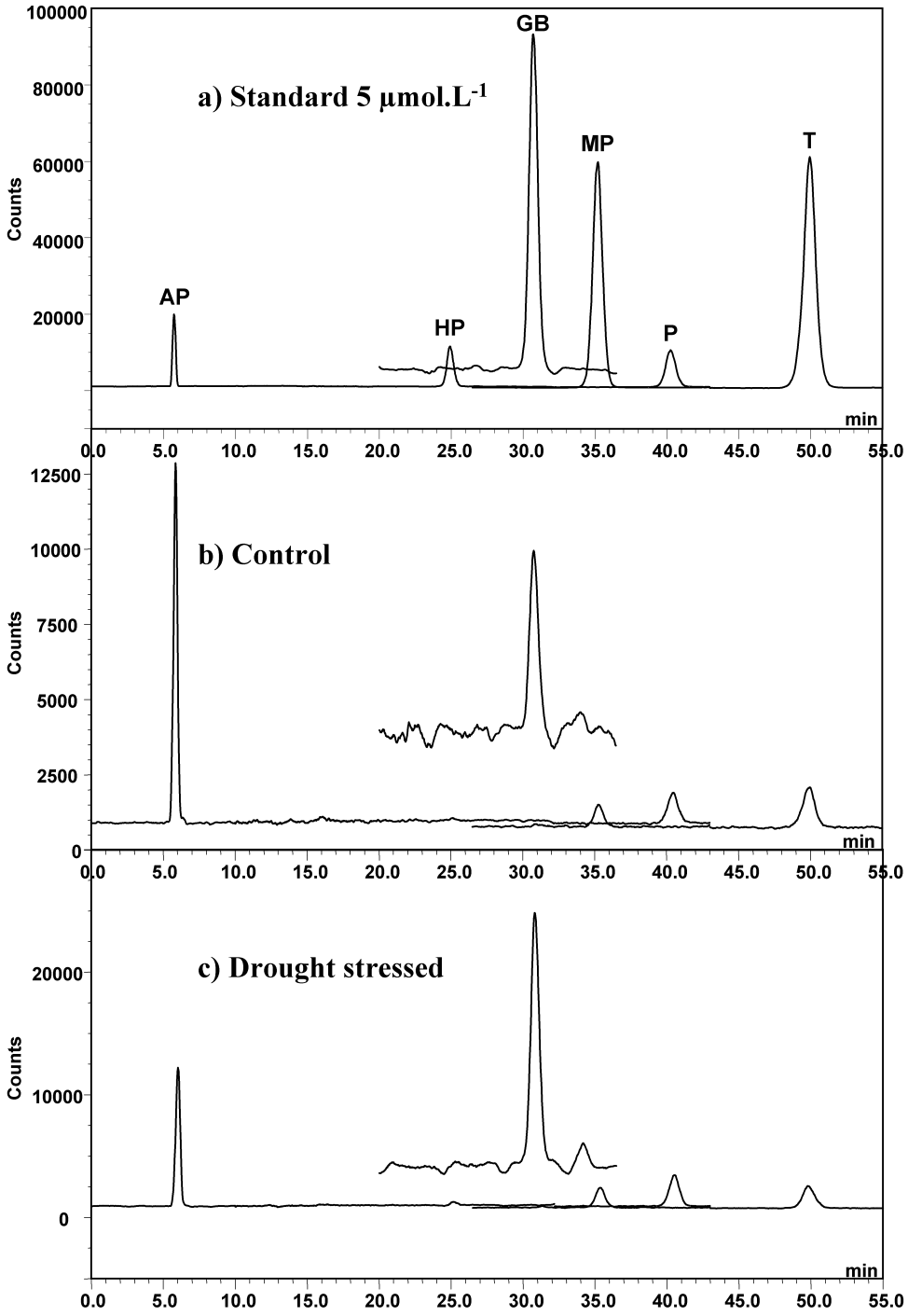


**Figure 6.4:** HPLEC-MS chromatograms of nitrogenous osmoprotectants (AP: N-Acetylproline; HP: Hydroxyproline; GB: Glycine Betaine; MP: N-Methylproline; P: Proline and T: Trigonelline). a) Standard solution at 5 µM, b) Control sample, c) Drought stressed sample.

#### 6.3.2.3 Sensitivity and linearity

Selectivity and sensitivity of MS detection in SIM mode allows quantification of very low amounts of osmolytes. The limits of quantification (Table 6.2), estimated as ten times the standard deviation of the noise, are comprised between 0.1 and 0.6 µmol/l, which allows the dilution of raw extracts before HPLC analysis, reducing by the same way the amount of interfering compounds from the matrix injected on the column. For all target compounds, the response of the MS detection follows a linear calibration curve between 0.3 and 10 µmol/l, with a correlation coefficient greater than 0.99 (see Table 6.2). All calibration curves were obtained from a mixture of all six compounds of interest. Moreover, in the case of highly concentrated compounds, the samples were diluted in order to stay in the linear calibration range.

Within-day recovery tests were carried out by adding various amounts (0.01 and 1 mM) of N-acetylproline to ten replicates. As shown in Table 6.3, the recoveries were 86.24- 116.68% for 1 mM and 103.15- 117.16% for 0.01 mM. Ideally, a significant improvement could be afforded by using an isotopically labeled (D or 13C) analogues as internal standards. However, each osmoprotectant is not available as deuterated compound or, if commercially available, is often too costly for routine analyses. On the whole, this new method allows a very specific and sensitive quantification of proline, glycine betaine and analogues. By using HPLEC in combination with single-quadrupole MS instead of classical UV detection, the detection limits were decreased by a factor of 1000. Nevertheless, pre-colum derivatisation with fluorescent labelling reagents coupled to RP-HPLC-FLD can give lower detection limits (down to fmol/injection) (Inoue et al., 1998; Varchola et al., 1999). Thus, this

**Table 6.2:** Calibration data (unweighted linear regression without offset) of target analytes.

| Analyte | N[1] | S[2] | I[3] | RSD[4] (%) | r[5] | Linear range (µM) | LoQ[6] (µM) |
|---|---|---|---|---|---|---|---|
| P | 8 | 594.18 | 0 | 1.1889 | 0.99991 | 0.3125-10 | 0.4 |
| MP | 8 | 3799.93 | 0 | 0.6487 | 0.99999 | 0.3125-10 | 0.1 |
| HP | 8 | 444.12 | 0 | 1.569 | 0.99994 | 0.3125-10 | 0.6 |
| GB | 8 | 4497.42 | 0 | 1.5431 | 0.99995 | 0.3125-10 | 0.3 |
| T | 8 | 4681.20 | 0 | 3.8945 | 0.99961 | 0.3125-10 | 0.1 |
| AP | 8 | 171.68 | 0 | 1.4569 | 0.99997 | 0.3125-10 | - |

[1]Number of calibration points [2]Slope [3]Intercept [4]Relative standard deviation [5]Corelation coefficient [6]Limit of quantification

sensitive technique makes it now possible to follow with a great accuracy the variations of endogenous nitrogenous osmoprotectants in plants submitted to abiotic stress in a single chromatographic run without extensive sample preparation.

### 6.3.3 Effect of drought exposure

Variations of endogenous nitrogenous osmoprotectants during water deficit in oak leaves have been assessed. According to Table 6.4, at time III, proline and glycine betaine were the major nitrogenous osmoprotectants in *Quercus robur* with an average content of 155.17 ±24.55 µmol/g FW and 174.61 ±74.13 µmol/g FW, respectively. At this last time, all nitrogenous osmoprotectants were significantly higher ($P < 0.05$) during drought exposure (soil moisture <17.4%) than in control conditions (soil moisture >40 %). In fact, significant early accumulation of trigonelline (time I) and proline (time II) and significant late (time III) accumulation of glycine betaine, methylproline and hydroxyproline were observed, and can be considered as consequences of a drought tolerance response with the increase of osmoprotectants from the nitrogen metabolism. Thus, after 56 days of drought stress exposure, *Quercus robur* showed a significant increase compared to control (Table 6.4) for these two major osmolytes: proline (2.4-fold, $P < 0.001$) and glycine betaine (2.5-fold, $P < 0.05$) By comparison with literature data, it can be noticed that the level of proline accumulation in plants is variable from species to species. Factually, higher accumulation of these two osmoprotectants was already observed in sugar beets leaves (*Beta vulgaris* L.) when exposed to an osmotic stress induced by PEG (11-fold increase compared to control, $P < 0.05$) (Gzik, 1996), in cotton plants leaves (*Gossypium hirsutum* L.) when exposed to a water deficit stress (49.9-fold increase compared to control, $P < 0.001$) (Showler, 2002) and also in young barley plants leaves (*Hordeum vulgare* ssp. *Vulgare*) when exposed to an osmotic stress induced by PEG (13-fold increase compared to control, $P < 0.05$) (Kocheva and Georgiev, 2008).

**Table 6.3:** Within-day recovery of N-acetyl proline added to oak leave extracts

| Content in sample (mM) | Added IS[a] (mM) | Recovery mean (%) (n=10) | SD (%) (n=10) |
|---|---|---|---|
| 0 | 1 | 101.46 | 15.222 |
| 0 | 0.01 | 110.16 | 7.004 |

[a]Amount of internal standard added to final extract (10 µl each)

**Table 6.4:** Effect of drought exposure on endogenous proline (P), glycine betaine (GB), N-methylproline (MP), hydroxyproline (HP) and trigonelline (T) levels in leaves of *Quercus robur*.

| | | P | | | GB | | | MP | | | HP | | | T | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Time | Treatment | Mean | SD | P | Mean | SD | P | Mean | SD | P | Mean | SD | P | Mean | SD | P |
| I | Control | 100.68 | 46.69 | | 18.27 | 2.51 | | 5.3 | 1.16 | | 6.24 | 2.51 | | 48.71 | 15.91 | |
| | Drought | 86.45 | 13.67 | 0.58 | 24.06 | 3.77 | 0.043 | 10.22 | 5.06 | 0.11 | 5.49 | 1.57 | 0.63 | 71.89 | 17.48 | 0.1 |
| II | Control | 163.89 | 110.59 | | 14.86 | 1.78 | | 4.24 | 0.79 | | 6.91 | 1.83 | | 24.69 | 10.94 | |
| | Drought | 279.51 | 156.18 | 0.27 | 18.52 | 2.08 | 0.037 | 7.27 | 3.00 | 0.099 | 6.42 | 1.6 | 0.7 | 40.70 | 14.72 | 0.13 |
| III | Control | 64.96 | 16.79 | | 68.24 | 28.16 | | 11.93 | 3.09 | | 8.17 | 2.37 | | 13.07 | 7.87 | |
| | Drought | 155.17 | 24.55 | <0.001 | 174.61 | 74.13 | 0.036 | 36.05 | 10.84 | 0.005 | 13.42 | 2.37 | 0.02 | 53.06 | 19.91 | 0.01 |

Each value is the mean (µmol/g FW) ±SD of measurements from oak plants (From each oak tree, a pool of four leaves per sampling was selected). At each sampling time, significance was calculated with a pairwise t-test (two-sided, alpha level = 0.05) with control/ drought as factors. All statistically significant differences between treatments were tested at the P <0.05 level (JMP IN 5.1).

## 6.4 Conclusion

Proline, glycine betaine and analogues as well as quaternary ammonium compounds like trigonelline are frequently involved in the plants' response to abiotic stresses. The classically and frequently used colorimetric and spectrophotometric methods: Ninhydrin reagent for free proline quantification (Bates et al., 1973) and cold KI-I2 for water soluble quaternary ammonium compounds (glycine betaine) (Grieve and Grattan, 1983) as well as UV detection approaches (Naidu, 1998) are not selective, respectively sensitive enough for the analysis of nitrogenous osmoprotectants in plant extracts. When analyzing complex mixtures, such as plant extracts or food, the main purpose is to keep the sample preparation as simple as possible. Indeed, multiple steps (pre-column derivatization, SPE, chemical conversion) introduce uncertainties in the accurate quantification of the analytes. Although other sensitive (down to fmol for RP-HPLC-FLD) and selective analytical methods for the determination of nitrogenous osmoprotectants determination have been developed over the years, we developed and proposed an alternative analytical method for a one-step quantification of five stress associated solutes, based on the coupling of an electrospray ionization mass spectrometer and high performance ligand-exchange chromatography without extensive preparation. This technique, which offers an excellent selectivity and low quantification limits (down to 2 pmol/injection for trigonelline and methylproline) for all endogenous nitrogenous osmoprotectants, was applied on *Quercus robur* leaves exposed to drought stress, in order to get more information on the pool sizes of these compounds. Assays of these compounds were correlated to both genomic (micro-array and real time RT-PCR) and proteomic approaches in order to define the possible role of these endogenous nitrogenous osmoprotectants during a drought stress and to study the impact of this stress as one of the causes of oak decline in Central Europe (Thomas et al., 2002). This integrative approach with both genomic and proteomic studies is still underway to complement our biochemical results (data not shown). At last, this reliable and sensitive HPLEC-ESI-MS method which simplifies more and more the sample preparation can be routinely used to quantify simultaneously endogenous nitrogenous osmoprotectants and opens wide possibilities as an additional tool for a metabolomic approach of different abiotic and biotic stresses in plants (Ashraf and Foolad, 2007; Schauer and Fernie, 2006).

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# CHAPTER 7

## Greenhouse

### 7.1 Experimental Setup

Five year old clonally propagated oak plants (*Quercus robur*) from tissue culture (clone P28 H9, figure 7.1) were subjected to controlled drought stress and subsequent rewatering in a three-year glasshouse experiment. Control (14 plants), drought stressed (15 plants), and rewatered oaks (15 plants) were set up in a randomised block design (figure 7.2).

**Figure 7.1:** Five year old plants of *Quercus robur* clone P28 H9 in the greenhouse.

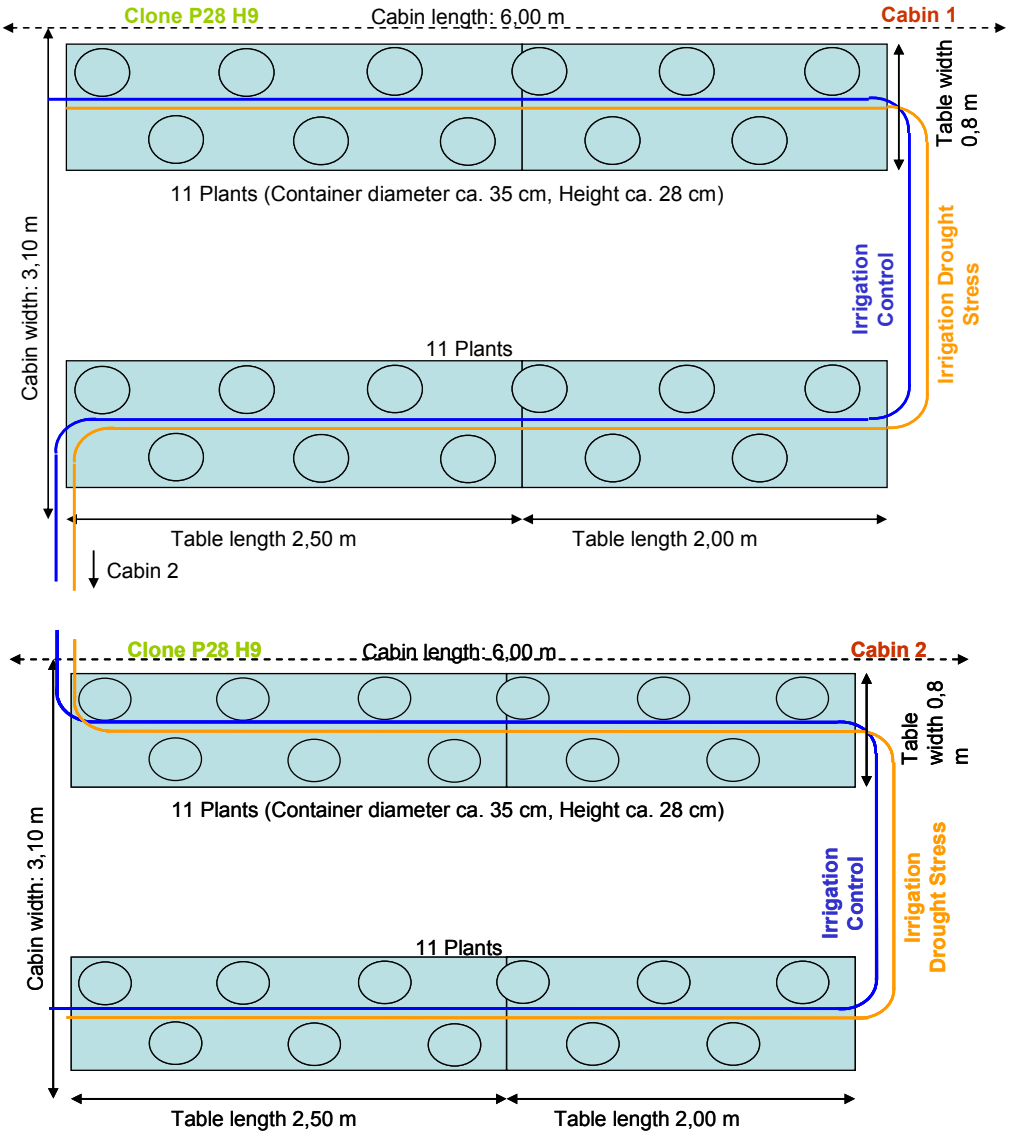


**Figure 7.2:** Experimental Setup of oak plants distributed over two greenhouse cabins including irrigation system for controls (and rewatered plants) and drought stressed oak plants.

## 7.2 Water Regime and Leaf Sampling

At the beginning of the growing season all plants were watered up to the saturation point of the soil (55-60%). The set of plants to be stressed were treated by withholding water. Soil water content for control plants (C) was kept at 30-50%. For the drought stress group (DS) soil moisture was set to 22-30% at the beginning of the first year. Later on, during summer soil moisture was decreased to 13% in order to achieve similar visual symptoms (leaf angle) (see Chapter 2 on page 21, Results). Usually, the difference between controls and drought stressed plants was 15-20% points. Rewatered plants (RW) were treated like controls with soil moisture content set to 30-50% (Figure 7.3). Leaves were sampled two times during the first flush and two times during the second flush. In the second year of the experiment (2007) an additional leaf sampling was included at the end of the growing season.

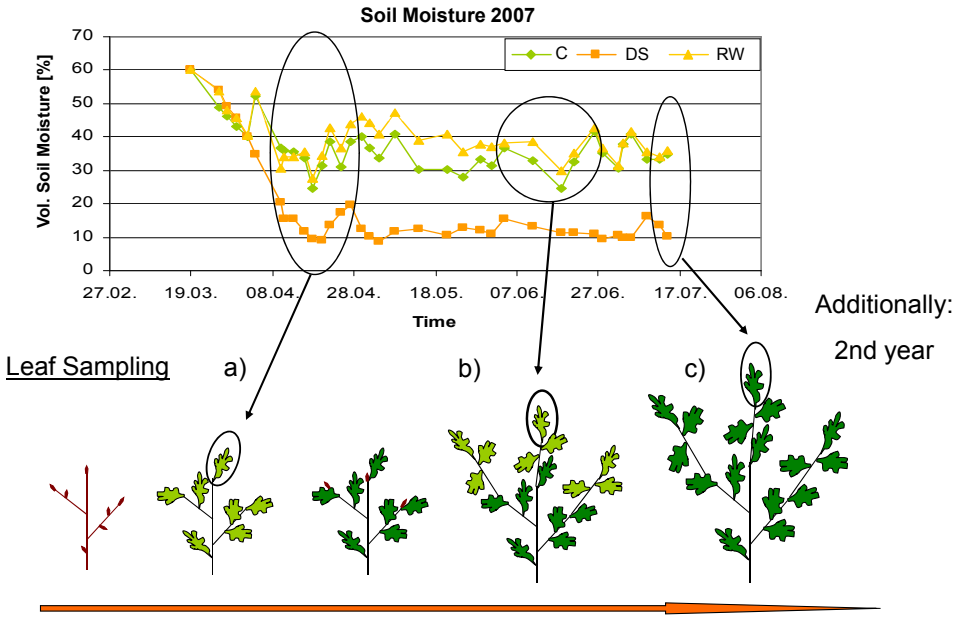


**Figure 7.3:** Volumetric soil moisture content (%) for controls (C), drought stressed (DS), and rewatered plants (RW) during the second year of the experiment (2007). Leaves were sampled during the a) first and b) second flush in the first (2006) and second year (2007) of the experiment. c) In 2007 an additional leaf sampling was included at the end of the growing season.

