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DISSERTATION

Titel der Dissertation

Drought response strategies mediated by plant-rhizobia interaction in *Medicago truncatula*

verfasst von:
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Acronyms

Aa amino acid

ABA abscisic acid

C carbon

c. circa

DW dry weight

EA elemental analyser/analysis

GC gas chromatography

Hepes 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IR infra red

IR isotope ratio

LC liquid chormatography

LTQ Linear Trap Quadrupole

Mbp mega base pairs

MDA malondialdehyde

MS mass spectrometry

MSTFA *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide

Myr million years

N nitrogen

NN non-nodulated

NOD(e) *Sinorhizobium medicae* nodulated

NOD(i) *Sinorhizobium meliloti* nodulated

NOD nodulated

PGPR plant growth promoting rhizobacteria

PUFAs polyunsaturated fatty acids

ROS reactive oxygen species

SWC substrate/soil water content

TBA thiobarbituric acid

TBARS thiobarbituric acid reactive species

TOF time-of-flight

VAM vesicular-arbuscular mycorrhiza

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Chapter 1

General introduction

1.1 Plant responses to drought

According to several models of the Intergovernmental Panel on Climate Change (IPCC) the global surface temperatures will rise in future, potentially leading to more extreme drought events which may develop faster and with greater intensity (IPCC, 2007; Trenberth et al., 2013). Water deficits develop in the tissues of higher plants, when water uptake by the roots is lower than the evapotranspirative demand. Water scarcity is a major factor limiting agricultural productivity (Boyer, 1982; Araus et al., 2002). As water is involved in every aspect of plant life, plants have evolved several strategies to overcome the damaging effects of drought. These strategies combine changes in morphological, phenological and physiological traits. Traditionally, drought responses are categorized into strategies mainly preventing tissue dehydration, i.e. drought escape and avoidance, and strategies mitigating the effects of low tissue water potentials, once they have established, i.e. drought tolerance (Levitt, 1980; Ludlow, 1989).

Plants that predominantly use the *drought escape* strategy tend to be rapidly growing species completing their life cycle before the dry season. Furthermore drought escape can also be obtained by developmental plasticity. Several accessions of the annual model plant *Arabidopsis thaliana* show accelerated flowering when exposed to drought stress (Verslues and Juenger, 2011) and *Brassica rapa* plants experiencing drought are early flowering when compared to plants grown under well-watered conditions (Franks, 2011).

Drought avoidance is characterized by the maintenance of high tissue hydration while water availability in the environment decreases. This internal water conservation can be obtained by multiple mechanisms. On the one hand, plants can maximize water uptake by ameliorating diverse root traits such as hydraulic conductance, root density and rooting depth (Price, 2002). On the other hand, plants limit water

loss and preserve soil water by restricted stomatal conductance and mechanisms reducing radiation absorption. Some examples are modified leaf surface characteristics (more trichomes, thicker cuticula), leaf rolling and reduced leaf area. Under slowly developing water limitations often smaller leaves emerge. Leaf senescence and subsequent leaf abscission of older leaves act to minimize the transpirational area, especially when droughts are severe and rapidly imposed. In addition, the nutrients contained in older leaves are transferred to younger parts or used for storage in order to resprout when environmental conditions are more favorable (Pinheiro and Chaves, 2011).

Drought tolerance describes the ability to maintain physiological processes or viability when tissue water potential decreases and to postpone excessive tissue dehydration. A widespread response to water deficits is the accumulation of osmotically active compounds (Serraj and Sinclair, 2002) by which turgor pressure and cell volume are maintained when water potentials are decreasing. Higher concentrations of osmolytes may also have other, so-called osmoprotective effects: stabilization of protein and membrane structure, scavenging of free radicals, or they may function as storage compounds of carbon (C), nitrogen (N) and reducing power for periods, when the environmental conditions for growth are more favorable (Smith, 2010; Szabados and Savouré, 2010).