## 7.3 Irrigation and Soil Moisture Measurement

In the first year, water was applied manually, in the second year a combination of automatic and manual watering was used. See figure 7.4 for an illustration of the irrigation system in the greenhouse.

The volumetric soil moisture content was measured with a ThetaProbe ML2x FD-Probe (Delta-T Devices, Cambridge, UK) usually three times a week. Soil moisture was measured at 10 and 15 cm soil depth and at the soil surface (figure 7.5).

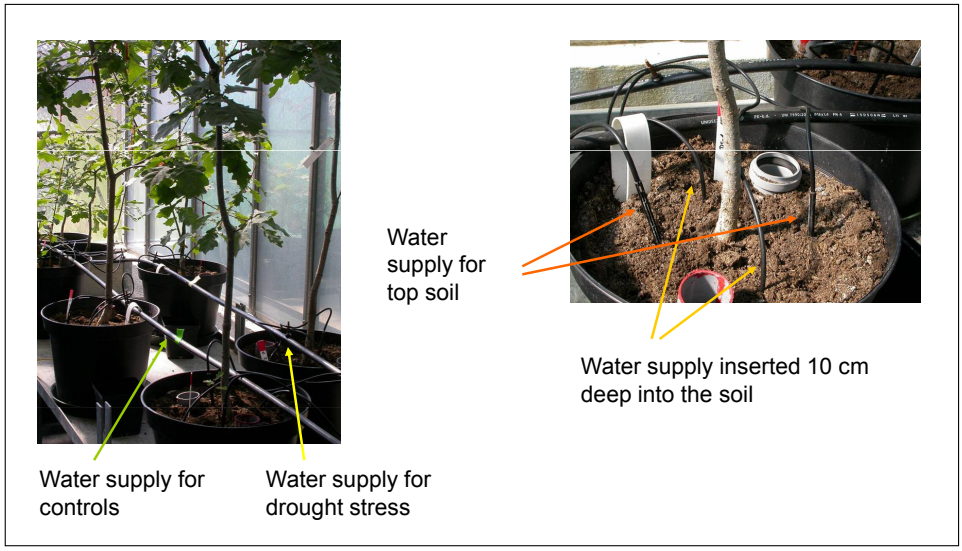


**Figure 7.4:** Irrigation system for controls and drought stressed plants. Water is applied at the soil surface and at 10 cm soil depth to facilitate homogenous/uniform water supply.

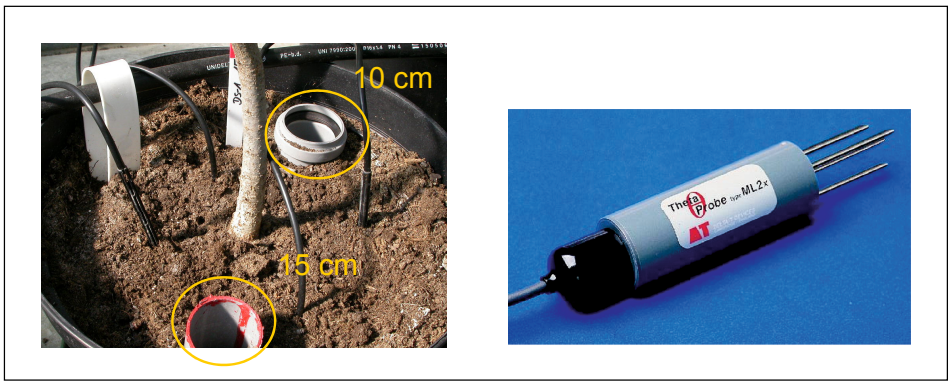


**Figure 7.5:** Measurement of volumetric soil moisture was conducted with a ThetaProbe ML2x FD-Probe at 10 cm and 15 cm soil depth as well as on the soil surface.

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# Part III

# Appendix

# APPENDIX A

## Supplemental Material

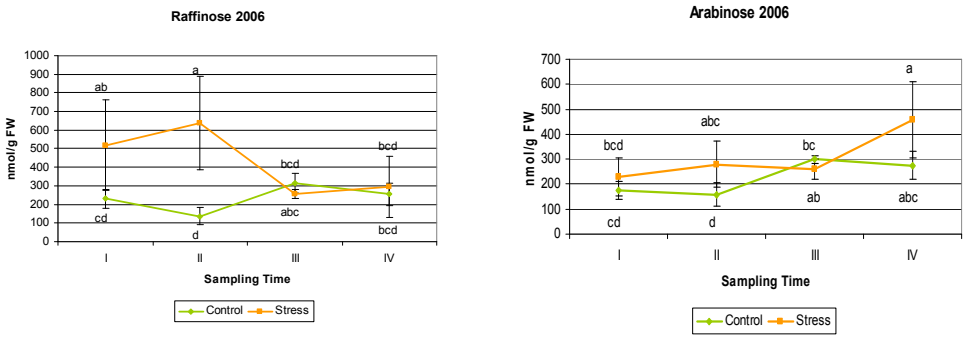


**Figure A.1:** Concentrations of Arabinose and Raffinose in oak leaves of drought stress treated plants and controls in 2006. Points not marked with the same letter are significantly different ($p < 0.05$).

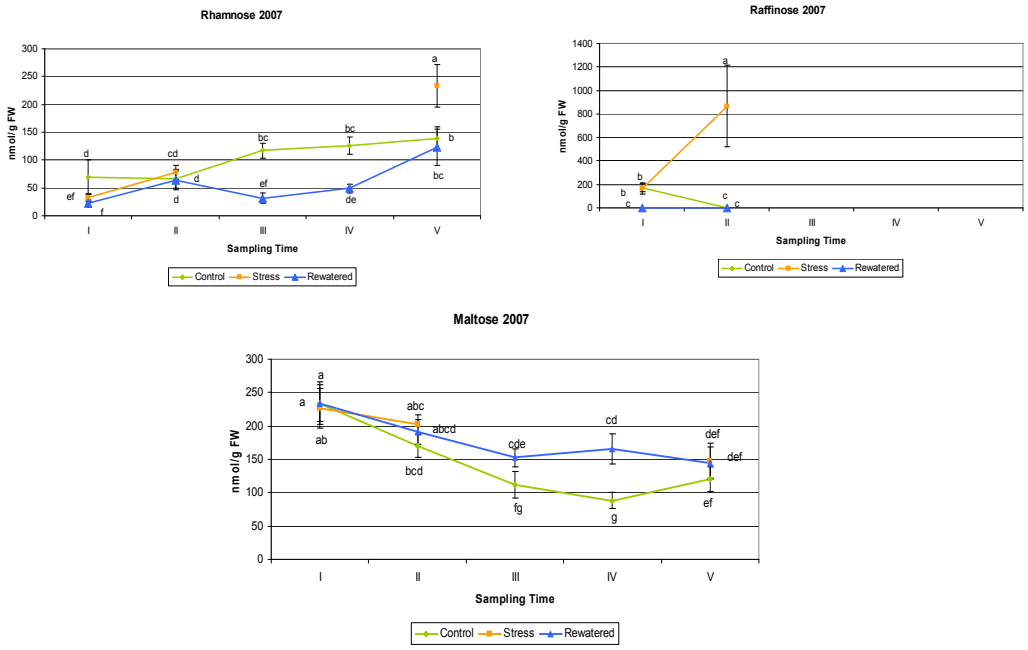


**Figure A.2:** Concentrations of Rhamnose, Raffinose and Maltose in oak leaves of drought stress treated plants, re-watered plants, and controls in 2007. Points not marked with the same letter are significantly different ($p < 0.05$).

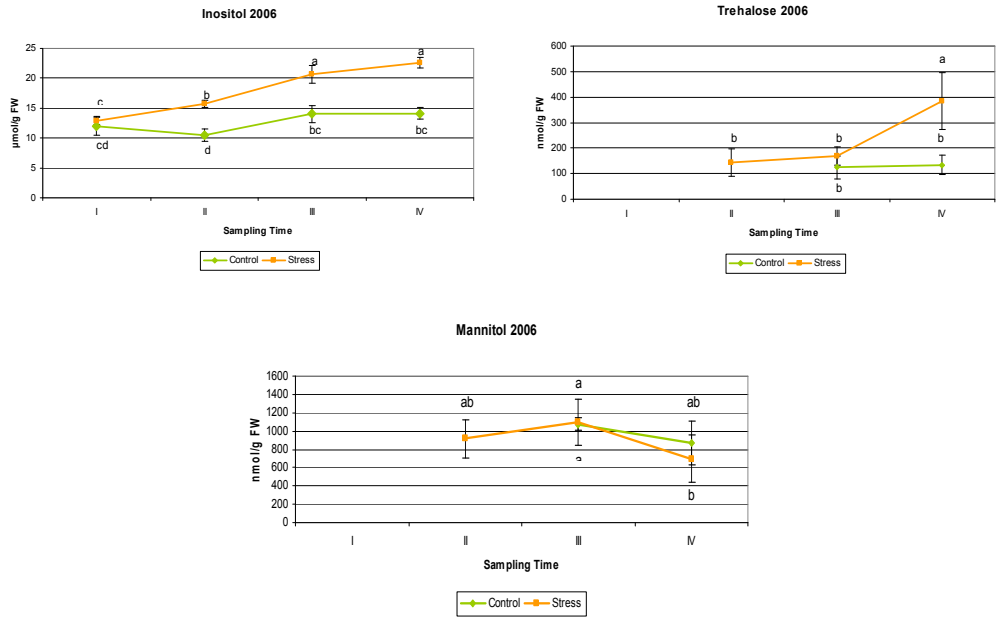


**Figure A.3:** Concentrations of Inositol, Trehalose, and Mannitol in oak leaves of drought stress treated plants and controls in 2006. Points not marked with the same letter are significantly different ($p < 0.05$).

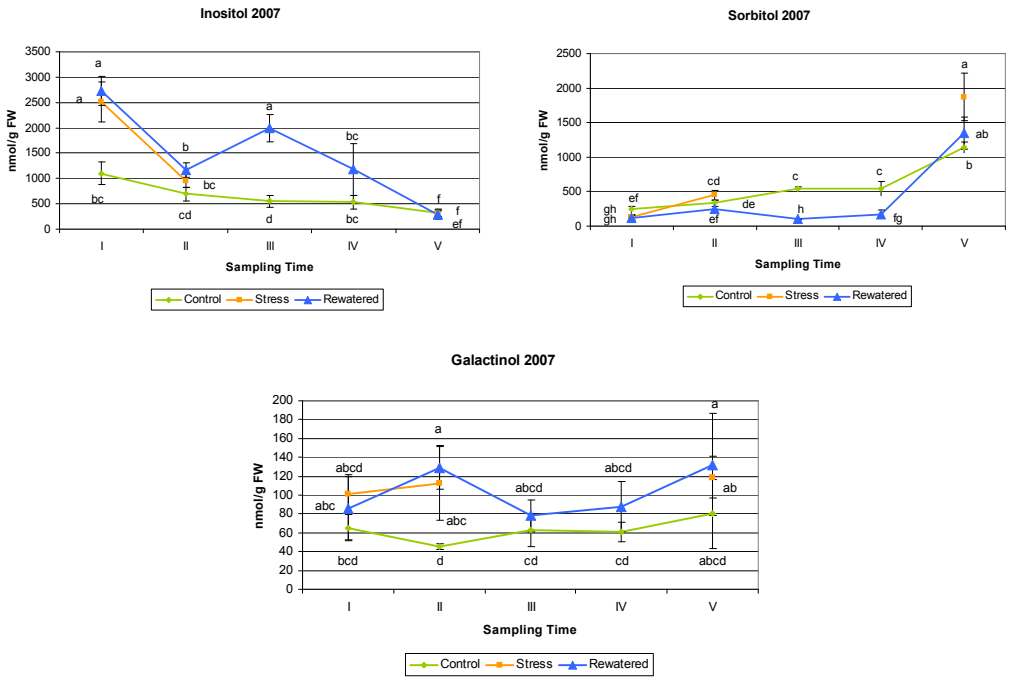


**Figure A.4:** Concentrations of Inositol, Sorbitol, and Galactinol in oak leaves of drought stress treated plants, re-watered plants, and controls in 2007. Points not marked with the same letter are significantly different ($p < 0.05$).

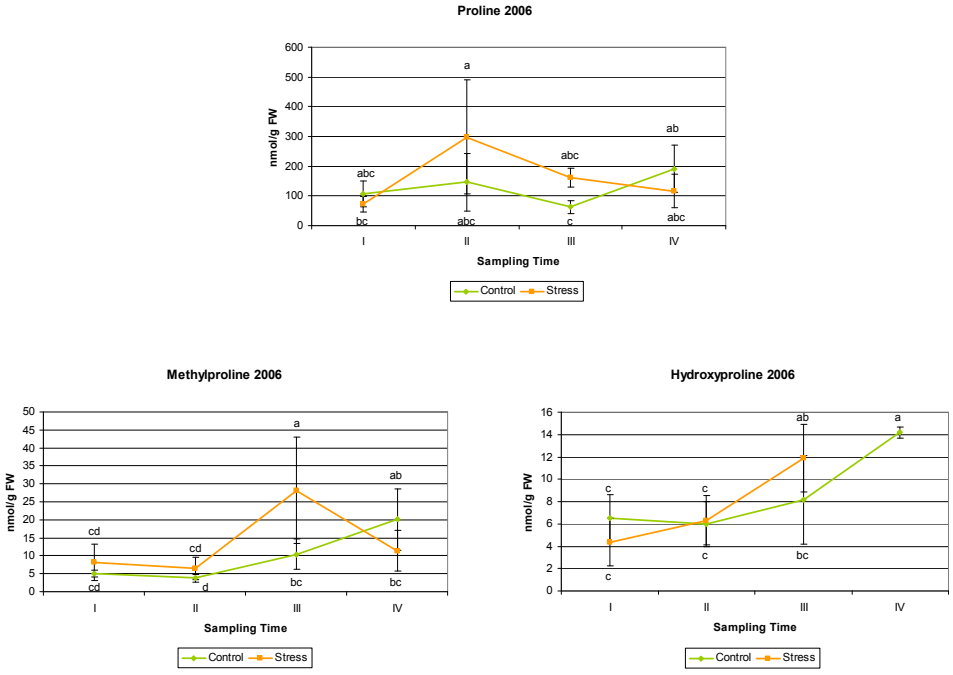


**Figure A.5:** Concentrations of Proline, Methyl-, and Hydroxyproline in oak leaves of drought stress treated plants and controls in 2006. Points not marked with the same letter are significantly different ($p < 0.05$).

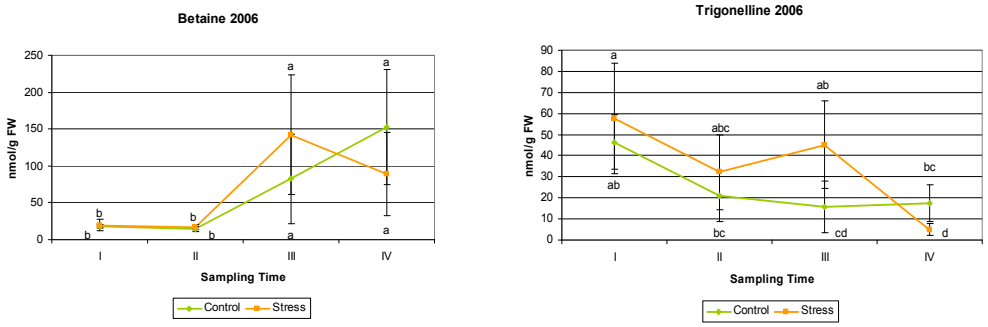


**Figure A.6:** Concentrations of Betain and Trigonelline in oak leaves of drought stress treated plants and controls in 2006. Points not marked with the same letter are significantly different ($p < 0.05$).

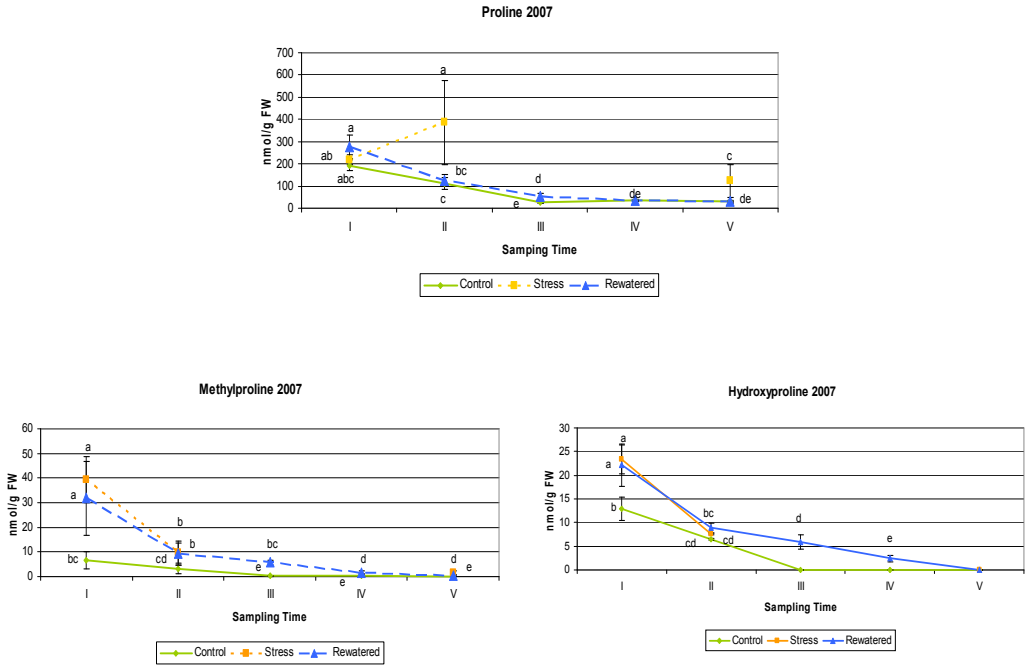


**Figure A.7:** Concentrations of Proline, Methyl-, and Hydroxyproline in oak leaves of drought stress treated plants, re-watered plants, and controls in 2007. Points not marked with the same letter are significantly different ($p < 0.05$).

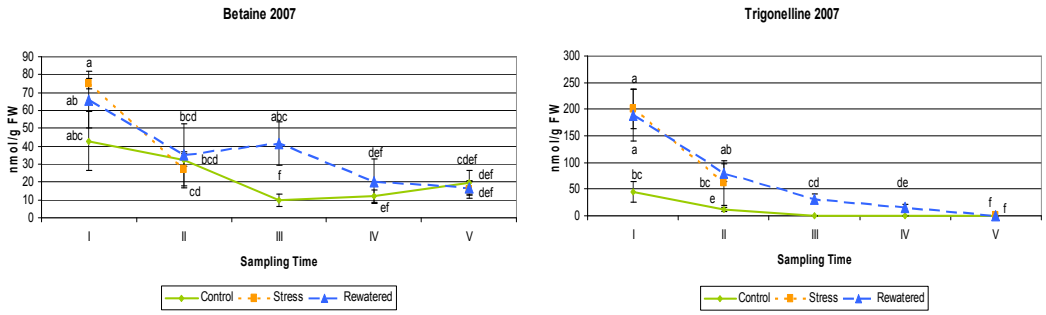


**Figure A.8:** Concentrations of Betain and Trigonelline in oak leaves of drought stress treated plants, re-watered plants, and controls in 2007. Points not marked with the same letter are significantly different ($p < 0.05$).

**Table A.1:** Differentially expressed genes (adjusted p-Values <0.05) allocated to functional groups at sampling times I-V in 2006 and I, II and V in 2007 for drought stressed plants (DS) and sampling times I, II, III, and V in 2007 for rewatered Plants (RW). For each gene accession number, annotation (est2uni) and log-fold change are given. Continued in table A.2.

| | | | 2006 DS | | | | 2007 DS | | | 2007 RW | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FG | Name | Annotation | I | II | III | IV | I | II | V | I | II | III | V |
| carbohydrate metabolism | LG0AAA19M15 | acidic endochitinase (CHIB1) | | -1,57 | | -2,19 | | -1,92 | | | | | |
| | LG0AAD28G23 | ATPGMP, PGM1, STF1, PGM PGM (PHOSPHOGLUCOMUTASE) | | 1,31 | | | | | | | | | |
| | LG0AAD2O3 | ATPHS2, PHS2 ATPHS2/PHS2 (ALPHA-GLUCAN PHOSPHORYLASE 2); phosphorylase/ transferase, transferring glycosyl groups | | | | | -1,38 | | | -1,27 | | | |
| | LG0AAD22O3 | ATTPPA ATTPPA (Arabidopsis thaliana trehalose-6-phosphate phosphatase); trehalose-phosphatase | | | | | | -1,45 | | | | | |
| | LG0AAD50L11 | Alpha-amylase precursor - Streptomyces thermoviolaceus | | 2,38 | 1,79 | | | 3,64 | 3,05 | | | | |
| | LG0AAB25G15 | BMY3 BMY3 (beta-amylase 3); beta-amylase | 1,22 | 1,66 | | | | | | | | | |
| | LG0AAD7M4 | BMY7, TR-BAMY BMY7/TR-BAMY (beta-amylase 7); beta-amylase | | | | | | 1,04 | | | | | |
| | LG0AAD36D20 | BMY7, TR-BAMY BMY7/TR-BAMY (beta-amylase 7); beta-amylase | | 1,46 | | | | | 2,21 | | | | |
| | LG0AAA20D4 | BMY7, TR-BAMY BMY7/TR-BAMY (beta-amylase 7); beta-amylase | | | | | | | | | | -2,19 | |
| | LG0AAD7O22 | glycosyl hydrolase family 1 protein | | | -1,02 | | | | | | | | |
| | LG0AAA4E21 | ISA1, ATISA1, ARA1 ARA1 (ARABINOSE SENSITIVE 1); ATP binding / galactokinase | | | | | | -1,23 | | | | | |
| | LG0AAA20P10 | MDH MDH (malate dehydrogenase); malate dehydrogenase | | | | | -1,16 | | | | | | |
| | LG0AAC6J14 | ribulose-phosphate 3-epimerase, cytosolic, putative / pentose-5-phosphate 3-epimerase, putative | | | | | | | | | 0,97 | | |
| | LG0AAD27N24 | starch synthase, putative | | | | | -1,39 | | | | | | |
| | LG0AAD21J11 | starch synthase, putative | | | | | | | | -1,29 | | | |
| | LG0AAB19I13 | SUS4 SUS4; UDP-glycosyltransferase/ sucrose synthase/ transferase, transferring glycosyl groups | | | | | | | | | | -2,77 | |
| growth, cellwall modification | LG0AAB16A4 | XTH9 XTH9 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9); hydrolase, acting on glycosyl bonds | | | | | | 1,16 | | | | | |
| | LG0AAB29P15 | XTH9 XTH9 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9); hydrolase, acting on glycosyl bonds | | | | | | 1,85 | | | 1,48 | | |
| | LG0AAC22P21 | XTH9 XTH9 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9); hydrolase, acting on glycosyl bonds | -0,87 | | | | | | | | | | |
| | LG0AAD9N21 | arabinogalactan-protein | | | | | | 1,95 | | | | | |
| | LG0AAD33K16 | putative arabinagalactan protein [Lotus japonicus] | | | | | | | 2,13 | | | | |
| | LG0AAC9G7 | fasciclin-like arabinogalactan protein 10 [Gossypium hirsutum] | | | | | 1,12 | | | | | | |
| | LG0AAD7A22 | ATPME1 ATPME1 (Arabidopsis thaliana pectin methylesterase 1); pectinesterase | | | | | | 1,33 | | | | | |
| | LG0AAA16C1 | ATPSK3, PSK1 PSK1 (PHYTOSULFOKINE 3 PRECURSOR); growth factor | | | | | | 1,51 | | | | | |
| | LG0AAC20N18 | BURP domain-containing protein / polygalacturonase, putative | | | | | | | | | | | -2,09 |
| | CU656890 | BURP domain-containing protein / polygalacturonase, putative | | -1,90 | | | | | | | -1,82 | | 1,46 |
| | LG0AAA3C11 | caffeoyl-CoA 3-O-methyltransferase, putative | | | | | | | | | | 2,92 | |
| | LG0AAB22L24 | CESA8, IRX1, ATCESA8, LEW2 CESA8 (CELLULASE SYNTHASE 8); cellulose synthase/ transferase, transferring glycosyl groups | | | | | | | | | | 3,77 | |
| | LG0AAB14O2 | chitinase | | | | | | | | | | 3,41 | |
| | LG0AAB6L15 | chitinase | | | | | | | | | | 3,23 | |
| | LG0AAC14E10 | cinnamoyl-CoA reductase-related | | | | | | | 1,69 | | | | |
| | LG0AAD29B22 | cinnamyl-alcohol dehydrogenase, putative | | | | | | | | 1,25 | | | |
| | LG0AAD30N15 | CYP84A1, FAH1 FAH1 (FERULATE-5-HYDROXYLASE 1); ferulate 5-hydroxylase | | | | | | | | | | 3,29 | |
| | LG0AAB19K10 | CYP84A1, FAH1 FAH1 (FERULATE-5-HYDROXYLASE 1); ferulate 5-hydroxylase | | | | | | | | | | 2,19 | |
| | LG0AAD9C10 | EXP8, ATEXP8, ATHEXP ALPHA 1.11, ATEXPA8 ATEXPA8 (ARABIDOPSIS THALIANA EXPANSIN A8) | | -2,78 | | | | -1,89 | | -1,69 | | 2,79 | |
| | LG0AAC14O14 | FLP1, YRE, WAX2 WAX2; catalytic | | 0,92 | | | | 1,37 | | | | | |
| | LG0AAB5I12 | LAC10 LAC10 (laccase 10); copper ion binding / oxidoreductase | | | | | | | | | | 2,90 | |
| | LG0AAD25G23 | Ligninase H2 precursor - Phanerochaete chrysosporium (White-rot fungus) (Sporotrichum pruinosum) | | | | | | -1,52 | | | | | |
| | CR628186 | Ndr family protein | | | | | | | | | | 1,70 | |
| | LG0AAD27O5 | OMT1, ATOMT1 ATOMT1 (O-METHYLTRANSFERASE 1) | | | | | | | | | | 2,51 | |
| | LG0AAD37D10 | pectinesterase family protein | | | | | | | | | | 1,84 | |
| | LG0AAD22F1 | plant cell wall protein SITFR88 [Lycopersicon esculentum] | | | | | | | | | | 2,10 | |
| | LG0AAD37H24 | proline-rich protein PRP1 [Castanea sativa] | | | | | | | | | -0,95 | | |
| chromosome org. | LG0AAD45O17 | DRM1 DRM1 (DORMANCY-ASSOCIATED PROTEIN 1) | 1,38 | | | | | | | | | | |
| | LG0AAB3P19 | DRM1 DRM1 (DORMANCY-ASSOCIATED PROTEIN 1) | 1,28 | | | | | | | | | | |
| | LG0AAA3B24 | histone H4 | | | | | | | | | -1,35 | | |
| | LG0AAC10F21 | histone H4 | | | -1,04 | | | | | | | | |
| | LG0AAC20F3 | histone H4 | | | | | | | | | -1,00 | | |
| | LG0AAB9B21 | NAP1;2 NAP1;2 (NUCLEOSOME ASSEMBLY PROTEIN1;2); DNA binding | | | | | | | | | | 2,55 | |
| | LG0AAD30K18 | SMC1, ATSMC1, TTN8 TTN8 (TITAN8); ATP binding | | | | | -1,50 | | | | | | |
| cytoskel. | LG0AAA13M9 | kinesin motor protein-related | | -1,18 | | | | | | | | | |
| | LG0AAB26F1 | TUA6 TUA6 (tubulin alpha-6 chiain) | | | | | | | | | | 2,18 | |
| | LG0AAC27D10 | TUA6 TUA6 (tubulin alpha-6 chiain) | | | | | | | | | | 2,86 | |
| | LG0AAB8N20 | TUA6 TUA6 (tubulin alpha-6 chiain) | | | | | | | | | | 3,41 | |
| | LG0AAD24D1 | ACT3 ACT3 (ACTIN 3); structural constituent of cytoskeleton | | | | | | | | | 1,94 | | |
| development | LG0AAC23J10 | ATCOP1, DET340, FUS1, EMB168, COP1 COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1) | | | | | | | | | | -2,44 | |
| | LG0AAD2I3 | ATLEA5, SAG21 SAG21 (SENESCENCE-ASSOCIATED GENE 21) | | | | | | | | | -1,48 | | |
| | LG0AAC4H12 | CCL CCL (CCR-LIKE) | | | | | | -2,60 | | | -1,95 | | |
| | LG0AAB13H3 | EAT1, EFE EFE (ethylene forming enzyme) | | | | | | -1,32 | | | | | |
| | LG0AAD44H15 | EAT1, EFE EFE (ethylene forming enzyme) | | -1,12 | | | | -1,07 | | | -0,87 | | |
| | LG0AAB17G13 | EAT1, EFE EFE (ethylene forming enzyme) | | | | | | -1,34 | | | | | |
| | LG0AAD13L6 | GASA1 GASA1 (GAST1 PROTEIN HOMOLOG 1) | | 1,75 | | | | 1,53 | | | 1,36 | | |
| | LG0AAD13L23 | GASA1 GASA1 (GAST1 PROTEIN HOMOLOG 1) | | 2,11 | | 1,61 | | | | | | | |
| | LG0AAC20O13 | GASA1 GASA1 (GAST1 PROTEIN HOMOLOG 1) | | | | | | 1,80 | 3,40 | | | | |
| | CU657317 | GASA1 GASA1 (GAST1 PROTEIN HOMOLOG 1) | | 2,57 | | | | 1,87 | | | | | |
| | LG0AAD50O15 | gibberellin-regulated family protein | | | | | | 1,11 | | | | | |
| | LG0AAD3M7 | GR-RBP7, CCR2, ATGRP7 ATGRP7 (COLD, CIRCADIAN RHYTHM, AND RNA BINDING 2); RNA binding | | | | | -1,59 | | | | -1,35 | | |
| | LG0AAA20H19 | GR-RBP7, CCR2, ATGRP7 ATGRP7 (COLD, CIRCADIAN RHYTHM, AND RNA BINDING 2); RNA binding / double-stranded DNA binding / single-stranded DNA binding | | | | | -1,14 | | | | | | |
| | LG0AAD35I5 | GR-RBP7, CCR2, ATGRP7 ATGRP7 (COLD, CIRCADIAN RHYTHM, AND RNA BINDING 2); RNA binding / double-stranded DNA binding / single-stranded DNA binding | | | | | -2,43 | | | | | | |
| | LG0AAD3M7 | GR-RBP7, CCR2, ATGRP7 ATGRP7 (COLD, CIRCADIAN RHYTHM, AND RNA BINDING 2); RNA binding | | | | | -1,59 | | | | -1,35 | | |
| | LG0AAB25I24 | LSH1 LSH1 (LIGHT-DEPENDENT SHORT HYPOCOTYLS 1) | | | | | | -0,95 | | | | | |
| | LG0AAD48O4 | oxidoreductase, acting on the CH-CH group of donors | | | | | | | | | | | 1,36 |
| | LG0AAC26K7 | putative senescence-associated protein [Cupressus sempervirens] | | | | | | | 2,97 | | | | |
| | CU657048 | UNE3, PGA2, TATC, APG2 APG2 (ALBINO AND PALE GREEN 2) | -1,58 | | | | | | | | | | |
| metabolism | LG0AAA5A24 | 2-dehydro-3-deoxyphosphoheptonate aldolase, putative / 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, putative / DAHP synthetase, putative | | | | | | | | | | 2,66 | |
| | LG0AAB29M4 | aconitate hydratase, cytoplasmic / citrate hydro-lyase / aconitase (ACO) | | | | | | | | | 1,30 | | |
| | LG0AAD30J9 | ALDH11A3 ALDH11A3 (Aldehyde dehydrogenase 11A3); 3-chloroallyl aldehyde dehydrogenase/ glyceraldehyde-3-phosphate dehydrogenase (NADP+) | 0,93 | | | | | | | | | | |
| | LG0AAD26C1 | AMI1, ATAMI1 ATAMI1 (AMIDASE-LIKE PROTEIN 1); amidase | | | | | | | | | | -2,75 | |
| | LG0AAB6C15 | AMI1, ATAMI1 ATAMI1 (AMIDASE-LIKE PROTEIN 1); amidase | | | | | | | | | | -2,04 | |
| | LG0AAD46H15 | AMP-dependent synthetase and ligase family protein | | | | | | | | | -1,34 | | |
| | LG0AAA10O7 | ATGPAT6, GPAT6 ATGPAT6/GPAT6 (GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE 6); 1-acylglycerol-3-phosphate O-acyltransferase/ acyltransferase | | | -1,09 | | | | | | 2,03 | | |
| | LG0AAD24F12 | ATPDX1.2, A37 A37 (PYRIDOXINE BIOSYNTHESIS 1.2); protein heterodimerization | | | | | | 0,98 | | | | | |
| | LG0AAC20F9 | AWI31, VEP1 VEP1 (VEIN PATTERNING 1) | | 1,49 | | | | 1,04 | 1,72 | | | | |
| | LG0AAB12H15 | AXS1 AXS1 (UDP-D-APIOSE/UDP-D-XYLOSE SYNTHASE 1) | | | | -1,28 | | | | | | | |
| | LG0AAA10B5 | B29, NIA2-1, CHL3, NR, NR2, NIA2 NIA2 (NITRATE REDUCTASE 2) | | | | | | | | | | | -1,63 |
| | LG0AAB26C8 | carbonic anhydrase family protein | | | | | | | | | | | -3,00 |
| | LG0AAD4L4 | CYP88A4, KAO2 KAO2 (ENT-KAURENOIC ACID HYDROXYLASE 2); oxygen binding | | | | | | 1,03 | | | | | |
| | LG0AAD30A8 | DIN6, AT-ASN1, ASN1 ASN1 (DARK INDUCIBLE 6) | | | | | | 1,80 | | | | | |
| | LG0AAC14O21 | EMB3004, MEE32 EMB3004 (EMBRYO DEFECTIVE 3004, maternal effect embryo arrest 32); 3-dehydroquinate dehydratase/ NADP binding / shikima | -1,37 | | | | | | | | | | |
| | LG0AAD12N20 | EPC1 EPC1 (ECTOPICALLY PARTING CELLS); transferase, transferring glycosyl groups | | | | | | | | | | | 1,67 |
| | LG0AAD19F16 | FAD2 FAD2 (FATTY ACID DESATURASE 2) | | | | | | | | | 0,91 | | |
| | LG0AAA14B10 | ferrochelatase | | | | | | | | | -1,33 | | |
| | LG0AAA16L4 | GDSL-motif lipase/hydrolase family protein | | | | | | | | 1,86 | | | |
| | LG0AAD10F20 | GLIP2 GLIP2 (GDSL-motif lipase 2); carboxylic ester hydrolase | | | | | | 1,12 | | | | | |
| | LG0AAD4C17 | GLIP5 GLIP5 (GDSL-motif lipase 5); carboxylic ester hydrolase | 1,13 | | | | | | | | | | |
| | LG0AAD36A13 | GLN2, ATGSL1, GS2 GS2 (GLUTAMINE SYNTHETASE 2) | | | | | | | | | | -1,83 | |
| | LG0AAA2P4 | P11, GLB1 GLB1 (glutamine synthetase B1) | | | 1,19 | | | | | | | -1,81 | |
| | LG0AAD21F9 | haloacid dehalogenase-like hydrolase family protein | | | | 1,36 | | | | | | | |
| | LG0AAD26H3 | hydrolase, alpha/beta fold family protein | | | | | | | | | | | 3,03 |
| | LG0AAD28J11 | ln2-1 protein, putative | | | | | | -1,26 | | | | | |
| | LG0AAB17F9 | KAS2, FAB1 FAB1 (FATTY ACID BIOSYNTHESIS 1); fatty-acid synthase | | | -1,52 | | | | | | | | |
| | LG0AAC23J14 | lipase class 3 family protein | | | | | | | | | | -2,99 | |
| | LG0AAA8G19 | lipase class 3 family protein [Arabidopsis thaliana] Os01g0243000 [Oryza sativa (japonica cultivar-group)] | | | | | | | | | -1,36 | | |
| | LG0AAD27I8 | long-chain-fatty-acid--CoA ligase family protein / long-chain acyl-CoA synthetase family protein (LACS8) | | | | | | | 1,65 | | | | |
| | LG0AAC5C24 | MEE59 MEE59 (maternal effect embryo arrest 59) | | | | | | | | -2,19 | -0,97 | | |
| | LG0AAD10D4 | PGDH PGDH (3-PHOSPHOGLYCERATE DEHYDROGENASE); phosphoglycerate dehydrogenase | | | | | | | | | | 4,22 | |
| | LG0AAD50M2 | phosphoribulokinase/uridine kinase family protein | | | | | | | | | | 3,24 | |
| | LG0AAB16P13 | RHM3 RHM3 (RHAMNOSE BIOSYNTHESIS 3); catalytic | | | | | -1,98 | | | | | | |
| | LG0AAB26O8 | SHM3 SHM3 (SERINE HYDROXYMETHYLTRANSFERASE 3); glycine hydroxymethyltransferase | | | | | | | | | | | 5,73 |
| | LG0AAB8K16 | SHM4 SHM4 (SERINE HYDROXYMETHYLTRANSFERASE 4); glycine hydroxymethyltransferase | | | | | | | | 1,06 | | | |
| | LG0AAD21D22 | short-chain dehydrogenase/reductase (SDR) family protein | | | | | | | | | | -1,74 | |
| | LG0AAD15H16 | succinyl-CoA ligase (GDP-forming) beta-chain, mitochondrial, putative / succinyl-CoA synthetase, beta chain, putative / SCS-beta, putative | | -0,87 | | | | | | | | | |
| | CU639611 | transferase family protein | | | | | | | | | | 3,10 | |
| | LG0AAD39F9 | TZ, THI1 THI1 (THIAZOLE REQUIRING) | | | | | -1,46 | | | | | | |
| | LG0AAD14A7 | UDP-glucoronosyl and UDP-glucosyl transferase, putative [Aspergillus clavatus NRRL 1] | | | | | | | | | -0,91 | | |
| | LG0AAD4J10 | UXS5 UXS5 (UDP-Xyl synthase 5); catalytic | | | | | | | | | | 3,58 | |
| | LG0AAD51C2 | WNK1 [Glycine max] | | | | | | | | | | -3,06 | |
| oxidation, reduct. | LG0AAC14E13 | ACD1-LIKE ACD1-LIKE; electron carrier | | | | | | | | | -1,20 | | |
| | LG0AAD45D13 | ATH9 ATH9 (thioredoxin H-type 9) | | | | | | | | | | | -2,33 |
| | LG0AAD46L5 | ATM4, TRX-M4, ATHM4 ATHM4 (Arabidopsis thioredoxin M-type 4); thiol-disulfide exchange intermediate | | | | | | | | | | -2,14 | |
| | LG0AAB24C1 | thioredoxin family protein | | 1,93 | | 1,66 | | | 3,12 | | | | |
| | LG0AAC7D2 | thioredoxin family protein | 1,29 | | | | | | | | | | |
| | LG0AAD1N6 | ATNTRB, NTRB NTRB (NADPH-dependent thioredoxin reductase B) | 1,02 | | | | | | | | | | |
| | LG0AAD49A16 | FQR1 FQR1 (FLAVODOXIN-LIKE QUINONE REDUCTASE 1) | | | | | | | | | | 1,88 | |
| | LG0AAD41H20 | oxidoreductase, 2OG-Fe(II) oxygenase family protein | | | 0,98 | | | 1,78 | | | | | |
| | LG0AAA9K18 | oxidoreductase/ zinc ion binding | | | | | | | | | | | -1,44 |
| | LG0AAB25E8 | UDP-glucose 6-dehydrogenase, putative | | | | | | | | | | 1,95 | |