1.2 Biotic factors influencing drought responsiveness

Many of the above mentioned drought responses are mechanistically related to each other and orchestrated by hormone signaling pathways. As plants are exposed to changing environmental conditions, they tune their stress responses and commonly use a combination of avoidance, tolerance and sometimes escape strategies (Ludlow, 1989). Which strategies are used preferentially is strongly influenced by abiotic and also biotic factors.

In natural and managed environments plants interact with microorganisms living in the rhizosphere, which may impact on the plant's hormonal balance and source-sink relationships. This interaction therefore has the potential to alter the plant's responsiveness to drought (Augé, 2001; Yang et al., 2009; Dimkpa et al., 2009; Atkinson and Urwin, 2012). Several studies showed that the interaction with a subset of free-living soil bacteria, referred to as plant growth promoting rhizobacteria (PGPR), can act as an elicitor of tolerance to drought. For instance, bacteria expressing ACC deaminase changed plant ethylene levels leading to better performance under drought and better recovery from water deficit (Mayak et al., 2004;

Belimov et al., 2009), nitric oxide producing microbes enhanced root growth via IAA signalling (Molina-Favero et al., 2008) and finally, PGPR promoted plant grain yield under drought as a result of increased cell wall elasticity (Creus et al., 2004). Mycorrhizal fungi are also reported to alter plant hormonal balances (Goicoechea et al., 1997; Aroca et al., 2008; Plett et al., 2014) which might impact on the host's drought responsiveness.

Another economically important interaction with soil microbes is the endosymbiosis formed by legumes and nitrogen-fixing rhizobacteria (Graham and Vance, 2003). After the infection process bacteroids are accommodated in root nodules, provided with photoassimilates in order to fuel nodule maintenance and symbiotic nitrogen fixation. Relative to the fields of PGPR and mycorrhiza research, comprehensive studies dealing with the impact of root nodule symbiosis on plant drought stress tolerance are rare. In one of the first studies on the subject, Antolín et al. (1995) reported that leaves of nodulated (NOD) alfalfa plants were less sensitive to decreasing leaf relative water content than nitrogen fertilized, non-nodulated (NN) plants, since they maintained higher net photosynthesis and chlorophyll content at moderate stress. In a cyclic drought experiment, NOD alfalfa accumulated more biomass as a result of altered leaf ABA/cytokinin balance relative to NN plants (Goicoechea et al., 1997). Others also observed an enhanced drought tolerance in nodulated *Phaseolus vulgaris* and *Pisum sativum* on the basis of pod yield or biomass accumulation relative to NN, nitrate-fed plants (Lodeiro et al., 2000; Frechilla et al., 2000) or an increased proline accumulation in NOD *Lotus corniculatus* when experiencing a dehydration shock (Borsani et al., 1999). Frechilla and coworkers proposed that an improved photorespiratory regulation during drought conditions partially explains the better performance of NOD plants. Taken together these studies show that nodulation with rhizobacteria can affect the drought response strategies employed by plants, however they raise questions about molecular mechanisms underlying altered performance.

1.3 The legume family and the model *Medicago truncatula*

Nitrogen (N) is the fourth most prevalent element in the biosphere. In the form of highly unreactive dinitrogen it represents 78% of the atmosphere. The vast majority of taxa within the legume family have the ability to use this unreactive form by establishing a symbiosis with N-fixing soil bacteria. The fixation of atmospheric N by rhizobial nitrogenase is energy costly and needs a low-oxygen environment. Both requirements are met in the root nodule which is produced by the host after infection

with bacteria. Due to their ability to fix atmospheric N, legumes reduce the need for N fertilizer applications in agro-ecosystems and are thus of high socio-economic value. Legume derived N covers on average one third of the human and livestock N demand (Graham and Vance, 2003).