**Table A.2:** Continued from table A.1. Continued in table A.3.

| FG | Name | Annotation | 2006 DS | | | | 2007 DS | | | 2007 RW | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | I | II | III | IV | I | II | V | I | II | III | V | |
| photosynthesis | LG0AAA13I12 | AB140, CAB140, LHCB1.3, CAB1 CAB1 (CHLOROPHYLL A/B BINDING PROTEIN 1); chlorophyll binding | | | | | | | | 1,45 | | | | |
| | LG0AAD45L18 | ATPPC1 ATPPC1 (PHOSPHOENOLPYRUVATE CARBOXYLASE 1); phosphoenolpyruvate carboxylase | | | | | | 2,30 | | | | | | |
| | LG0AAC31J6 | ELIP, ELIP1 ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN); chlorophyll binding | | | | 2,39 | | | | | -1,51 | | | |
| | LG0AAA8H2 | ELIP, ELIP1 ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN); chlorophyll binding | | | | | | | | -1,64 | | | | |
| | LG0AAA18E23 | ELIP, ELIP1 ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN); chlorophyll binding | | | | | | | | | | -2,37 | | |
| | LG0AAA7N24 | ELIP, ELIP1 ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN); chlorophyll binding | | | | | | | | | | -2,38 | | |
| | LG0AAA10J24 | ELIP, ELIP1 ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN); chlorophyll binding | | | | | | | | | | -3,24 | | |
| | LG0AAA2I22 | ELIP, ELIP1 ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN); chlorophyll binding | | | | | | | | -2,16 | | | | |
| | LG0AAA19E24 | ELIP, ELIP1 ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN); chlorophyll binding | | | | | | | | -1,69 | | | | |
| | LG0AAC28K8 | LHCB1.4, LHB1B1 LHB1B1 (Photosystem II light harvesting complex gene 1.4); chlorophyll binding | | | | | 1,37 | | | 1,54 | | | | |
| | LG0AAA14O9 | LHCB2.2 LHCB2.2 (Photosystem II light harvesting complex gene 2.2); chlorophyll binding | | | | -1,46 | | | | | | | | |
| | LG0AAA20L8 | LHCB3*1, LHCB3 LHCB3 (LIGHT-HARVESTING CHLOROPHYLL BINDING PROTEIN 3) | | | | | | | | 2,14 | | | | |
| | LG0AAA15H8 | OEE2, PSBP-1 PSBP-1 (OXYGEN-EVOLVING ENHANCER PROTEIN 2); calcium ion binding | | | | | | | | 1,17 | | | | |
| | LG0AAA4O9 | PSAF PSAF (photosystem I subunit F) | | | | -1,05 | | | | | | | | |
| | LG0AAA8E4 | PSAO PSAO (photosystem I subunit O) | | | | | | | | 1,23 | | | | |
| | LG0AAA11B16 | PSBQ, PSBQ-2 PSBQ/PSBQ-2 (photosystem II subunit Q-2); calcium ion binding | | | | -1,50 | | | | | | | | |
| | LG0AAC14I16 | PSBX PSBX (photosystem II subunit X) | | | | -1,51 | | | | | | | | |
| | CR628050 | PSBX PSBX (photosystem II subunit X) | | | | -1,43 | | | | | | | | |
| PA-s) | LG0AAD26C21 | adenosylmethionine decarboxylase | -1,53 | | | | | | | | | | | |
| | LG0AAA4E11 | adenosylmethionine decarboxylase | -1,15 | | | | | | | | | | | |
| protein turnover | LG0AAB15K18 | 26S proteasome regulatory subunit, putative (RPN7) | | | | | | | | | | 2,42 | | |
| | LG0AAA4H11 | 40S ribosomal protein S11 (RPS11B) | | | | | | | | | -1,20 | -2,45 | | |
| | LG0AAA2J21 | 40S ribosomal protein S9 (RPS9C) | | | | | | 1,78 | | | | | | |
| | LG0AAC25F3 | 60S ribosomal protein L28 (RPL28C) | -0,84 | | | | | | | | | | | |
| | LG0AAD49C12 | armadillo/beta-catenin repeat family protein / U-box domain-containing protein | | | | | | | | | | 1,62 | | |
| | CU640080 | ATBAG1 ATBAG1 (ARABIDOPSIS THALIANA BCL-2-ASSOCIATED ATHANOGENE 1); protein binding | | | | | | | | | | 2,52 | | |
| | LG0AAD43C12 | ATMKK9 ATMKK9 (Arabidopsis thaliana MAP kinase kinase 9); kinase | | | | | -1,39 | | | | -1,33 | | | |
| | CU639524 | ATVPS45, VPS45 VPS45 (VACUOLAR PROTEIN SORTING 45); protein transporter | | | | | | | | | | -1,49 | | |
| | LG0AAC15D15 | cell death associated protein-related [Arabidopsis thaliana] (TAIR:AT1G49650.1); Esterase/lipase/thioesterase [Medicago truncatula] (GB:ABE83375.1) | -0,94 | | | | | | | | | | | |
| | LG0AAC28I21 | chaperonin, putative | | | | | | | | | -1,16 | | | |
| | LG0AAD35L16 | cysteine proteinase, putative / thiol protease, putative | | | | | | -1,08 | | | | | | |
| | LG0AAA1E16 | DIN6, AT-ASN1, ASN1 ASN1 (DARK INDUCIBLE 6) | | | | | | | 2,39 | | | | | |
| | LG0AAD9A21 | dual specificity protein phosphatase family protein | | | | | | | | | | -1,79 | | |
| | LG0AAB12F14 | eukaryotic translation initiation factor 4A, putative / eIF-4A, putative / DEAD box RNA helicase, putative [Arabidopsis thaliana] Centrin [Oryza sativa (japonica cultivar-group)] | | | | | | | | | 1,27 | | | |
| | LG0AAD35G14 | J8 J8; heat shock protein binding / unfolded protein binding | | 1,05 | | | | | | | | | | |
| | LG0AAC5C24 | MEE59 MEE59 (maternal effect embryo arrest 59) | | | | | | | | -2,19 | -0,97 | | | |
| | LG0AAA10O12 | NCLPP5, CLPR1 CLPR1 (Clp protease proteolytic subunit 5); endopeptidase Clp | | | | 1,84 | | | | -2,33 | | -2,97 | | |
| | LG0AAD2N24 | Opticosapeptide/Phox/Bem1p [Medicago truncatula] | | | | | | | 1,88 | | | | | |
| | LG0AAA20P6 | PAG1 PAG1 (20S proteasome alpha subunit G1); peptidase | | | | | | | | | -1,62 | -2,18 | | |
| | LG0AAA10H22 | ribosomal protein L18 family protein | | | | | | 1,48 | | | | | | |
| | LG0AAB19G1 | ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein | | | | | -1,57 | | | -1,34 | | | | |
| | LG0AAD30L11 | RLK4, CRK10 CRK10 (CYSTEINE-RICH RLK10); kinase | | | | | | -1,27 | | | | | | |
| | LG0AAC15C16 | RNA cyclase family protein | | -1,01 | | | | | | | | | | |
| | LG0AAC14J20 | RPL24A RPL24A (RIBOSOMAL PROTEIN L24); structural constituent of ribosome | | | | | | | | | | 2,12 | | |
| | CU657604 | RPS12, RPS12C chloroplast gene encoding ribosomal protein s12. The gene is located in three distinct loci on the chloroplast genome and is transpliced to make or | | | -1,25 | | | | | | | | | |
| | LG0AAC25L13 | SCPL18 SCPL18 (serine carboxypeptidase-like 18); serine carboxypeptidase | | | | | | 1,04 | | | | | | |
| | LG0AAD44B4 | signal peptidase subunit family protein | | | | | | | | | | | 2,49 | |
| | LG0AAA17E3 | transcription initiation factor IID (TFIID) 28 kDa subunit (TAFII-28) family protein | | | -1,07 | | | | | | | | | |
| | LG0AAB2G20 | XSP1 XSP1 (XYLEM SERINE PEPTIDASE 1); subtilase | | | | | | -1,57 | | | | | | |
| regulation of gene expression | LG0AAA5E12 | AGL14 (AGAMOUS-LIKE 14); DNA binding / transcription factor | | | | | | | | | | -2,33 | | |
| | LG0AAC14D8 | ARAC7, ATROP9, RAC7, ROP9 ARAC7/ATROP9/RAC7/ROP9 (rho-related protein from plants 9); GTP binding | | | | 1,31 | | | | | | | | |
| | LG0AAB17D11 | AtMYB85, MYB85 MYB85 (myb domain protein 85); DNA binding / transcription factor | | | | | | | | | | | -2,14 | |
| | LG0AAD24N12 | ATRDRP1, RDR1 RDR1 (RNA-DEPENDENT RNA POLYMERASE 1); RNA-directed RNA polymerase/ nucleic acid binding | | | 1,39 | | | | | | | | | |
| | LG0AAD22E19 | ATWRKY53, WRKY53 WRKY53 (WRKY DNA-binding protein 53); transcription factor | | 2,68 | 1,81 | | | 3,99 | 3,26 | | | | | |
| | LG0AAD41M10 | basic helix-loop-helix (bHLH) family protein | | | | | | 1,31 | | | | | | |
| | LG0AAD29N12 | cleavage and polyadenylation specificity factor (CPSF) A subunit C-terminal domain-containing protein | | | | | | | | 1,38 | | | | |
| | LG0AAB19G13 | CPL4 (C-TERMINAL DOMAIN PHOSPHATASE-LIKE 4) | | | -1,63 | | | | | | | | -2,20 | |
| | CR627819 | DNA-binding protein-related | | | | | | | 2,52 | | | | | |
| | LG0AAD18N22 | glycine-rich RNA binding protein [Cryptococcus neoformans var. neoformans JEC21] | | | | -2,31 | | | | | | | | |
| | CU657114 | GTE6 GTE6 (GENERAL TRANSCRIPTION FACTOR GROUP E6); DNA binding | | | | | | | | | | -1,82 | | |
| | LG0AAB28M3 | HAP5C HAP5C (HEME ACTIVATED PROTEIN 5C); DNA binding / transcription factor | | | | | | | | | 1,24 | | | |
| | LG0AAD5A4 | harpin-induced protein-related / HIN1-related / harpin-responsive protein-related | | | | | | | | | | 1,64 | | |
| | LG0AAA13I11 | HIT-type zinc finger-containing protein C1orf181 homolog - Mus musculus (Mouse) | | | | | | | | | | 2,27 | | |
| | LG0AAD31A16 | hydroxyproline-rich glycoprotein DZ-HRGP [Volvox carteri f. nagariensis] | | | | | | | | | -1,10 | | | |
| | LG0AAD37E11 | LHY1, LHY LHY (LATE ELONGATED HYPOCOTYL) | | | | | | | | | | -1,99 | | |
| | CR627988 | Light-inducible protein CPRF-2 - Petroselinum crispum (Parsley) (Petroselinum hortense) | 1,19 | | | | | 2,56 | | | | | | |
| | LG0AAD42M1 | myb family transcription factor | | | | | | 1,61 | | | | | | |
| | LG0AAB25A16 | MYB transcription factor MYB177 [Glycine max] | | | | 1,77 | | | | | | | | |
| | LG0AAD37N7 | transcription factor | | | | | | | | | | 2,39 | | |
| | LG0AAA3A14 | transcriptional factor B3 family protein | | | 1,04 | | | | | | | | | |
| | LG0AAC10N4 | VIP2 VIP2 (VIRE2 INTERACTING PROTEIN2); transcription regulator | | | | | | | | | 1,36 | | | |
| | CU656661 | zinc finger (C3HC4-type RING finger) family protein | | -0,76 | | | | | | | | | | |
| | LG0AAC30E6 | zinc finger (C3HC4-type RING finger) family protein | | | | | | | | | | 3,20 | | |
| | LG0AAA14K19 | zinc finger (ZPR1-type) family protein | | | | | | | | | | 2,11 | | |
| | LG0AAD35I13 | zinc finger protein-related | | | | | | | | | | | 2,01 | |
| RNA- | LG0AAC3O14 | RRP41 RRP41 (Ribosomal RNA Processing 41); 3'-5'-exoribonuclease/ RNA binding | -1,30 | | | | | | | | | | | |
| | LG0AAD5D5 | SCL30 SCL30 (SC35-like splicing factor 30); RNA binding | -0,84 | | | | | | | | | | | |
| signaling | LG0AAB3N12 | ARF16 ARF16 (AUXIN RESPONSE FACTOR 16); miRNA binding / transcription factor | | | | -1,13 | | | | | | | | |
| | LG0AAB6E2 | ARR12 ARR12 (ARABIDOPSIS RESPONSE REGULATOR 12); transcription factor/ two-component response regulator | | | | | | | | | 1,27 | | | |
| | LG0AAA10K11 | ASK1, SNRK2-4, SNRK2.4, SRK2A ASK1 (ARABIDOPSIS SERINE/THREONINE KINASE 1); kinase | | | -1,60 | | | | | | | | | |
| | LG0AAD43C12 | ATMKK9 ATMKK9 (Arabidopsis thaliana MAP kinase kinase 9); kinase | | | | | -1,39 | | | | -1,33 | | | |
| | LG0AAB7J11 | ATPI4K ALPHA ATPI4K ALPHA (Arabidopsis thaliana phosphatidylinositol 4-kinase alpha); inositol or phosphatidylinositol kinase | | 2,54 | | 3,47 | | | | | | -2,99 | | |
| | LG0AAB24D5 | ATPIN1, PIN1 PIN1 (PIN-FORMED 1); transporter | | | | | | -1,28 | | | | | | |
| | LG0AAD6L14 | CML24, TCH2 TCH2 (TOUCH 2); calcium ion binding | | | | | | | | | -0,90 | | | |
| | LG0AAC22K9 | DC1 domain-containing protein | | 1,67 | | | | 2,20 | | | | | | |
| | CU639821 | FBL6, EBF1 EBF1 (EIN3-BINDING F BOX PROTEIN 1); ubiquitin-protein ligase | | | | | | | 2,39 | | | | | |
| | LG0AAB18M24 | GTP-binding protein-related | | | | | | | | | 1,09 | | | |
| | LG0AAD48E14 | leucine-rich repeat transmembrane protein kinase, putative | | | | | | -1,81 | | | -2,14 | | | |
| | LG0AAD15C24 | leucine-rich repeat transmembrane protein kinase, putative | | | | | | -1,44 | | | -1,42 | | | |
| | CU656459 | leucine-rich repeat transmembrane protein kinase, putative | | | -1,07 | | | | | | | | | |
| | LG0AAA6D23 | NTF2A NTF2A (NUCLEAR TRANSPORT FACTOR 2A); Ran GTPase binding / protein transporter | | | | | | | | | | -1,86 | | |
| | LG0AAB24E22 | OST1, SNRK2-6, SNRK2.6, SRK2E, P44 OST1 (OPEN STOMATA 1); kinase/ protein kinase | | 1,64 | | | | 1,25 | | | | | | |
| | CU639700 | phosphatidic acid phosphatase-related / PAP2-related | | | | | | | | 1,91 | | | | |
| | LG0AAB8E2 | phosphatidic acid phosphatase-related / PAP2-related | | | | | | | | | 1,17 | | | |
| | LG0AAD43L16 | protein kinase family protein | | | | | | -1,26 | | | -1,26 | | | |
| | LG0AAB27F17 | protein kinase family protein | | | | | | | | | 1,04 | | | |
| | LG0AAD15H15 | protein kinase family protein | | | | | | 1,06 | | | | | | |
| | LG0AAD43L16 | protein kinase family protein | | | | | | -1,26 | | | -1,26 | | | |
| | CU640516 | protein kinase family protein | | | | | -1,74 | | | -1,34 | -1,12 | | | |
| | LG0AAD12P23 | protein kinase, putative | | | | | | | | | -1,49 | | | |
| | LG0AAC21F15 | protein phosphatase 2C, putative / PP2C, putative | | | | | | -1,26 | | | | | | |
| | LG0AAB13J11 | CPK8, CDPK19 CDPK19 (CALCIUM-DEPENDENT PROTEIN KINASE 19); calcium- and calmodulin-dependent protein kinase/ kinase | | | | | | | | | 1,73 | | | |
| | CU640322 | RLK4, CRK10 CRK10 (CYSTEINE-RICH RLK10); kinase | | | | | | | | | | -1,91 | | |
| | LG0AAB19J18 | SIP3, SnRK3.14, CIPK6 CIPK6 (CBL-INTERACTING PROTEIN KINASE 6); kinase | 1,13 | | | | | | | | | | | |
| | LG0AAA3K5 | SIP3, SnRK3.14, CIPK6 CIPK6 (CBL-INTERACTING PROTEIN KINASE 6); kinase | | 1,15 | | | | | | | | | | |
| | LG0AAD3J10 | SIP3, SnRK3.14, CIPK6 CIPK6 (CBL-INTERACTING PROTEIN KINASE 6); kinase | | | | | | | 2,45 | | | | | |
| | LG0AAA10G19 | XLG2 XLG2 (extra-large GTP-binding protein 2); signal transducer | | | | | | -1,20 | | | | | | |
| stress | LG0AAA11N5 | AGP17 (ARABINOGALACTAN PROTEIN 17) [Arabidopsis thaliana] AGP17_ARATH Lysine-rich arabinogalactan protein 17 precursor (Lys-rich AGP 17) | | | | | | 1,05 | | | | | | |
| | CU656278 | ALDH7B4 ALDH7B4 (ALDEHYDE DEHYDROGENASE 7B4); 3-chloroallyl aldehyde dehydrogenase | | | | | | 1,08 | | | | | | |
| | CR627971 | ATCHITIV, CHIV, ATEP3 ATEP3 (Arabidopsis thaliana chitinase class IV); chitinase | | | | | | | | | | | 3,03 | |
| | LG0AAB10E20 | ATCOR413-PM2 COR413-PM2 (cold regulated 413 plasma membrane 2) | | | | | | 1,23 | | | | | | |
| | LG0AAD9O16 | ATLOX2, LOX2 LOX2 (LIPOXYGENASE 2) | | | | | | | | | | | 1,80 | |
| | LG0AAD16O9 | ATPER1 ATPER1 (Arabidopsis thaliana 1-cysteine peroxiredoxin 1); antioxidant | | | | | | | | | | 2,02 | | |
| | LG0AAB15C11 | ATPP2CA, AHG3 AHG3/ATPP2CA (ARABIDOPSIS THALIANA PROTEIN PHOSPHATASE 2CA); protein binding / protein phosphatase type 2C | | 1,57 | | | | 1,67 | | | | | | |
| | LG0AAD8A20 | ATPP2CA, AHG3 AHG3/ATPP2CA (ARABIDOPSIS THALIANA PROTEIN PHOSPHATASE 2CA); protein binding / protein phosphatase type 2C | | 1,73 | | | | | | | | | | |
| | LG0AAA7C15 | CYP77A4 CYP77A4 (cytochrome P450, family 77, subfamily A, polypeptide 4); oxygen binding | -1,27 | | | | | | | | | 3,29 | | |
| | CR627776 | DI21, ATDI21 ATDI21 (Arabidopsis thaliana drought-induced 21) | | 3,17 | | | | | 3,17 | | | | | |
| | LG0AAC20B18 | disease resistance-responsive family protein / dirigent family protein | | | | | | | 2,87 | | | | | |
| | CU657271 | DNAJ heat shock N-terminal domain-containing protein | | | | | -2,97 | 2,06 | | | | | | |
| | LG0AAD11D18 | EDS1 EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1); signal transducer/ triacylglycerol lipase | | | | | | -1,77 | | | -1,74 | | | |
| | LG0AAA16L11 | GAPC-2 GAPC-2; glyceraldehyde-3-phosphate dehydrogenase | | | | | | | | | -0,85 | | | |
| | LG0AAA12B13 | GLP1 GLP1 (GERMIN-LIKE PROTEIN 1); manganese ion binding / metal ion binding / nutrient reservoir | | | | | | | | | | 2,17 | | |
| | LG0AAA16M9 | GLP3A, GLP3B, GLP3 GLP3 (GERMIN-LIKE PROTEIN 3); manganese ion binding / metal ion binding / nutrient reservoir | | | | -3,24 | -2,17 | -2,08 | | | | 4,24 | | |
| | LG0AAA1E14 | GLP3A, GLP3B, GLP3 GLP3 (GERMIN-LIKE PROTEIN 3); manganese ion binding / metal ion binding / nutrient reservoir | | -1,53 | | -2,30 | -1,69 | | | | | | | |
| | LG0AAA2B20 | GLP3A, GLP3B, GLP3 GLP3 (GERMIN-LIKE PROTEIN 3); manganese ion binding / metal ion binding / nutrient reservoir | -1,70 | | | | | | | | | 1,92 | | |
| | LG0AAA16M9 | GLP3A, GLP3B, GLP3 GLP3 (GERMIN-LIKE PROTEIN 3); manganese ion binding / metal ion binding / nutrient reservoir | | | | -3,24 | -2,17 | -2,08 | | | | 4,24 | | |
| | LG0AAA1E14 | GLP3A, GLP3B, GLP3 GLP3 (GERMIN-LIKE PROTEIN 3); manganese ion binding / metal ion binding / nutrient reservoir | | -1,53 | | -2,30 | -1,69 | | | | | | | |
| | CU656866 | 17.6 kDa class I small heat shock protein (HSP17.6B-CI) | | | | | | | | | | | 3,21 | |
| | LG0AAA15B17 | HSP70 HSP70 (heat shock protein 70); ATP binding | -1,43 | | | | | | | | | | | |
| | LG0AAA5K19 | HSP70 HSP70 (heat shock protein 70); ATP binding | -1,40 | | | | | | | | | | | |
| | CU639824 | HSP70-1, AT-HSC70-1, HSC70, HSC70-1 HSC70-1 (heat shock cognate 70 kDa protein 1); ATP binding | -1,12 | | | | | | | | | | | |
| | LG0AAC22H21 | HSP70-1, AT-HSC70-1, HSC70, HSC70-1 HSC70-1 (heat shock cognate 70 kDa protein 1); ATP binding | -0,95 | | | | | | | | | | | |

**Table A.3:** Continued from table A.2. Continued in table A.4.

| FG | Name | Annotation | 2006 DS | | | | 2007 DS | | | 2007 RW | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | I | II | III | IV | I | II | V | I | II | III | V |
| stress | LG0AAA11F23 | inositol-3-phosphate synthase isozyme 2 / myo-inositol-1-phosphate synthase 2 / MI-1-P synthase 2 / IPS 2 | | | | | | | | 1,01 | | | |
| | LG0AAD26H5 | isoflavone reductase, putative | -0,98 | | | | | | | | | | |
| | LG0AAD21G11 | isoflavone reductase, putative | | | | | -1,90 | | | | | | |
| | CU657720 | lactoylglutathione lyase | | -1,51 | | -1,39 | | | | | | | |
| | LG0AAC18B20 | late embryogenesis abundant protein [Catharanthus roseus] | | | | | | 4,50 | 3,28 | -1,58 | | | |
| | CR627993 | Late embryogenesis abundant protein Lea5 - Citrus sinensis (Sweet orange) | | 2,67 | 2,59 | | | 5,39 | 3,14 | | 2,64 | | |
| | LG0AAC15L2 | late embryogenesis abundant protein, putative / LEA protein, putative | | 1,38 | | | | | | | | | |
| | LG0AAD45D14 | lipocalin, putative | | | | | | 1,20 | | | | | |
| | LG0AAB14E16 | maturation-associated SRC1-like protein [Carica papaya] | | | | | | 1,07 | 1,82 | | | | |
| | LG0AAD46K14 | maturation-associated SRC1-like protein [Carica papaya] | | | 1,59 | | | 2,59 | | | | | |
| | LG0AAC19F10 | maturation-associated SRC1-like protein [Carica papaya] | | | | | | 1,76 | | | | | |
| | LG0AAD21M21 | Metallothionein-like protein 1 - Pisum sativum (Garden pea) | | | | | | 1,09 | | | | | |
| | LG0AAA5F10 | Metallothionein-like protein 1 - Pisum sativum (Garden pea) | | | | | | 0,78 | | | | | |
| | LG0AAA10A6 | MLP423 Bet v I allergen family protein | | | | | | | | 1,90 | | 4,54 | |
| | LG0AAA2I21 | MLP423 Bet v I allergen family protein | | | | | | | | | | 1,99 | |
| | LG0AAD18B20 | MLP423 Bet v I allergen family protein | | | | | | -1,64 | | | | | |
| | CR628213 | MT3 MT3 (METALLOTHIONEIN 3) | 1,90 | | | | | | | | | | |
| | LG0AAA20P6 | PAG1 PAG1 (20S proteasome alpha subunit G1); peptidase | | | | | | | | | -1,62 | -2,18 | |
| | LG0AAA7F9 | peroxidase, putative | | | | | | | | | | 2,22 | |
| | LG0AAD25D17 | PR3, PR-3, CHI-B, B-CHI, ATHCHIB ATHCHIB (BASIC CHITINASE); chitinase | | | | | | | | | | 1,90 | |
| | LG0AAD49A3 | PR3, PR-3, CHI-B, B-CHI, ATHCHIB ATHCHIB (BASIC CHITINASE); chitinase | | | | | -1,41 | | | | | | |
| | LG0AAD48P10 | pyruvate decarboxylase PDC2 [Laccaria bicolor S238N-H82] | | | 1,57 | | | | | | | | |
| | LG0AAB10A20 | RCI2B RCI2B (RARE-COLD-INDUCIBLE 2B) | | 1,42 | | | | 1,34 | 2,43 | | | | |
| | CU656821 | RCI2B RCI2B (RARE-COLD-INDUCIBLE 2B) | | 2,27 | | | | 2,57 | 3,04 | | | | |
| | LG0AAC24C5 | RCI2B RCI2B (RARE-COLD-INDUCIBLE 2B) | 1,09 | 1,50 | | | | 1,40 | | | | | |
| | CU656691 | RCI2B RCI2B (RARE-COLD-INDUCIBLE 2B) | | | | | | | | | -0,83 | | |
| | LG0AAD10L19 | RD19A, EMB3005, RD19 RD19 (RESPONSIVE TO DEHYDRATION 19); cysteine-type peptidase | | | | | | -0,89 | | | | | |
| | LG0AAA6M12 | RD22 RD22 (RESPONSIVE TO DESSICATION 22) | | | | | | 1,41 | | | | | |
| | LG0AAD37E3 | AT1G56075.1, LOS1 LOS1 (Low expression of osmotically responsive genes 1); translation elongation factor/ translation factor, nucleic acid binding | | | | | | 0,89 | | | | | |
| | LG0AAD37M8 | SEN2, CAT3 CAT3 (CATALASE 3); catalase | | | | | 4,45 | | | | | | |
| | LG0AAA15D7 | SH1, FAD8 FAD8 (FATTY ACID DESATURASE 8); omega-3 fatty acid desaturase | | | | | | | -1,72 | | | | |
| | LG0AAD51A12 | SRC2, (AT)SRC2 (AT)SRC2/SRC2 (SOYBEAN GENE REGULATED BY COLD-2); protein binding | | | | | | | | | | 3,08 | |
| | LG0AAD16D23 | universal stress protein (USP) family protein | | | | | | | | | -1,04 | | |
| | LG0AAD9B5 | peroxidase 64 (PER64) (P64) (PRXR4) | | | -1,39 | | | | | | | | |
| | LG0AAA1O17 | SH1, FAD8 FAD8 (FATTY ACID DESATURASE 8); omega-3 fatty acid desaturase | | | | -1,83 | | | | | | | |
| transport | LG0AAD31O19 | (VACUOLAR ATP SYNTHASE SUBUNIT B2); hydrogen ion transporting ATP synthase, rotational mechanism | | | | | | 1,00 | | | | | |
| | LG0AAB25J6 | AAC1 AAC1 (ADP/ATP CARRIER 1); binding | | | | | | 1,04 | | | | | |
| | LG0AAD36J11 | AAC2 AAC2 (ADP/ATP CARRIER 2); binding | -1,09 | | | | | | | | | | |
| | LG0AAA20I17 | AHA8 AHA8 (ARABIDOPSIS H(+)-ATPASE 8); ATPase | | | | | | -0,90 | | | | | |
| | LG0AAB5G2 | anion exchange family protein | | | | | | -1,13 | | | | | |
| | LG0AAA4P24 | ATCNGC2, CNGC2, DND1 DND1 (DEFENSE NO DEATH 1); cation channel/ cyclic nucleotide binding / inward rectifier potassium channel | | -1,08 | | | | | | | | | |
| | LG0AAA2D3 | ATG8D ATG8D (autophagy gene 8-related); microtubule binding | | | | | | 1,55 | | | | | |
| | LG0AAA4I2 | ATP synthase gamma chain, mitochondrial (ATPC) | | | | -1,04 | | | | | | | |
| | LG0AAB5M4 | AVPL1, AVP2 AVP2 (ARABIDOPSIS VACUOLAR H+-PYROPHOSPHATASE 2) | | | | | -2,09 | | | | | | |
| | LG0AAD44I22 | BOU BOU (A BOUT DE SOUFFLE); binding | | 1,12 | | | | | | | | | |
| | LG0AAD24P1 | cytochrome c oxidase family protein | | | | | | | | | 1,62 | | |
| | LG0AAA9O18 | ELD1, ABI8, KOB1 KOB1 (KOBITO) | | | | | | | | | 1,07 | | |
| | CU657855 | forisome [Medicago truncatula] | | | | | | | 1,90 | | | | |
| | LG0AAD7P10 | haloacid dehalogenase-like hydrolase family protein | | | 1,22 | | | | | | | | |
| | LG0AAD14N3 | heavy-metal-associated domain-containing protein | | | 1,34 | | | | | | | | |
| | LG0AAC21E21 | Encodes a Protease inhibitor/seed storage/LTP family protein | | | | -1,99 | | | | | | | |
| | LG0AAA14O8 | LTP1, ATLTP1, LP1 LP1 (nonspecific lipid transfer protein 1) | | | | | | 0,95 | | | | | |
| | CU657255 | LTP1, ATLTP1, LP1 LP1 (nonspecific lipid transfer protein 1) | | | | | | 1,25 | | | | | |
| | LG0AAA7B19 | LTP1, ATLTP1, LP1 LP1 (nonspecific lipid transfer protein 1) | | | | | | 1,66 | | | | | |
| | LG0AAC4C24 | LTP3 LTP3 (LIPID TRANSFER PROTEIN 3); lipid binding | | | | | | 1,98 | | | | | |
| | LG0AAC27F5 | protease inhibitor/seed storage/lipid transfer protein (LTP) family protein | -2,38 | -1,35 | | | | | | 1,72 | | | |
| | LG0AAD50M24 | protease inhibitor/seed storage/lipid transfer protein (LTP) family protein | | | 2,26 | | | | | | | | |
| | LG0AAD27N10 | nascent polypeptide-associated complex (NAC) domain-containing protein / BTF3b-like transcription factor, putative | | | | | | | 2,75 | | | | |
| | LG0AAC16P20 | mechanosensitive ion channel domain-containing protein / MS ion channel domain-containing protein | | | | | | | | | | 3,11 | |
| | LG0AAD43D15 | NIP1;2, NLM2 NIP1;2/NLM2 (NOD26-like intrinsic protein 1;2); water channel | | | | | | -1,14 | | | | | |
| | LG0AAA18F13 | PIP2D, PIP2;5 PIP2;5/PIP2D (plasma membrane intrinsic protein 2;5); water channel | | | | -2,51 | | | | | | | |
| | CU640246 | TIP2;1, DELTA-TIP1, AQP1, ATTIP2;1, DELTA-TIP DELTA-TIP (delta tonoplast integral protein); water channel | | | | | | | | 1,11 | | | |
| | LG0AAC2B21 | TOM6 TOM6 (TRANSLOCASE OF THE OUTER MITOCHONDRIAL MEMBRANE 6) | | | | | -1,53 | | | | | | |
| | LG0AAC11H12 | integral membrane protein, putative | | | | | | | | | | | -2,00 |
| | LG0AAB26H1 | sugar transporter, putative | | | | | | | | | 1,41 | | |
| | LG0AAD45E20 | ATINT1 ATINT1 (INOSITOL TRANSPORTER 1); carbohydrate transporter/ sugar porter | 1,74 | 2,83 | | 2,51 | | 2,11 | | | | | |
| | LG0AAA14C3 | ATSEC22, SEC22 SEC22 (secretion 22); transporter | 2,18 | 2,24 | | | | 3,11 | | | | | |
| | LG0AAD45N16 | ATKUP3, ATKT4, KUP3 KUP3 (K+ uptake permease 3); potassium ion transporter | | | | | | | | | | -2,10 | |
| | LG0AAA13N22 | transporter-related | | | | | | | | | | -3,31 | |
| unknown | LG0AAC15N10 | 2-oxoglutarate-dependent dioxygenase, putative | | | | | | -1,10 | | | | | |
| | LG0AAD15P15 | 4D11_29 [Brassica rapa subsp. pekinensis] | | | | | | 1,11 | | | | | |
| | LG0AAD13K5 | AAA-type ATPase family protein | | | | | | | | | 1,31 | | |
| | LG0AAA3B7 | ACI13 [Lycopersicon esculentum] (GB:AAY97863.1); contains InterPro domain tRNA-binding arm; (InterPro:IPR010978) | | -1,07 | | | | | | | | | |
| | LG0AAB28C1 | AGP41 AGP41 | -1,29 | | | | | | | | | | |
| | LG0AAA15D10 | ankyrin repeat family protein | | | | | | | | | | -1,81 | |
| | LG0AAB18L9 | At2g47010/F14M4.16 [Arabidopsis thaliana] >gb\|AAN31092.1\|At2g47010/F14M4.16 [Arabidopsis thaliana] | | | | | | | | | | | 2,15 |
| | LG0AAB2M11 | AT4g27350 [Arabidopsis thaliana] | | | | | | | | | | 2,67 | |
| | LG0AAD29M6 | ATCFIM-25/CFIM-25 (ARABIDOPSIS HOMOLOG OF CFIM-25) [Arabidopsis thaliana] | | | | | | | | | -0,99 | | |
| | LG0AAD12K16 | ATGSTU25 ATGSTU25 (Arabidopsis thaliana Glutathione S-transferase (class tau) 25); glutathione transferase | | | | | | | | | | -1,81 | |
| | LG0AAC18I5 | AtRABG3f, AtRab7B AtRABG3f/AtRab7B (Arabidopsis Rab GTPase homolog G3f); GTP binding | | | | | | | | | | 2,68 | |
| | LG0AAB24H13 | ATROPGEF7, ROPGEF7 ATROPGEF7/ROPGEF7 (KINASE PARTNER PROTEIN-LIKE); Rho guanyl-nucleotide exchange factor | | | -1,37 | | | | | | | | |
| | LG0AAA12O1 | basic helix-loop-helix (bHLH) protein-related | | | | | | | | | | | 1,57 |
| | LG0AAD39P14 | binding | | | 1,20 | | | | | | | | |
| | CU657688 | binding | | | | -1,57 | | | | | | | |
| | LG0AAD47F17 | calcium-binding EF hand family protein | | | | | | | | | | | -3,97 |
| | LG0AAD8F21 | catalytic | | -0,79 | | | | | | | 0,88 | | |
| | LG0AAD39G17 | CBL3 ATCBL3 (CALCINEURIN B-LIKE 3) | | | 1,05 | | | | | | | | |
| | LG0AAA13F16 | CDH1-D [Gallus gallus] | | | | | | | | | | | 2,23 |
| | LG0AAD16K2 | CipC1 protein, concanamycin induced protein C [Laccaria bicolor S238N-H82] | | | | | | 2,48 | | | | | |
| | LG0AAC14G16 | circadian clock coupling factor-related | | | | | | | | | -1,25 | | |
| | LG0AAD6K22 | COP1-interacting protein-related | | | | | | | | | 1,25 | | |
| | CU657037 | CP12, CP12-1 CP12-1 (CP12 domain-containing protein 1) | | | | | | | | -2,07 | | | |
| | LG0AAC22K9 | DC1 domain-containing protein | | 1,67 | | | | 2,20 | | | | | |
| | LG0AAD27I1 | DNAJ heat shock N-terminal domain-containing protein | | | | | -2,97 | 2,06 | | | | | |
| | LG0AAC15D1 | EMB1441 EMB1441 (EMBRYO DEFECTIVE 1441); nucleic acid binding | | | | -1,40 | | | | | | | |
| | LG0AAD46D4 | embryo-abundant protein-related | | | | | | | | | -0,94 | | |
| | LG0AAD13G14 | epsin N-terminal homology (ENTH) domain-containing protein | | | | | | | | 1,31 | | | |
| | LG0AAB2O3 | ethylene-responsive family protein | | | | | | | | | | -1,90 | |
| | CU657167 | FAD-binding domain-containing protein | | | | | | | | 1,14 | | | |
| | LG0AAA17C9 | F-box family protein | | | | | | | | | | 2,09 | |
| | LG0AAD19P4 | fiber protein Fb34 [Gossypium barbadense] | | | | | | | | | | 1,95 | |
| | LG0AAA20B19 | hydrolase, alpha/beta fold family protein | | | | -1,13 | | | -2,51 | | | | |
| | CU657795 | kelch repeat-containing F-box family protein | | | | | | | | | | -1,94 | |
| | LG0AAD37M9 | LSM14 protein homolog B - Mus musculus (Mouse) | | | | | | | | | | | -1,67 |
| | LG0AAA8O4 | membrane bound O-acyl transferase (MBOAT) family protein | | | | | | | | 2,03 | | | |
| | LG0AAD11G5 | mucin | | | | | | | | | -1,10 | | |
| | LG0AAD45E12 | novel protein [Danio rerio] | | | | | | 1,12 | | | | | |
| | LG0AAA9A3 | NSL1 (NECROTIC SPOTTED LESIONS 1) [Arabidopsis thaliana] Membrane attack complex component/perforin/complement C9 [Medicago truncatula] | | | | | | | | | 1,22 | | |
| | CU639550 | nuclear matrix constituent protein-related | | | | | | | | | -1,53 | | |
| | LG0AAD8C1 | ORF124 [Pinus koraiensis] >gb\|ABP35456.1\|ORF124 [Pinus koraiensis] | | | -0,98 | | | | | | | | |
| | LG0AAC14J13 | Os03g0807900 [Oryza sativa (japonica cultivar-group)] chaperonin-like RbcX [Trichodesmium erythraeum IMS101] | | | -0,93 | | | | | | | | |
| | LG0AAD36E10 | Os04g0549400 [Oryza sativa (japonica cultivar-group)] (GB:NP_001053483.1) | | | | | | | | | | | 2,53 |
| | CR627770 | Os08g0432500 [Oryza sativa (japonica cultivar-group)] ATP-dependent Clp protease adaptor protein ClpS [Trichodesmium erythraeum IMS101] | | | | | | | | | | -2,61 | |
| | LG0AAC24J10 | Os08g0566100 [Oryza sativa (japonica cultivar-group)] >dbj\|BAF24454.1Os08g0566100 [Oryza sativa (japonica cultivar-group)] | | | | | | | | | 1,19 | | |
| | LG0AAD24E7 | Os11g0616200 [Oryza sativa (japonica cultivar-group)]; putative protein [Zea mays] (GB:AAN40032.1); contains domain Protozoan pheromone proteins (SSF47014) | | | | | | | | 1,57 | | | |
| | LG0AAC16D9 | OSIGBa0132E09-OSIGBa0108L24.12 [Oryza sativa (indica cultivar-group)] | | | | 2,31 | | | | | | | |
| | LG0AAD44I9 | phase-change related protein [Quercus robur] | | | -1,16 | | | | | | | | |
| | LG0AAD19C21 | phosphate-responsive protein, putative (EXO) | | | | | -1,21 | | | | | | |
| | CU656326 | phosphate-responsive protein, putative (EXO) | | | | | | -1,09 | | | | | |
| | LG0AAD33D15 | PRLI-interacting factor, putative | | | | | | | | 1,69 | | | |
| | LG0AAB9B20 | proline-rich cell wall protein | | | | | | | | | | 3,35 | |
| | LG0AAA15N18 | protease-related | | | | | | | | | -1,52 | | |
| | LG0AAC28A22 | putative protein [Arabidopsis thaliana] | | | | 1,09 | | 0,97 | | | | | |
| | LG0AAB19L2 | putative protein [Arabidopsis thaliana] | | | | | | | | | | 3,45 | |
| | LG0AAA9B12 | putative protein [Arabidopsis thaliana] >emb\|CAB81389.1\|putative protein [Arabidopsis thaliana] | | | | -1,12 | | | | | | | |
| | LG0AAC7G4 | putative protein [Arabidopsis thaliana] >gb\|AAL47372.1\|putative protein [Arabidopsis thaliana] | | | | | | 0,90 | | | | | |
| | LG0AAD32I13 | pyridoxal-dependent decarboxylase family protein | | | | | | | | | | 3,07 | |
| | LG0AAD38M11 | Quinate permease - Neurospora terricola | | | | | | | | | | -1,76 | |
| | LG0AAC26E11 | tetratricopeptide repeat (TPR)-containing protein | | | | | | | | | | -1,50 | |
| | LG0AAD51C24 | Tropomyosin - Schizosaccharomyces pombe (Fission yeast) | | | | | | | | | 1,04 | | |
| | LG0AAD25E24 | UBX domain-containing protein | | | | | | | | | | -2,36 | |
| | LG0AAB7D22 | UDP-N-acetylglucosamine transferase subunit ALG14 homolog - Rattus norvegicus (Rat) | | | | | 1,19 | | | | | | |