Legumes evolved about 60 million years (Myr) ago during the Paleocene period (Lavin et al., 2005). To date, about 20000 legume species are described, constituting one of the most diverse and geographically widespread clades in the plant kingdom (Doyle and Luckow, 2003). It still remains an open question what factors contributed to the evolutionary success of this family. In the most diverse and species-rich legume subfamiliy, the Papilionoidea, there was a whole-genome duplication event about 58 Myr ago (Young et al., 2011). Some authors propose that ancestral polyploidy may be an important driver of phenotypic diversification (Freeling and Thomas, 2006). In addition, the non-nodulating species (c. 10%) interestingly are restricted to the tropics and only half as diverse as the rest of the family (Sprent, 2007). It is thus tempting to assume that nodulation is at least a contributing factor influencing legume expansion. McKey (1994) postulated the hypothesis that legume evolutionary success is due to a 'N rich lifestyle', i.e. N rich leaves and a diverse secondary metabolism. However it is still unclear, whether the symbiotic association with rhizobia contributes to the spread of the family, by for example altering plant responses to various stresses.

About 12-15 % of the earth's arable land are cultivated with legume crops (Graham and Vance, 2003). The perennial lucerne, *Medicago sativa*, is economically the third most important crop in the USA, after corn and soybean (Bouton, 2007). Since *M. sativa* is autotetraploid and obligate outcrossing, its close relative *M. truncatula* cv. Jemalong A17 was chosen as a model species for the investigation of indeterminate nodule formation (Barker et al., 1990; Cook, 1999), with its genome being published in 2011 (Young et al., 2011). *M. truncatula* is a self-fertile diploid species with short life cycle, a relatively small genome of about 500 Mbp and it can be easily transformed using *Agrobacterium tumefaciens* (Colebatch et al., 2002). This annual medic is indigenous to the Mediterranean basin (Lesins and Lesins, 1979). Due to its high nutritive value (Brand et al., 1991) it is also cultivated as a grassland species in other Mediterranean-type climates such as those encountered in South Australia, South Africa and Chile or as a cover crop in mid-western USA (Fisk et al., 2001). *M. truncatula* grows best in warm and temperate conditions with an annual precipitation of 250 – 600 mm on well drained neutral-alkaline soils with a high water table. Thus, *M. truncatula* is well adapted to dry hot summers and mild moist winters.

1.4 Proteomics in the context of drought stress research

Analogoue to 'genome', the term 'proteome' describes the entire protein complement of an organism. Proteomics is now defined as the large-scale study of proteins present in a given tissue or cell at a given time point (Schulze and Usadel, 2010). By definition, proteomics thus bears the potential to gain a holistic understanding of a biological system (Glinski and Weckwerth, 2006). However, the approach faces substantial analytical challenges. The concentration range of distinct proteins within a cell can span seven to twelve orders of magnitude (Corthals et al., 2000) and many proteins are subject to post-translational modifications, such as phosphorylation, glycosylation or acetlyation.

Proteomic approaches can be categorized into three different aspects: protein profiling aims at the description of an entire protein complement, at qualitatively identifying the proteins present in a given sample. This approach is particularly successful in model species with completely sequenced genomes such as *Arabidopsis thaliana* (Wienkoop et al., 2004; Ytterberg et al., 2006). Comparative proteomic techniques allow to go beyond qualitative description and provide semiquantitative information. Here, protein abundance changes are assessed when plants are exposed to environmental or internal stimuli. Finally, functional proteomics aims at combining the identification and the functional characterisation of proteins.