**Table A.4:** Continued from table A.3. Continued in table A.5.

| FG | Name | Annotation | 2006 DS | | | | 2007 DS | | | 2007 RW | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | I | II | III | IV | I | II | V | I | II | III | V |
| unknown | LG0AAB4O10 | UNC-50 family protein | | | | | | | | | | 2,91 | |
| | LG0AAB14F23 | Uncharacterized protein ART2 - Saccharomyces cerevisiae (Baker's yeast) | | | | | | | 2,11 | | | | |
| | LG0AAD5J18 | Uncharacterized protein ART2 - Saccharomyces cerevisiae (Baker's yeast) | | | | | | | | | | 2,38 | |
| | LG0AAD12L9 | Uncharacterized protein C22orf19 - Homo sapiens (Human) | | | | | 2,5 | | | 3,3 | | | |
| | LG0AAA14F15 | Uncharacterized protein L630 - Mimivirus | | | | | | | | | -1,13 | | |
| | LG0AAC11M7 | UPF0041 protein R07E5.13 - Caenorhabditis elegans | | | | | | | | -1,64 | -1,16 | | |
| | CU657680 | yippee family protein | | | | | | | | | | -2,30 | |
| | LG0AAA9I9 | yrdC protein-related | | | | | | | | | -1,07 | | |
| | LG0AAC23M14 | | | | | | | | | | -1,00 | | |
| | LG0AAD7M21 | | | | | | | 0,92 | | | | | |
| | LG0AAA17C10 | | | 0,92 | | | | | | | | | |
| | LG0AAC14B10 | | | -0,90 | | | | | | | | | |
| | LG0AAD23N5 | | | | | | | | | | -0,94 | | |
| | LG0AAD3A11 | | | | 1,21 | | | | | | | | |
| | LG0AAD15N9 | | | | | | | | | | -0,96 | | |
| | LG0AAD26I2 | | | | | | | | | | 1,00 | | |
| | LG0AAD40I18 | | -1,01 | | | | | | | | | | |
| | LG0AAD24P24 | | | | | 2,32 | | | | | -1,02 | | |
| | LG0AAD18N24 | | | | | | | -1,04 | | | | | |
| | LG0AAD24K3 | | | | | | | | | | -1,07 | | |
| | DN949957 | | | | | | | | | | -1,09 | | |
| | DN950065 | | | | | | | | | -1,13 | | | |
| | LG0AAA1G20 | | -0,95 | | | | | | | | | | |
| | LG0AAD33H18 | | | | | | | | | | 1,10 | | |
| | LG0AAC11K6 | | | | | | | | | | 1,20 | | |
| | LG0AAD26E5 | | | 1,19 | 1,36 | | | | | | | | |
| | LG0AAA10O19 | | | | | | | 1,17 | | | | | |
| | LG0AAD10O16 | | | | | | | | | | 1,16 | | |
| | LG0AAD49G10 | | | | | | | 1,14 | | | | | |
| | LG0AAD42M6 | | | | | | | | | | 0,97 | | |
| | LG0AAD24M18 | | | 1,11 | | | | | | | | | |
| | LG0AAC22I8 | | | | | | | 0,99 | | | | | |
| | LG0AAC15O4 | | | | 1,08 | | | | | | | | |
| | LG0AAD25K16 | | | 1,05 | | | | | | | | | |
| | LG0AAD2E4 | | | | | | | 1,04 | | | | | |
| | LG0AAD43H19 | | | | | | | 1,03 | | | | | |
| | LG0AAD32O16 | | | | 1,03 | | | | | | | | |
| | LG0AAD34B11 | | | | | | | | | | -1,15 | | |
| | LG0AAD46O21 | | | | 1,13 | | | | | | | | |
| | LG0AAA4A22 | | | | | | -1,45 | | | | | | |
| | LG0AAD7M15 | | | | | | | -1,35 | | | | | |
| | DN950586 | | | | | | | | | | | | -1,36 |
| | LG0AAD24M24 | | | | | | | | | -1,38 | | | |
| | LG0AAD22C24 | | | | | | | | | | -1,41 | | |
| | LG0AAA1H16 | | | | | | | | | | | | -1,42 |
| | LG0AAD44J12 | | | | | | | | | | -1,33 | | |
| | LG0AAB26G15 | | | | | -1,44 | | | | | | | |
| | LG0AAA10P10 | | | | | | | 1,22 | | | | | |
| | LG0AAB10G8 | | | | | | | -1,46 | | | | | |
| | DN949948 | | | | | | | | | | -1,48 | | |
| | LG0AAA13A11 | | | | | | | | | | | -1,48 | |
| | LG0AAD13D11 | | -1,48 | | | | | | | | 1,18 | | |
| | LG0AAC15E7 | | | | | -1,49 | | | | | | | |
| | DN950278 | | | | | | -1,50 | | | | | | |
| | LG0AAD5J19 | | | | | | | -1,44 | | | | | |
| | LG0AAD15H22 | | | | | | | | | | -1,28 | | |
| | LG0AAB16J5 | | | | | | | -1,19 | | | | | |
| | LG0AAD40F18 | | | | | | -1,20 | | | | | | |
| | CU657502 | | | | -1,22 | | | | | | | | |
| | LG0AAA1L23 | | | | | | | | | | -1,24 | | |
| | LG0AAB17B13 | | | | | -1,25 | | | | | | | |
| | LG0AAC4F8 | | | | | | | -1,26 | | | | | |
| | LG0AAD26H13 | | 1,39 | | | | | 1,40 | | | | | |
| | DN950344 | | | | | | | | | | | | -1,27 |
| | LG0AAB8P23 | | | | -1,34 | | | | | | 1,08 | | |
| | LG0AAD28H20 | | | | | | | | | | -1,29 | | |
| | LG0AAC31A7 | | -1,29 | | | | | | | | | | |
| | LG0AAD18H16 | | | | | | | -1,30 | | | -1,12 | | |
| | DN950578 | | | | | | | | | | | | -1,31 |
| | LG0AAD41M13 | | | | | | -1,31 | | | | | | |
| | CU656250 | | | | | | -1,17 | | | | | | |
| | LG0AAD27B15 | | | | | | | | | | -1,26 | | |
| | CU640071 | | | 1,88 | | | | | | | | | |
| | LG0AAD21I21 | | | | | | | | | | | 2,31 | |
| | CR627740 | | | 2,29 | | | | 5,85 | 3,33 | | | | |
| | LG0AAD38E10 | | | | | | | | 2,28 | | | | |
| | LG0AAC21P12 | | | | | | 2,26 | | | | | | |
| | LG0AAD29K4 | | | | | | | | | | | | 2,22 |
| | LG0AAD48M9 | | | 1,67 | | | | | | | | | |
| | CR627795 | | 1,90 | | | | | 2,59 | 4,70 | | | | |
| | LG0AAC23H2 | | | | | | | | 2,53 | | | | |
| | LG0AAA9L10 | | | | | | | | | 1,85 | | | |
| | LG0AAB10H16 | | | | | | | | | | 1,85 | | |
| | LG0AAC30L6 | | 1,85 | | | | | | | | | | |
| | LG0AAD7P8 | | 1,73 | 2,95 | 2,23 | 2,85 | | 2,64 | | | | | |
| | LG0AAD35G19 | | | | | | | 1,71 | | | | | |
| | LG0AAA1J12 | | | | | | | 1,39 | | | | | |
| | LG0AAD8L2 | | | | | | | | | | | 1,96 | |
| | CR627518 | | | | | 2,81 | | | | | | | |
| | DN950518 | | | | | | | | | | | | 11,10 |
| | DN950328 | | | | | | | | | | | | 8,51 |
| | LG0AAA5C21 | | | | | | | | | | | 5,12 | |
| | LG0AAB13I7 | | | | | | | | | | | 3,52 | |
| | LG0AAA1A6 | | | | | | | | | | | 3,12 | |
| | LG0AAC23J4 | | | | | | | | | | | 3,05 | |
| | LG0AAB18H14 | | | | | | | | | | | 2,32 | |
| | LG0AAD17A6 | | | | | | | | | | | 2,87 | |
| | DN949681 | | | | | 2,34 | | | | | | | 3,20 |
| | LG0AAB15C16 | | | | | | | | | | | 2,75 | |
| | LG0AAD34B5 | | | | | | | | | | | 2,71 | |
| | LG0AAB2G24 | | | | | | | | | | | 2,64 | |
| | LG0AAB23E15 | | | | | | | | | | | 2,60 | |
| | LG0AAD18M11 | | | | | | | | | | | 2,55 | |
| | DN949985 | | | | | | | 1,64 | | | | | |
| | LG0AAC18J17 | | | | | | | | 2,93 | | | | |
| | LG0AAD41I9 | | | 1,29 | | 1,81 | | | | | | | |
| | LG0AAC6C4 | | | | | | | | | | -1,51 | | |
| | LG0AAA10K9 | | | | | | | 1,38 | | | | | |
| | LG0AAB4K22 | | | | | | | | | | 1,36 | | |
| | LG0AAD50K14 | | | | | | | 1,34 | | | 1,40 | | |
| | LG0AAD18F10 | | | 1,34 | | | | | | | | | |
| | DN950275 | | | | 1,67 | | | 2,23 | | | | | |
| | LG0AAD28O1 | | 1,31 | 2,66 | | | | 2,83 | | | | | |
| | LG0AAD22D7 | | | | | | | | | | | | 1,41 |
| | LG0AAD16M10 | | | | | | | | | | 1,29 | | |
| | LG0AAB8I24 | | | | | | | | | 1,26 | | | |
| | DN949992 | | | | 1,25 | | | | 1,79 | | | | |
| | LG0AAC24C7 | | | | | | | | | | 1,24 | | |
| | LG0AAB26C7 | | 1,23 | | | | | | | | | | |
| | LG0AAB21M11 | | | | | | | 1,22 | | | | | |
| | LG0AAC9I21 | | | | | | | 1,31 | | | | | |
| | LG0AAD22G3 | | | | | | | | | | | | 1,48 |
| | LG0AAD15E24 | | | 1,71 | | | | 1,63 | | | | | |
| | LG0AAC25L1 | | | | | | | | | 1,59 | | | |
| | LG0AAC12P10 | | | | | | | 1,55 | | | | | |
| | LG0AAD35H11 | | | | | | | 1,51 | | | | | |
| | LG0AAD15D15 | | | | | | | | | | | | 1,50 |
| | LG0AAB27H22 | | | | | 1,49 | | | | | | | |
| | LG0AAD16G9 | | | | | | | | | | | | 1,39 |
| | LG0AAD47E8 | | | | | | | | | | 1,48 | | |
| | LG0AAD2C9 | | | | | | | | | | | -1,80 | |
| | LG0AAC5M15 | | | | | | 1,48 | | | 1,74 | | 3,95 | |

**Table A.5:** Continued from table A.4.

| | | | 2006 DS | | | | 2007 DS | | | 2007 RW | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FG | Name | Annotation | I | II | III | IV | I | II | V | I | II | III | V |
| unknown | LG0AAD45K16 | | | 1,56 | 1,54 | | | 1,46 | | | | | |
| | LG0AAD23C11 | | | | | | | 1,45 | | | | | |
| | LG0AAD19O21 | | | | | | | 1,44 | | | | | |
| | LG0AAA19J10 | | | | | | | | | | 1,42 | | |
| | DN951035 | | | | | | | 1,22 | 2,72 | | | | |
| | LG0AAD32F2 | | | 1,99 | 1,48 | | | | | | | | |
| | CU657113 | | | | | | | | | | | -2,51 | |
| | LG0AAD48H21 | | | | | | | | | | | -2,05 | |
| | LG0AAD10L8 | | | | | | | | | | | | -1,52 |
| | LG0AAC22C15 | | | | | | | | | | | -2,12 | |
| | CR628094 | | | | | | | | | | | | -2,14 |
| | LG0AAD26D21 | | | | | | | | | | | -2,17 | |
| | LG0AAD45E22 | | | | | | | | | | | -2,25 | |
| | LG0AAD43G13 | | | | | | | | | | | -2,28 | |
| | LG0AAD51M13 | | | | | | | | | | | -2,30 | |
| | CU656334 | | | | | | | | | | | -2,30 | |
| | LG0AAD36P17 | | | | | | | | | | | -2,32 | |
| | LG0AAD15N5 | | | | | | | | | | | -2,36 | |
| | LG0AAD18N13 | | | | | | | | | | -1,13 | -2,01 | |
| | CU656213 | | | | | -2,47 | | | | | | | |
| | LG0AAC8K13 | | | | | | | -2,03 | | | -2,02 | | |
| | LG0AAD44I20 | | | | | | | | | | | -2,59 | |
| | LG0AAC18G17 | | | | | | | | | | | | -2,60 |
| | LG0AAC24K24 | | | | | | | | | -1,34 | | -2,63 | |
| | DN950486 | | | | | | -3,11 | | | -2,66 | | | |
| | LG0AAD42K4 | | | | | | | | | | | | -3,11 |
| | LG0AAC28L3 | | | | | | | -3,18 | | | -1,94 | | |
| | LG0AAA17A4 | | -2,05 | | | -1,68 | | | -3,29 | | | | |
| | LG0AAB8H13 | | | | | | | | | | | -4,29 | |
| | LG0AAD44H7 | | | | | | | | | | | | -4,59 |
| | LG0AAC31B19 | | | | | | | | | | | | -5,48 |
| | DN950519 | | | | -10,59 | | | | | | | | |
| | DN950804 | | | | | -1,77 | | | | | | | |
| | DN949808 | | | | | | | | | | | -2,40 | |
| | LG0AAD1K14 | | | | | | | | | | | -1,71 | |
| | LG0AAC23I22 | | | | | | | | | | -1,53 | | |
| | LG0AAD35B10 | | | | | | | -1,53 | | | | | |
| | LG0AAD47C3 | | | | | | | | | | | -1,55 | |
| | CU657862 | | | | | | | | | | | -1,56 | |
| | LG0AAD45H14 | | | | | | | | | | -1,56 | | |
| | LG0AAA1N17 | | | | | | | -1,65 | | | | | |
| | DN950100 | | | | | | | | | | | -1,65 | |
| | LG0AAD43E12 | | | | | | | | | | | -1,66 | |
| | LG0AAA5D22 | | | | | | | -1,67 | | | -1,33 | | |
| | LG0AAB4C10 | | | | | | | -1,08 | | | -1,68 | | |
| | LG0AAA4L10 | | | | | | | -2,06 | | | | | |
| | CU656889 | | | | -1,69 | | | | | | | | |
| | LG0AAD10G8 | | | | | | | | | | | -1,98 | |
| | LG0AAA3E9 | | | | | | | | | | | -1,91 | |
| | LG0AAB5D11 | | | | | | | | | | | -1,95 | |
| | CU657199 | | | | | | | -1,94 | | | | | |
| | LG0AAC3K3 | | | | | | | | | -1,68 | | | |
| | DN950621 | | | | | | | -1,92 | | | | | |
| | DN950171 | | | | | | | -1,73 | | | -1,62 | | |
| | LG0AAA11N24 | | | | | | | | | | | -1,91 | |
| | LG0AAA17E12 | | | | | | -1,91 | -0,86 | | | | | |
| | CU657602 | | | | | | | -1,91 | | | | | |
| | DN950583 | | | | | | | | | | | -1,88 | |
| | LG0AAD4E5 | | | | | | | | | | -1,43 | -1,86 | |
| | LG0AAD32F16 | | | | | | | | | | | | -1,81 |
| | LG0AAD46G5 | | | | | | | | | | | -1,77 | |
| | LG0AAD15C14 | | | | | | | 1,06 | | | | -1,92 | |

**Table A.6:** Sample R results table.

| Name | logFC | AveExpr | t | P.Value | adj.P.Val | B |
|---|---|---|---|---|---|---|
| LG0AAA14C3 | 2,18 | 8,35 | 8,92 | 0,000001 | 0,005025 | 5,61 |
| CR627795 | 1,90 | 7,48 | 5,53 | 0,000120 | 0,048666 | 1,44 |
| CR628213 | 1,90 | 13,44 | 5,55 | 0,000117 | 0,048651 | 1,48 |
| LG0AAC30L6 | 1,85 | 5,29 | 7,96 | 0,000003 | 0,009921 | 4,31 |
| LG0AAD45E20 | 1,74 | 8,15 | 8,01 | 0,000003 | 0,009921 | 4,54 |
| LG0AAD7P8 | 1,73 | 8,25 | 5,77 | 0,000081 | 0,040866 | 1,82 |
| LG0AAD26H13 | 1,39 | 8,72 | 6,30 | 0,000036 | 0,040866 | 2,57 |
| LG0AAD45O17 | 1,38 | 8,11 | 6,87 | 0,000015 | 0,026506 | 3,31 |
| LG0AAD28O1 | 1,31 | 6,07 | 5,90 | 0,000067 | 0,040866 | 1,94 |
| LG0AAC7D2 | 1,29 | 5,46 | 6,44 | 0,000029 | 0,040093 | 2,48 |
| LG0AAB3P19 | 1,28 | 6,74 | 6,00 | 0,000056 | 0,040866 | 2,09 |
| LG0AAB26C7 | 1,23 | 5,89 | 5,74 | 0,000086 | 0,040866 | 1,66 |
| LG0AAB25G15 | 1,22 | 6,20 | 5,98 | 0,000059 | 0,040866 | 2,02 |
| CR627988 | 1,19 | 6,86 | 5,61 | 0,000105 | 0,046664 | 1,56 |
| LG0AAB19J18 | 1,13 | 9,46 | 5,79 | 0,000079 | 0,040866 | 1,84 |
| LG0AAD4C17 | 1,13 | 5,14 | 5,83 | 0,000074 | 0,040866 | 1,83 |
| LG0AAC24C5 | 1,09 | 7,13 | 5,73 | 0,000087 | 0,040866 | 1,70 |
| LG0AAD1N6 | 1,02 | 5,41 | 6,20 | 0,000042 | 0,040866 | 2,33 |
| LG0AAC25F3 | -0,84 | 7,74 | -5,68 | 0,000095 | 0,043280 | 1,65 |
| LG0AAD5D5 | -0,84 | 7,55 | -5,82 | 0,000075 | 0,040866 | 1,86 |
| LG0AAC22P21 | -0,87 | 13,31 | -5,80 | 0,000077 | 0,040866 | 1,83 |
| LG0AAD30J9 | -0,93 | 8,00 | -5,75 | 0,000084 | 0,040866 | 1,80 |
| LG0AAC15D15 | -0,94 | 7,21 | -5,88 | 0,000069 | 0,040866 | 1,95 |
| LG0AAC22H21 | -0,95 | 8,62 | -6,11 | 0,000048 | 0,040866 | 2,26 |
| LG0AAA1G20 | -0,95 | 7,05 | -6,42 | 0,000030 | 0,040093 | 2,67 |
| LG0AAD26H5 | -0,98 | 8,15 | -5,76 | 0,000083 | 0,040866 | 1,81 |
| LG0AAD40I18 | -1,01 | 6,35 | -6,15 | 0,000045 | 0,040866 | 2,28 |
| LG0AAD36J11 | -1,09 | 7,88 | -6,29 | 0,000036 | 0,040866 | 2,54 |
| CU639824 | -1,12 | 7,38 | -5,83 | 0,000074 | 0,040866 | 1,87 |
| LG0AAA4E11 | -1,15 | 7,66 | -6,58 | 0,000024 | 0,037270 | 2,90 |
| LG0AAA7C15 | -1,27 | 6,49 | -6,09 | 0,000049 | 0,040866 | 2,16 |
| LG0AAC31A7 | -1,29 | 6,85 | -6,01 | 0,000056 | 0,040866 | 2,13 |
| LG0AAB28C1 | -1,29 | 7,09 | -8,82 | 0,000001 | 0,005025 | 5,43 |
| LG0AAC3O14 | -1,30 | 5,31 | -5,58 | 0,000110 | 0,047848 | 1,35 |
| LG0AAC14O21 | -1,37 | 8,01 | -7,23 | 0,000009 | 0,017747 | 3,78 |
| LG0AAA5K19 | -1,40 | 9,68 | -5,84 | 0,000072 | 0,040866 | 1,93 |
| LG0AAA15B17 | -1,43 | 9,40 | -7,37 | 0,000008 | 0,016469 | 3,94 |
| LG0AAD13D11 | -1,48 | 6,05 | -5,54 | 0,000117 | 0,048651 | 1,43 |
| LG0AAD26C21 | -1,53 | 9,52 | -8,82 | 0,000001 | 0,005025 | 5,43 |
| CU657048 | -1,58 | 8,69 | -6,23 | 0,000040 | 0,040866 | 2,48 |
| LG0AAA2B20 | -1,70 | 10,07 | -5,82 | 0,000075 | 0,040866 | 1,90 |
| LG0AAA17A4 | -2,05 | 7,66 | -10,15 | 0,000000 | 0,004360 | 6,46 |
| LG0AAC27F5 | -2,38 | 8,60 | -7,38 | 0,000007 | 0,016469 | 3,95 |

# APPENDIX B

## Abstract / Zusammenfassung

## B.1 Abstract

For the next decades a climate change with longer drought periods during the summer months is expected. This will increase the need, with regard to sustainable forest management and afforestation purposes, for trees that posses the potential to adapt easily to longer drought periods. The objectives of this study were to characterize the response of *Quercus robur* to long-term drought stress and rewatering on the whole plant level.