Proteomics is a valuable tool to investigate conceivable relationships between plant stress tolerance and protein abundance, modifications or protein-protein interactions (Hu et al., 2015). Using a label-free shotgun proteomics approach, Larrainzar et al. (2007) studied the plant proteome response to water deficit of the *M. truncatula* root nodule, which was distinct from the response in the bacteroid part of the nodule. In another study, the relative abundance changes of 159 leaf proteins during variation in water availability were tracked in three wheat cultivars, that differed in drought tolerance (Ford et al., 2011). The changes observed occured at different time points within each cultivar. As a common response the authors revealed increases in proteins related to oxidative stress metabolism and decreases in photosynthetic enzymes and enzymes of the Colvin cycle. When plants are exposed to water deficit, growth is rapidly inhibited. Bonhomme et al. (2012) recently explored the changes in protein phosphorylation occuring in the growing zone of maize leaves. By applying a stable-isotope labeling approach, 138 phosphopeptides could be identified that responded to drying-rewetting cycles. Many of them originate from proteins known as plant growth regulators.

1.5 Metabolomic techniques to study drought responses in plants

The term metabolomics was first used in 1998 (Oliver et al., 1998). As with proteins the analytical techniques are challenged by the number and chemical diversity of compounds present in plants. Up to 200000 distinct metabolites are predicted to exist in the plant kingdom (Fiehn, 2002). To cope with this level of complexity a vast diversity of chromatographic and mass spectrometric techniques was developed within the past years. To date, gas chromatography (GC) coupled to mass spectrometry is the most widely employed technique to quantify small molecules with masses up to 1 kDa (Fiehn et al., 2000). By combining compound-specific retention time and mass spectral information this technique allows for the identification of hundreds of metabolites (Roessner-Tunali, 2007). Certainly the public availability of spectral libraries contributed to the success of this technology (Halket et al., 2005).

The main effect of drought on metabolites is a general accumulation of small molecules, as evidenced by spectrophotometric (e.g. Hummel et al., 2010) as well as metabolomic analyses (e.g. Sanchez et al., 2008; Silvente et al., 2012). Which molecules preferentially accumulate at which timepoint is highly context-dependent. For example, Skirycz et al. (2010) used a targeted metabolomics approach to illustrate the developmental stage-specificity in leaf amino acid and sugar accumulation in response to osmotic stress. Furthermore, Sanchez et al. (2012) discovered by metabolite profiling that several *Lotus* species exhibit a high metabolic plasticity when exposed to drought. Only one-fourth to one-third of the detected changes were common between closely related species.

1.6 Aim of this thesis

This study aimed at (i) determining whether nodulation alters the performance of *Medicago truncatula* during drought and rehydration. As the symbiotic interaction with N-fixing rhizobia influences the plant N nutritional status and a positive relationship between plant N levels and drought tolerance has been observed, another aim was to (ii) test whether the anticipated changes depend on the initial leaf N concentration or other symbiotically mediated factors. Furthermore, this study focused on (iii) the major molecular mechanisms involved in a differential drought stress responsiveness in NOD(e) and NN *M. truncatula*.

Chapter 2

Symbiotic interaction modulates drought response strategies in *Medicago truncatula*

2.1 Introduction

From an ecological as well as agricultural point of view plant survival and productivity under water limitation are among the most relevant traits related to drought tolerance. Especially in the field of molecular biology, a plethora of studies used the scoring of survival rates to infer enhanced or decreased drought tolerance of one genotype relative to another (references in Skirycz et al., 2011). In arid regions presenting a high probability of intense and prolonged droughts, enhanced survival is a valuable trait, all the more so in perennial plants. The ability to survive extreme drought is however not necessarily related to improved plant productivity during moderate water limitation (Tardieu, 2012). Skirycz et al. (2011) tested the biomass accumulation of 25 *Arabidopsis* lines in a mild drought stress-assay. All of those lines previously showed superior survival under severe drought and were thus reported to be more drought tolerant than the wild-type plants. However, under mild drought conditions not a single mutation had a significant effect on plant shoot growth. A futile loss in biomass may represent a competitive disadvantage in natural environments and results in undesired yield losses in agricultural situations. Therefore, an in-depth understanding of the processes regulating biomass accumulation and preservation during drought represents promising avenues towards the development of drought tolerant cultivars for temperate regions (Tisne et al., 2010; Skirycz et al., 2011).

Numerous species of the genus *Medicago* are used as forage crops in temperate regions. When mineral N in the soil solution is scarce, medics build indetermi-

nate root nodules harboring the N-fixing soil bacteria *Sinorhizobium meliloti* and *S. medicae* (Graham, 2008). A survey of field isolates from several medics growing in the Mediterranean basin revealed that both, *S. meliloti* and *S. medicae*, were differentially associated with hosts and their corresponding soil types (Garau et al., 2005). This study showed that *S. meliloti* was mainly associated with hosts growing on neutral to slightly alkaline soils, whereas *S. medicae* was predominantly found in annual medics growing in regions with a lower soil pH. This is also reflected in their agronomic usage. Both rhizobia were contained in commercial inoculants distributed in Australia (Bullard et al., 2005): *S. meliloti* SU47 (Vincent, 1941), the parent strain of the subsequently isolated laboratory strains Sm1021 (Meade et al., 1982) and Sm2011 (Scherrer and Denarie, 1971), was used by farmers to inoculate perennial *M. sativa*. Annual medics, however, were inoculated with a commercial inoculant containing *S. medicae* WSM419. The publication of the complete genome of *S. melilot* 1021 (Galibert et al., 2001) paved the way for its predominant utilisation as a model species. In spite of the high number of publications focusing on nodule formation in the system *M. truncatula-S. meliloti*, it was only recently, that the poor N-fixation effectiveness of the association between *M. truncatula* A17 with the microsymbionts *S. meliloti* 1021 and 2011 was described (Terpolilli et al., 2008; Larrainzar et al., 2014). Since the parent strain SU47 appears to effectively nodulate *M. truncatula*, Terpolilli et al. (2008) propose that the poor efficiency arises from the long cultivation under laboratory conditions.

As outlined in chapter 1.2, there is some evidence that nodulation has the potential to alter the plant responsiveness to drought. However, it still remains unclear whether these alterations are related to differences in the N nutritional level under well-watered conditions, which could arise from the treatment with distinct N sources. *M. truncatula* thus constitutes a well-suited model to address this question as the host nodulates with two distinctly efficient rhizobia resulting in distinct plant N nutritional levels (Terpolilli et al., 2008; Larrainzar et al., 2014).

It is well known that the N nutritional level of plants depending on N from the soil solution exerts a strong effect on the sensitivity to water deprivation (Radin and Ackerson, 1981; Radin and Boyer, 1982). Notably, low N supply increased the stomatal sensitivity to water deficit and to ABA (Radin and Ackerson, 1981). N deficiency reduced root hydraulic conductivity under well-watered conditions (Clarkson, 2000). With increasing leaf N levels drought stressed *Agrostis palustris* showed traits associated with better stress adaptation such as cellular membrane stability, lower osmotic potentials and reduced concentrations of malonyl dialdehyde, the final product of lipid peroxidation (Saneoka et al., 2004).

This study therefore aimed at assessing potential differences in shoot biomass accumulation and maintenance of NOD and NN *M. truncatula* when exposed to

similar external stress doses. Another objective was to investigate whether these potential differences are independent of the initial leaf N concentration. To test these hypotheses, pot-grown plants were treated with distinct N regimes (rhizobia or N-fertilization) resulting in various leaf N concentrations and were then exposed to progressively decreasing substrate water contents.