In a three-year glasshouse experiment five year old clonally propagated oak plants from tissue culture were subjected to controlled drought stress and subsequent rewatering. Phenotypic parameters were monitored and leaf material was sampled in regular intervals for analyses of the transcriptome (cDNA-microarray), proteome and physiological parameters (carbohydrates, polyols, proline). The experiment comprised three treatments: 1) control, 2) one year drought - one year rewatering, 3) two years drought, one year rewatering.

On the phenotypic level, the application of drought stress led to a growth reduction in the first year and a growth cessation in the second (absence of lammes shoots). Flushing time changed in the second year for both treatment groups, plants under continued drought stress and rewatered plants, indicating the use of drought avoidance strategies by *Q. robur*. Rewatered oak plants further showed a strong compensation growth combined with a shifted growth pattern compared to the controls. While in control plants the main growth usually took place during the first flush, rewatered plants displayed their main growth in subsequent flushes.

On the physiological level, a significant increase in the concentrations of glucose, fructose and galactose under drought stress could be observed in both years. Furthermore, significantly higher concentrations of quercitol and mannitol were detected in the second

year of the drought stress treatment. Rewatered plants showed a similar pattern of sugar and quercitol concentrations compared to the controls while the concentration of mannitol returned to control level not before the last sampling. For proline and related compounds no significant correlation between concentration and stress treatment could be observed. It is suggested that in *Q. robur* carbohydrates and polyols play a major role in osmotic adjustment, stress adaptation and recovery where they may serve as energy source. Proline and related compounds seem to be of minor importance in the long-term stress response and adaptation of *Q. robur*.

For the analysis of plant responses on the transcriptome level a cDNA array comprising 18545 unigenes was used. In *Quercus robur* the long-term drought stress treatment (two growing seasons) activated a very specific response at each sampling time depending on intensity and duration of the water deficit with the majority of the genes (88%) being induced at only one time point.

Under drought stress an induction of genes involved in starch degradation, reinforcement of the cuticula, genes with protective function and senescence associated genes could be observed. A repression of photosynthesis could only be detected at the end of the first year of drought stress. In the second year no significant down-regulation of photosynthesis-related genes under drought stress occured. In general, the response to water deprivation seems to be stronger in the second year leading to a better defence and consequently protection of metabolic processes such as photosynthesis.

Rewatering resulted in the (early) recovery of photosynthesis, down-regulation of genes with protective function and senescence-associated genes, slow but strong induction of growth processes, and up-regulation of "rehydration-specific" genes.

Based on these results easily applicable functional markers for the selection of trees with increased drought resistance level will be developed. Suggested candidate genes for further selection of drought resistant oak trees are "WAX2" and lipid transfer protein (LTP), both being involved in cuticle biosynthesis. The induction of these genes could lead to reduced water loss under drought stress through a reinforcement of the cuticula resulting in decreased transpiration rates. Furthermore, lipid transfer proteins have a proposed function in the repair of stress-induced damage in membranes. Other potential candidates for selection would be genes encoding for late embryogenesis abundant protein (LEA protein) and rare cold inducible 2B (RCI2B) with proposed function in maintaining membrane function and/or integrity under water stress. Increased synthesis of these proteins might prevent damage and maintain metabolic processes even at low water potential.

## B.2 Zusammenfassung

Aktuellen Voraussagen zufolge wird in den nächsten Jahrzehnten ein Klimawandel mit längeren Trockenperioden während des Sommers erwartet. Dies macht eine Selektion von Bäumen für Aufforstungszwecke mit erhöhtem Adaptionspotential gegenüber Trockenstress notwendig. Ziel dieser Studie war die Charakterisierung der Stressantwort von *Quercus robur* auf Langzeittrockenstress und Wiederbewässerung auf Ebene der gesamten Pflanze. Dazu wurden in einem dreijährigen Glashausversuch 5-jährige Eichenklone Langzeittrockenstress mit anschließender Regenerationsphase (Kontrollbedingungen) ausgesetzt. Es wurden Analysen auf morphologischer (Pflanzenentwicklung und -wachstum), physiologischer (Kohlenhydrate, Polyole, Prolin) und RNA-Ebene (Microarray) durchgeführt. Das Experiment umfasste drei Behandlungen: 1) Kontrolle, 2) ein Jahr Trockenstress - ein Jahr Wiederbewässerung, 3) zwei Jahre Trockenstress, ein Jahr Wiederbewässerung.

Auf phenotypischer Ebene führte die Applikation von Trockenstress zunächst zu einer Wachstumsreduktion im ersten Jahre und schließlich zu einem Wachstumsstopp im zweiten Jahr. Zudem trat im zweiten Jahr der Austrieb von Trockenstress- und wieder bewässerten Pflanzen signifikant früher auf als bei der Kontrollgruppe was auf die Anwendung von Trockenstressvermeidungsstrategien durch *Q. robur* schließen lässt. Wieder bewässerte Pflanzen zeigten ein starkes Kompensationswachstum, jedoch ein anderes Wachstumsmuster/-verhalten als die Kontrollpflanzen. Während das Hauptwachstum in der Kontrollgruppe im ersten Austrieb stattfand war die Hauptwachstumsphase der wieder bewässerten Pflanzen auf die folgenden Austriebe verlagert.

Auf physiologischer Ebene konnte unter Trockenstress ein signifikanter Anstieg von Glukose, Fruktose und Galaktose in beiden Versuchsjahren festgestellt werden. Im zweiten Jahr konnten zudem signifikant erhöhte Quercitol- und Mannitolkonzentrationen unter Trockenstress gemessen werden. Wieder bewässerte Pflanzen zeigten bei den Zuckern sowie Quercitol ein ähnliches Muster wie die Kontrollen während die Konzentration von Mannitol erst bei der letzten Probenahme auf Kontrolllevel absank. Für Prolin, Trigonellin und Glycin-Betain konnte keine signifikante Korrelation zwischen Konzentration und Trockenstressbehandlung festgestellt werden. In *Q. robur* scheinen Kohlenhydrate und Polyole die Hauptrolle bei der osmotischen und Stressanpassung zu spielen und als Energiereserve in Erholungsphasen zu dienen. Prolin, Trigonellin und Glycin-Betain hingegen scheinen bei der Langzeit-Stressantwort und -Anpassung nur von untergeordneter Bedeutung zu sein.

Für die Analyse der Transkriptomebene wurde ein cDNA-Microarray mit 18545 “Uni-

genes" verwendet. Unter Langzeittrockenstress (2 Vegetationsperioden) konnte in *Q. robur* zu jedem untersuchten Zeitpunkt eine sehr spezifische Reaktion beobachtet werden. Die Mehrheit der Gene (88%) wurde nur zu einem Zeitpunkt aktiviert, abhängig von Intensität und Dauer des applizierten Trockenstresses. Unter Trockenstress wurden Gene induziert die involviert sind in Stärkeabbau, Verstärkung der Cuticula, Seneszenz und Gene mit Schutzfunktion (chaperones). Eine Herunterregulierung der Photosynhtese konnte nur am Ende des ersten Jahres unter Trockenstress festgestellt werden. Im zweiten Jahr fand keine signifikante Herunterregulierung der Photosynthese statt. Generell scheint die Stressantwort im zweiten Jahre stärker auszufallen und führte scheinbar zu einer besseren Stressabwehr und Schutz metabolischer Prozesse (wie z.B. der Photosynthese). Wiederbewässerung hingegen führte zu einer Induktion/Erholung der Photosynthese, Herunterregulation von Genen mit Schutzfunktion und Genen die in Seneszenz involviert sind, langsamer aber starker Induktion von Wachstumsprozessen und Hochregulation von "rehydration-specific genes".

Auf Grundlage dieser Ergebnisse werden stressinduzierte Gene auf ihre Eignung für die Erstellung eines funktionellen Markersystems zur Selektion trockenstresstoleranter Eichen untersucht. Als potentielle Kandidaten für die weitere Selektion trockenstressresistenter Eichen kämen die Gene "WAX2" und "lipid transfer protein" (LTP) in Frage, die beide in die Synthese der Cuticula involviert sind. Die Induktion dieser Gene könnte zu vermindertem Wasserverlust unter Trockenstress führen durch eine Verstärkung der Cuticula und somit reduzierten Transpirationsraten. Zudem wird LTPs eine Funktion in der Reparatur stressinduzierter Membranschäden zugeschrieben. Andere potentielle Kandidaten für die Selektion wären Gene die für "late embryogenesis abundant proteins" (LEA) codieren sowie "rare cold inducible 2B" (RCI2B) mit möglicher Funktion in der Erhaltung der Membranfunktion und/oder -integrität unter Trockenstress. Eine erhöhte Synthese dieser Proteine könnte demnach durch Trockenstress hervorgerufenen Schäden vorbeugen und metabolische Prozesse auch bei niedrigem Wasserpotential aufrechterhalten.

# APPENDIX C

## Curriculum Vitae

### Personal Data

**Name:** Nadine Schulz

**Place and date of birth:** Germany, Limburg/Lahn, 14. November 1980

### Education

**1987 – 1989** Grundschule Birlenbach

**1989 – 1991** Grundschule Lingenfeld

**1991 – 1997** Goethe-Gymnasium Germersheim

**1997 – 2000** Purrmann-Gymnasium Speyer

**Juni 2000** Abitur

### University Education

University of Hohenheim - Study of Agricultural Sciences

**10/2000 – 02/2004** Bachelor of Science, Major in Crop Science, Grade point average: 3.1 ("good")

- One Semester at the Iowa State University (08/2002 – 12/2002)
- Internship and Bachelor-Thesis Work at BASF, Agrarzentrum Limburgerhof, Herbizidforschung (02/2003 – 04/2003)
- Internship at the "Staatlichen Lehr- und Forschungsanstalt Neustadt/Weinstr." (05/2003 – 07/2003)

**03/2004 – 10/2005** Master of Science, Major in Crop Science, Specialization in Phytomedicine, Grade point average: 3.6 ("very good")

- Two Semesters at the University of Illinois at Urbana-Champaign (08/2004 – 05/2005)

University of Vienna - PhD Thesis in Biology/Ecology

**2006 – 2010** PhD Thesis conducted at the Austrian Institute of Technology Seibersdorf

## Scholarships

- DAAD Scholarship for one Semester at the Iowa State University
- Baden-Württemberg Scholarship for one academic year at the University of Illinois at Urbana-Champaign

## Research Experience

**Fall 2002** Independent Study "*Erwinia Tracheiphila*, Causal Agent of Bacterial Wilt in Cucurbits", conducted at the Iowa State University

**02/2003 – 04/2003** Bachelor Thesis "Vergleich von Testmethoden zur Erfassung von Herbizidresistenz bei *Alopecurus myosuroides* Huds.", conducted at BASF

**Fall 2004** Entomolgy Project: "Parasitoid Host Range – Important Physiological and Ecological Determinants", conducted at the Department of Entomolgy – University of Illinois at Urbana-Champaign

**03/2003 – 09/2005** Master Thesis "Glucosinolate governed tritrophic interactions of broccoli, *Myzus persicae*, and *Aphidius colemani*", conducted at the University of Illinois at Urbana-Champaign

**2006 – 2010** PhD Thesis "Monitoring of the evnironmental influence on stress regulated genes in oak", conducted at the Austrian Institute of Technology Seibersdorf

**2007 –2010** co-work at project "Heavy metal stress in Pea", at the Austrian Institute of Technology Seibersdorf

## Participation

- Conference on Genomics of Forest and Ecosystem Health in the Fagaceae (Beech Family); November 10-13, 2009, North Carolina, USA
- International Conference: Plant Abiotic Stress Tolerance; 08.-11.02.2009, Vienna Austria
- EVOLTREE Researcher training course "Response to abiotic and biotic stresses", September 22nd-26th, 2008, Technische Universität München, Helmholtz-Zentrum München
- SHE 2008 (FIRST SYMPOSIUM ON HORTICULTURE IN EUROPE); February 17th - 20th, Vienna
- PROTEOMLUX 2006, International Conference on Proteomics: "Bridging the Gap Between Gene Expression and Biological Function", 11-14 October 2006, Luxembourg
- 3$^{rd}$ student seminar of the department of Biogenetics and Natural Resources, University of Natural Resources and Applied Life Sciences, Vienna, November 2006
- Poster competition ARC Seibersdorf, June 2006

## Publications

Alleyne, M; Schulz, N; Dam, NM Van; and Waeckers, F (2005). Effect of glucosinolate content of broccoli leaves on development of the aphid parasitoid *Aphidius colemani*. In *The 2005 ESA Annual Meeting and Exhibition, December 15-18, 2005, Florida, USA; (Poster)*.

Fluch, S.; Homolka, A.; Burg, K.; Berenyi, M.; Schulz, N.; Wilhelm, E.; and Schüler, S. (2008). Simple methode for snp typing of drought stress related genes in european oak (quercus spp.). In *IUFRO-CITA 2008 Joint Conference, August 2008, Quebec (Poster)*.

Homolka, A.; Berenyi, M.; Schüler, S.; Schulz, N.; Fluch, S.; and Burg, K. (2009). Gene based diversity of austrian oak (*Q. robur*, *Q. petraea*) populations. In *Conference on Genomics of Forest and Ecosystem Health in the Fagaceae (Beech Family), November 10-13, 2009, North Carolina, USA; (Poster)*.

Homolka, A.; Burg, K.; Berenyi, M.; Fluch, S.; Schulz, N.; Wilhelm, E.; and Schüler, S. (2008a). Stress gene based diversity of austrian oak populations. In *The Joint Annual Meeting LIFESCIENCES 2008 of the ÖGBM, ÖGGGT, ÖGBT and ANGT, Sept. 08 Graz (Poster)*.

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Homolka, A.; Burg, K.; Berenyi, M.; Fluch, S.; Schulz, N.; Wilhelm, E.; and Schüler, S. (2008c). Stress gene based diversity of austrian oak populations. In *Molecular Mapping and Marker Assisted Selection in Plants, Februar 2008, Wien (Poster)*.

Oufir, Mouhssin; Schulz, Nadine; Vallikhan, Patan Shaik Sha; Wilhelm, Eva; Burg, Kornel; Hausman, Jean-Francois; Hoffmann, Lucien; and Guignard, Cedric (2009). Simultaneous measurement of proline and related compounds in oak leaves by high-performance ligand-exchange chromatography and electrospray ionization mass spectrometry for environmental stress studies. *J Chromatogr A*, 1216(7):1094–1099. doi:10.1016/j.chroma.2008.12.030.

Pirselová, B.; Schulz, N.; Libantová, J.; Moravcíková, J.; Fluch, S.; and Matusíková, I. (2009). Different effects of cadmium, lead and arsenic on roots of maize and soybean.

In *International Conference: Plant Abiotic Stress Tolerance; 08.-11.02.2009, Vienna Austria; (Poster).*

Schulz, N.; Burg, K.; Fluch, S.; Oufir, M.; Hausman, J.-F.; and Wilhelm, E. (2008a). Understanding oak drought stress response: A wholistic approach. In *International conference: Mountain Forests in a changing world; 02.04.-04.04.2008, Vienna Austria; (Poster).*

Schulz, Nadine; Burg, Kornel; Fluch, Silvia; Oufir, Mouhssin; Hausman, Jean-Francois; and Wilhelm, Eva (2009a). How oak responds to long term drought stress - a wholistic approach. In *Conference on Genomics of Forest and Ecosystem Health in the Fagaceae (Beech Family), November 10-13, 2009, North Carolina, USA; (Oral Presentation).*

Schulz, Nadine; Burg, Kornel; Fluch, Silvia; Oufir, Mouhssin; Hausman, Jean-Francois; and Wilhelm., Eva (2009b). Oak under long-term drought stress & recovery - morphological & physiological responses. In *International Conference: Plant Abiotic Stress Tolerance; 08.-11.02.2009, Vienna Austria; (Poster).*

Schulz, Nadine; Burg, Kornel; Fluch, Silvia; Oufir, Mouhssin; Sergeant, Kjell; Hausman, Jean-Francois; and Wilhelm., Eva (2010). A wholistic view on the effect of long-term drought stress and rewatering in oak. In *Conference on Forest ecosystem genomics and adaptation; 9-11 June, 2010, San Lorenzo de El Escorial (Madrid), Spain; (Abstract accepted).*

Schulz, Nadine; Wilhelm, Eva; Burg, Kornel; Fluch, Silvia; Oufir, Mouhssin; and Hausman, Jean-Francois (2008b). A wholistic approach in understanding oak drought stress response. In *SHE 2008 (First Symposium on Horticulture in Europe), February 17th -20th, Vienna; (Poster).*

Sergeant, Kjell; Schulz, Nadine; Oufir, Mouhssin; Hausman, Jean-François; Renaut, Jenny; and Wilhelm, Eva (2008). Drought stress in oak: a systems biology approach. In *Proteomlux 2008, International Conference on Proteomics in Plants, Microorganisms and Environment; 22.10.08-25.10.08, Luxemburg (Oral Presentation).*

# Oak under Long-term Drought Stress & Recovery – Molecular, Morphological & Physiological Responses

*Nadine Schulz[1], Kornel Burg[1], Silvia Fluch[1], Mouhssin Oufir[2], Jean-Francois Hausman[2], Eva Wilhelm[1]*

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[2]Centre de Recherche Public Gabriel Lippmann, Luxemburg

## Strategy

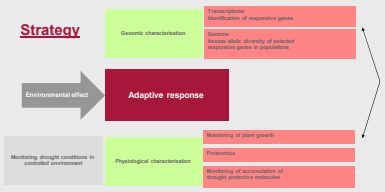
### Background

According to current predictions on climate change longer drought periods during summer months are expected. Therefore, there is a need for trees with higher adaptation potential to drought as planting material for afforestation purposes. In this study, the effect of long-term drought stress and rewatering on young oak trees (*Quercus robur*) is investigated. The final goal of the study is the development of easily applicable functional markers for selection of trees with a higher drought resistance level from natural populations.

### Plant Material & Experimental Design

In a three-year glasshouse experiment five year old clonally propagated oak plants from tissue culture were subjected to controlled drought stress and subsequent rewatering.

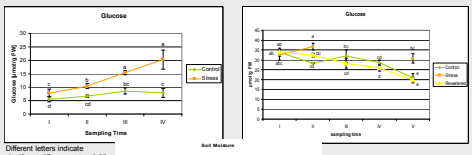
Treatments
1) Control (well watered)
2) one year drought - one year rewatering
3) two years drought - one year rewatering.




## Plant Physiology

### Carbohydrates/Polyols

Carbohydrates 2006 2007

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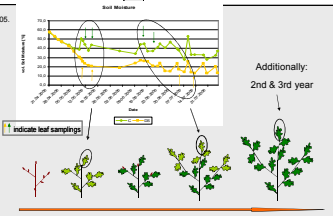
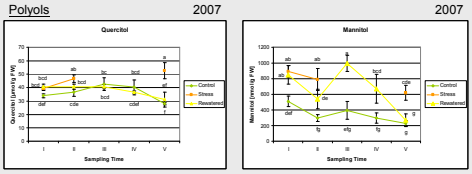



Polyols 2007 2007




#### Summary/Conclusions

**Drought Stress**

•Significantly higher levels of carbohydrates/polyols compared to controls (both years)

•Mannitol showed sign. higher concentrations only in the 2nd year

**Rewatering**

•Similar pattern as controls for glucose, fructose, sucrose, galactose & quercitol

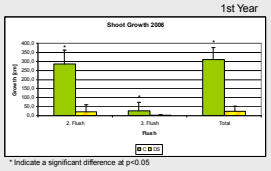
•Sign. higher mannitol level at sampling times I-IV

**Adaptation to drought stress through accumulation of osmolytes (carbohydrates, polyols)**

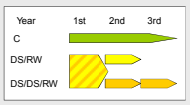
## Plant Morphology

### Plant Growth & Development

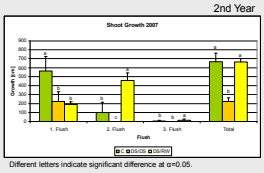
1st Year




* Indicate a significant difference at p<0.05

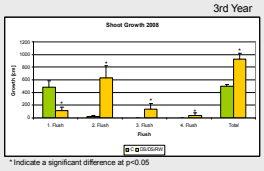



2nd Year




Different letters indicate significant difference at α=0.05.

3rd Year




* Indicate a significant difference at p<0.05

#### Summary/Conclusions

**Drought Stress (DS)**

•Reduced shoot growth
•no 2nd/3rd flush in 2nd year
•Reduced stem diameter
•Earlier 1st flush in 2nd year (compared to control)

**Rewatering (RW)**

•Compensation growth – increasing with preceeding stress duration

•Flushes earlier than in control

•Different growth pattern than controls – main growth during 2nd and 3rd flush

**Control (C)**

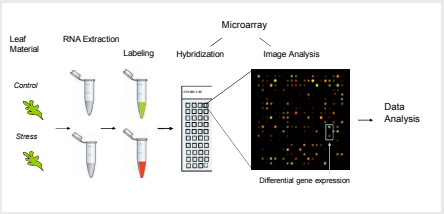
•Main growth during 1st flush (except 1st year – pattern like in rewatered plants)

**Growth reduction under drought stress but full recovery of plants when rewatered**

## Proteomics/ Microarray

### Current & Future Work

**Gene Expression Profiling - Microarray-Analysis**




**Microarray**

• ~30,000 oak ESTs from *Quercus robur* and *Quercus petraea*
• Gene expression analysis of control vs. drought treated plants

**Proteomics-Analysis**

•Fluorescense Difference Gel Electrophoresis (2D-DIGE)

•Comparison control vs. stress (plus internal standard)

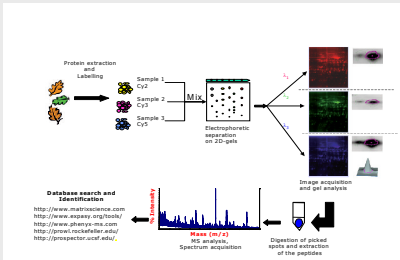



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**Figure C.1:** Poster at the Plant Abiotic Stress Conference 2008.

# Monitoring of the evnironmental influence on stress regulated genes in oak

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Biogenetics and Natural Resources, Biotechnology

## Introduction

During the following years/decades an increase in the annual temperature is expected, which will cause drought periods (in the summer) to arise more often and more severely.
This means, plants have to adapt to these changing conditions in order to survive. However, for trees with their long lifespan this might be more challenging than for short-lived plants.
Trees which will be planted now or in the near future will most probably be exposed to these changes in climate during their life span. For this reason, selecting trees for afforestation, which are adapted best to dry conditions would be reasonable.
One possibility to select for those trees which are adapted best to drought stress is the use of molecular markers or, more precisely, functional markers. These are functionally characterised genes with polymorph allele sequences which are linked to a certain phenotype (e.g. drought resistance). In the present study, single nucleotide polymorphisms (SNPs) will be used for the estimation of the genetic adaptability of oaks to drought stress.

## Preliminary Tests

**Aim:** Determination of sampling times for subsequent experiments

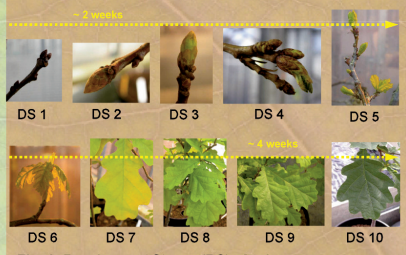


**Fig. 1:** Development Stages (DS) of oak

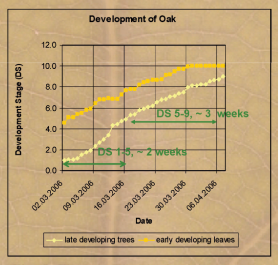


**Fig. 2:** Time needed for leaf development of oak

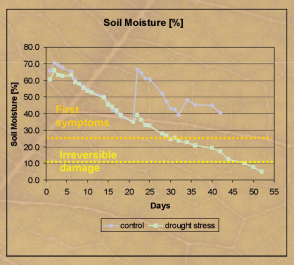


**Fig. 3:** Time needed for drought stress symptoms to occur

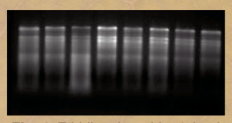

**Fig. 4:** Ethidium bromide stained 1.5% agarose gel of RNA from oak leaves

**Fig. 5:** Control plant with sufficient water supply

**Fig. 6:** Drought stress plant showing first symptoms

**Fig. 7:** Drought stress plant showing irreversible damage

## Approach

The project will be divided into two parts. In the first part of the project the effect of a two-year drought stress on gene expression, transcription of stress regulated genes and on physiology will be investigated. In the second part, suitable genes will be selected for marker development based on the results from the first part.
The first part of the project will subdivided into two experiments (Exp. I & II):

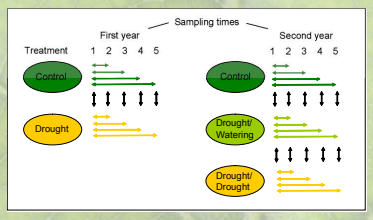


**Fig. 8:** Hybridization Scheme Exp. I

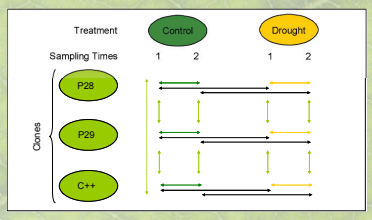


**Fig. 9:** Hybridization Scheme Exp. II

## Experimental Design

Experiment I
One oak clone will be used in a two-year experiment to compare the effects of drought on the transcriptome (cDNA-Microarray), on proteomics level and on physiological level. For this experiment three treatments will be applied: 1) Control (regular watering up to saturation point), 2) Drought Stress (repeated cycles of water stress from saturation to 20% soil moisture) in the first & second year, and 3) Drought stress in the first year but regular watering in the second year (Fig. 10).

Experiment II
Three clones, consisting of five year old oaks from somatic embryogenesis, will be exposed to drought stress during the summer months for two growing seasons while the control group (of each clone) will be sufficiently supplied with water.
Leaves of each group will be collected twice a year for RNA-isolation and subsequent cDNA-Microarray analysis in order to explore the gene expression profile under stress and control conditions, respectively.

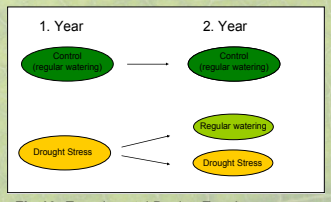


**Fig.10:** Experimental Design Exp. I

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**Figure C.2:** Poster for Poster Competition at the ARC Seibersdorf.

# Physiological & Molecular Responses of Oak to Drought Stress

*Nadine Schulz, Eva Wilhelm, Kornel Burg, Silvia Fluch,* Austrian Research Centers GmbH – ARC, Biogenetics and Natural Resources, Biotechnology

## INTRODUCTION

During the following decades a climate change with drought periods is expected. This means, trees with their long lifespan have to adapt to these changing conditions in order to survive. For this reason, selecting trees for afforestation which are adapted best to dry conditions would be reasonable. The final goal of the project is to develop functional markers for the selection of drought resistant oak varieties.

## Experimental Design

Experiment I

In a two-year experiment one oak clone (five year old plantlets) is used to compare the effects of drought on the transcriptome (cDNA-Microarray), on the proteomic level and on the physiological level (proline and carbohydrates). Three treatments are being applied: 1) Control (regular watering up to saturation point), 2) Drought Stress (repeated cycles of water stress from saturation to 15% soil moisture) in the 1st & 2nd year, and 3) Drought stress in the 1st year but regular watering in the 2nd year.

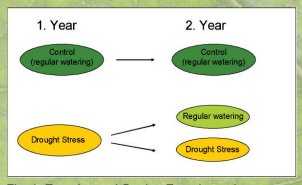


Fig. 1: Experimental Design Experiment I

Experiment II

Three clones (five year old oaks from somatic embryogenesis) are exposed to drought stress during the summer months for two growing seasons while the controls are sufficiently supplied with water.

Leaves of each group are collected twice a year for RNA-isolation and subsequent cDNA-Microarray analysis in order to explore the gene expression profile under stress and control conditions, respectively (Fig. 2). Furthermore, physiological parameters (shoot number and shoot length) are monitored.

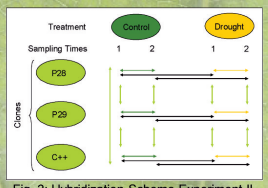


Fig. 2: Hybridization Scheme Experiment II

## Materials & Methods

Water regime: Controls are sufficiently supplied with water (volumetric soil moisture content of 30-50%) whereas drought stress treated plants are allowed to dry down to about 20%, after adaptation to about 15% volumetric soil moisture.

Soil moisture is measured with a FD-Probe via access tubes (figure 3 & 4) 3 times a week. Prior to leaf samplings measurements were conducted daily.

Fig. 3: Acces tubes for soil moisture measurement

Fig. 4: FD-Probe for soil moisture measurement

## Results

First symptoms of drought stress could be observed at around 25% volumetric soil moisture (Fig. 5b). When soil moisture (quickly) decreased below 10% the oak plants were irreversibly damaged (Fig. 5c). However, after several weeks of drought stress treatment the oak plants showed a certain degree of adaptation and soil moisture could be reduced to 10-15% (Fig. 6) without the occurrence of the symptoms described in Fig. 5.

Fig. 5: a) control, b) first symptoms, c) irreversible damage

As another effect of drought stress, however, partial leaf colouring and leaf fall could be observed in the drought stressed plants at the end of July. Yet, leaf shedding in autumn was delayed by several weeks in the drought stressed plants compared to the controls.

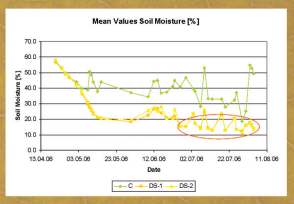


Fig. 6: Water regime for control (C) and drought stressed plants (DS).

At the end of May a second flush could be observed (Fig. 8) in the control plants of two of the three clones - P28 and P29. The controls (C) of clone C++ developed new shoots only ten days later. However, in all three clones the second flush of the drought stressed (DS) plants was delayed by four weeks compared to the controls (Fig. 7). Furthermore, the mean growth of the drought stressed plants was significantly reduced ($p<0.05$) compared to the controls in all clones (Fig. 9 & 10). The drought stressed plants of clone P28, however, grew significantly more ($p<0.05$) than the drought stressed plants of clones P29 and C++ . Additionally, the controls showed a third flush at the beginning of July (Fig. 7, 8, & 10). In the drought stressed plants a third flush did not occur apart from some isolated and short shoots at some trees of clone P28 (Fig. 10).

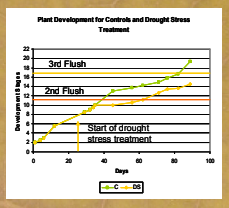


Fig. 7: Effect of drought stress on plant development of oak

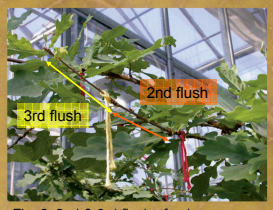


Fig. 8: 2nd & 3rd flush of oak

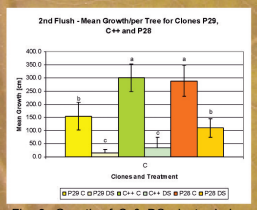


Fig. 9: Growth of C & DS plants during the 2nd growth period (student's t-test with $\alpha = 0.05$).

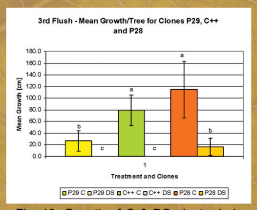


Fig. 10: Growth of C & DS plants during the 3rd growth period(student's t-test with $\alpha = 0.05$).

## Conclusion

In this study, the drought stress treatment significantly affected plant development ($p<0.05$) in all clones. This corresponds with Dils & Day (in "Physiologie und Ökologie der Gehölze", Ed. Lyr, 1992) who found, that plants exposed to a few weeks of drought during the summer show strongly reduced growth (30-70%) compared to trees with sufficient water supply. Regarding the physiological parameters only, clone P28 seems to be adapted best to drought stress conditions. During the second flush it showed significantly more growth per tree ($p<0.05$) than clones P29 and C++. Furthermore, it was the only clone which had a third flush under drought stress.

**Further work** to be done: RNA extractions for construction of cDNA microarrays, proteomic analysis and analysis of proline and carbohydrates.

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**Figure C.3:** Poster for BOKU Student Seminar.

# A wholistic approach in understanding oak drought stress response

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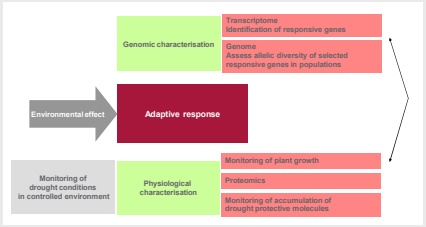
## Background

Drought periods due to climate changes, as being forecasted for the upcoming decades, will demand specific drought tolerating strategies of trees due to their long life cycle.

Selecting trees with the potential to more easily adapt to the changing environmental conditions would be reasonable, especially for afforestation purposes where sustainable forest management is a major issue.

Thus, the final goal of this project is to develop easy applicable functional markers for drought resistant oaks from natural poulations. These can support plant selection to enhance drought resistance of oaks (Quercus spp.).

## Strategy




## Plant Material & Experimental Design

In order to identify informative markers, four approaches are combined. Based on a two-year glasshouse experiment, five year old clonal oak plants originating from tissue culture are subjected to controlled drought stress conditions in a closed environment. During this time, in order to get a 'full picture' of the response to long-term drought stress on the "whole-plant level",

i) the transcriptome (cDNA-Microarray)

ii) the proteome

iii) physiological parameters such as proline and carbohydrate content as well as

iv) phenotypic paramters are monitored and plant material is sampled in regular intervals.

During the two years of the experiment, three treatments have been applied:

1) control: soil kept moist (vol. soil moisture > 30%),

2) drought stress: cycles of increasing water stress from 20% to <10% vol. soil moisture for two growing seasons and

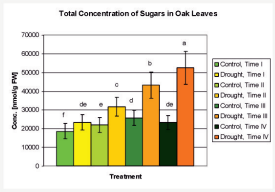
3) drought stress in the 1st year but "control conditions" in the 2nd year (rewatering).

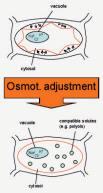
## Results

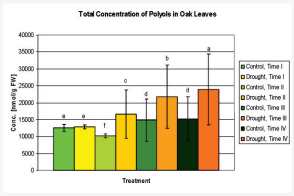
### Carbohydrate & Polyol Analyses – 1st Year

**Sugars**

- Significantly higher sugar concentrations in drought stressed plants compared to controls at each sampling time ($p<0.05$)
- Significant accumulation of carbohydrates with persisting drought stress treatment ($p<0.05$)



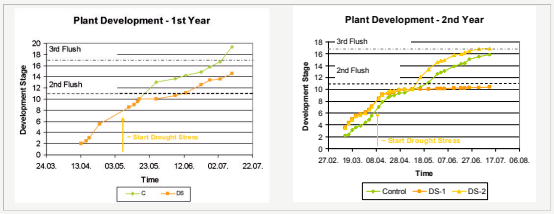



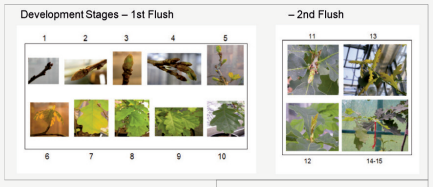




**Polyols (sugar alcohols)**

- Significantly higher polyol concentrations in drought stressed plants compared to controls at sampling times 2-4 ($p<0.05$)
- Significant accumulation of polyols with persisting drought stress treatment ($p<0.05$)

### Plant Development – 1st & 2nd Year




**1st Year**

- Significant growth reduction ($p<0.05$) under drought stress during 2nd & 3rd flush
- Significant delay ($p<0.05$) of plant development under drought stress - 2nd flush ~ 4 weeks later than in control plants

**2nd Year**

1st flush

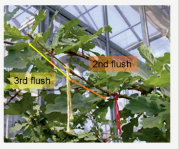
- Leaf development of DS-1 (1st & 2nd year drought) and DS-2 (1st year drought, 2nd year rewatered) starts significantly earlier than in controls ($p<0.05$)
- Continuously stressed plants (DS-1) and rewatered plants (DS-2) grow significantly less than controls ($p<0.05$)

2nd flush

- Oaks under continued drought stress (DS-1) do not show a 2nd or 3rd flush
- Rewatered plants (DS-2) show significantly stronger growth than controls ($p<0.05$)
- 2nd flush occurs significantly earlier in rewatered plants than in controls ($p<0.05$)

**Total growth 1st & 2nd year**

- Control (C) > rewatered plants (DS-2) > continuously drought stressed plants (DS-1)




Determination of length (cm) and number of shoots per tree (for each flush)

## Summary/Conclusions

- **Osmotic adjustment in drought stressed plants through accumulation of sugars and polyols**
- **1st year of drought stress: Growth delay and reduction**
- **2nd year of drought stress (DS-1): growth cessation after 1st flush**
- **After rewatering (2nd yr., DS-2): growth compensation during 2nd & 3rd flush**
- **2nd year: growth during 1st flush is influenced by stress conditions of preceding growing season**

## Further Steps

- Gene Expression Profiling
- Proteomic Analysis
- Carbohydrate Analysis for 2nd year

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**Figure C.4:** Poster at the SHE Conference 2008 in Vienna.