2.2 Materials and methods

2.2.1 Plant and rhizobial growth conditions

The temperate model legume *Medicago truncatula* Gaertn. cv Jemalong 17 was chosen to study the effect of plant-rhizobia interactions on drought acclimation. Seeds were incubated for 7 min in 98 % sulfuric acid and surface sterilized for 3 min in 5 % sodium hypochloride. After extensive washing with sterile water, the seeds were placed on 0.8 % agar plates and left 3 days for germination in darkness: one day at 4 °C and the following two days at room temperature. Seedlings were then transferred to 1 L pots containing a sterile vermiculite-perlite mixture (2:5, v:v). The plants grew in a climatic chamber equipped with metal halide lamps (Radium HRI-TS 250W/NDL) under controlled environmental conditions (500 µmol m^{-2} s^{-1} for 14 h photoperiod, 22 ± 2.8 °C and 16 ± 0.5 °C day and night temperatures; 59 ± 10 % relative humidity). Plants were watered daily with nutrient solution (Evans, 1974, see Table A.1). During the first week a solution containing 0.5 mmol NH_4NO_3 (pH 6.2) was used, during the subsequent two weeks plants received 2.5 mmol NH_4NO_3 (pH 7.4). Three week-old plants were then separated into three groups (Figure 2.1): non-nodulated (NN) plants receiving 2.5 mmol NH_4NO_3 and two nodulated (NOD) groups, which were inoculated with either *Sinorhizobium medicae* WSM419 (NOD(e)) or *S. meliloti* 2011 (NOD(i)) receiving 0.5 mmol NH_4NO_3 in order to ensure more similar growth performances. *S. medicae* and *S. meilioti* were chosen, as their N-fixation efficiencies differ when associated with *M. truncatula* resulting in distinct leaf N concentrations (Terpolilli et al., 2008; Larrainzar et al., 2014). The NN-fertilization was chosen on the basis of preliminary experiments.

Sinorhizobium medicae WSM419 and *S. meliloti* 2011 were grown separately at 27 °C for 72 h on a rotary shaker in yeast extract-mannitol medium which contained 10 g L^{-1} mannitol, 0.5 g L^{-1} K_2HPO_4, 0.4 g L^{-1} yeast extract, 0.2 g L^{-1} $MgSo_4 \cdot 7\,H_2O$, 0.1 g L^{-1} NaCl, pH adjusted to 6.8. Each plant designated for nodulation was inoculated with 2 mL of the culture.

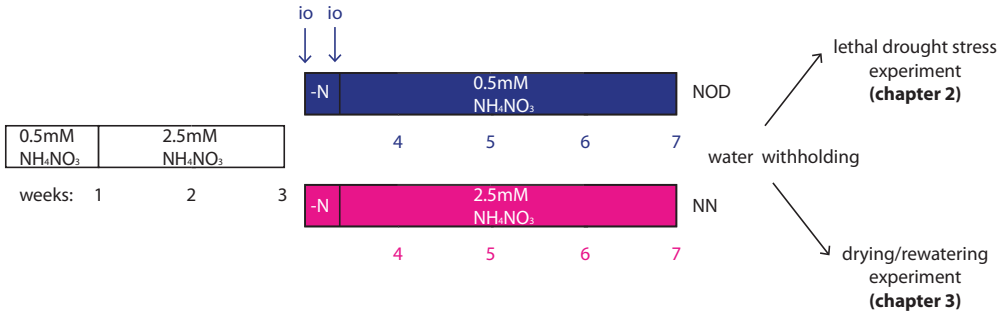

Figure 2.1: Schematic representation of the experimental setup in time.

2.2.2 Experimental design

After seven weeks of growth, plants were subjected to drought by withholding irrigation. Here, a lethal drought stress experiment was performed. NN plants and the two rhizobial treatments, NOD(e) and NOD(i), were exposed to lethal drought stress to assess the effect of nodulation and initial leaf N concentration on *M. truncatula* performance under drought, while control plants were watered daily. The time of death was scored, when all leaves were wilted and the plants did not recover upon rehydration.

2.2.3 Drought stress treatment and water status measurements

Water deficit was imposed by water-withholding for several days. Before the onset of the drying period, pots were watered to pot capacity in order to minimize heterogeneity in substrate water content (SWC). After one day, the pot weight was recorded and served as a reference point for the following experiment. Drought stress was then imposed by withholding irrigation. To estimate the daily water requirement of control plants, the pots were weighed before the photoperiod on days 0 and 1. The changes in substrate water content (SWC) of stressed plants were also estimated gravimetrically, presuming that the contribution of plant growth to pot weight was negligible compared to the total amount of water lost. For each plant, the measurements were performed on mature leaves which were cut and directly sealed in the pressure chamber. Predawn leaf xylem water potential was measured when the SWC reached *c.* 50% of the pot capacity (i.e. day seven in this experiment). For each plant, the measurements were performed on mature leaves which were cut and directly sealed in the pressure chamber, as described previously (Scholander et al., 1965).

2.2.4 Leaf senescence, chlorophyll content and photosynthesis

To quantify the extent of leaf senescence, fully developed leaves were labeled with small plastic badges before the beginning of the stress period. Leaves were considered dead when all three leaflets were abscised from the petiole. The degree of leaf senescence was then expressed as the number of dead leaves relative to the number of mature leaves developed at the beginning of the experiment.

Leaf chlorophyll content was estimated using the SPAD-502 chlorophyll meter (Minolta Camera Co., Osaka, Japan). For this purpose, the last fully developed leaf of the second side axis was labelled at day 0. Its chlorophyll content was then assessed after eight days of water withholding.

Net photosynthesis was measured at the last fully developed leave of the third side axis, using a portable infrared gas analyzer (LI-6200, Li-Cor, Lincoln, NE, USA).

2.2.5 Elemental analysis - Isotope Ratio/Mass Spectrometry Measurements (EA-IR/MS)

Natural abundances of C isotopes of bulk leaf material and the C and N content were measured by Elemental Analyzer coupled to Isotopic Ratio Mass Spectrometer (EA1110, CE Instruments; mass spectrometer DELTAplus Finnigan MAT, Thermo Fisher Scientific). For the analysis of bulk leaf, 1.5-2 mg of pulverized material were weighed into tin capsules and introduced into the EA-IR/MS. Tin capsules with sample were combusted at 1000 °C and oxidized by a chromium oxide column. Molecular oxygen was excluded. A subsequent cupreous column traps excess oxygen and reduces NO_x to N_2 at 600 °C. Water vapor was trapped by a column of Mg-perchlorate. Helium was used as carrier gas transporting the sample constituents through a packed column at 120 µL min^{-1} to separate CO_2 and N_2. The eluents were ionized by electron-impact and detected in the mass spectrometer.

2.2.6 Statistical analysis

Unless stated otherwise, statistical analysis was conducted using one-way ANOVA. The homogeneity of variances was tested employing Levene's test. All analyses were performed using MATLAB(R2010b) and STATGRAPHICS 16.1.11.

2.3 Results

2.3.1 Relationships between leaf senescence and rhizobial treatment during lethal drought

The objective of this study was (i) to assess differences in drought susceptibility and responsiveness of NN and NOD *M. truncatula* plants and (ii) to investigate whether these differences are influenced by initial differences in leaf N content or other parameters related to a symbiotic lifestyle. On the basis of a preliminary experiment (Figure A.1), three N regimes were chosen, which resulted in distinct leaf N concentrations under well-irrigated conditions. Inoculation with *S. medicae* resulted in the highest, inoculation with *S. meliloti* resulted in the lowest leaf N concentration. With 2.5 mmol NH_4NO_3-fertilization an intermediate N nutritional level was obtained (%N and C:N ratios in Table 2.1). The %C (Table 2.2) was unaffected by the N regime.

Table 2.1: C and N characteristics in bulk leaf material of seven week old non-nodulated (NN) and nodulated (NOD) *Medicago truncatula*, inoculated with *Sinorhizobium medicae* NOD(e) or *S. meliloti* NOD(i) under well-watered conditions.

	C (%)			N (%)			C/N			δ^{13}C (‰)		
NN	43.08	(0.45)	a	3.19	(0.26)	a	13.52	(1.56)	a	-32.19	(0.45)	a
NOD(e)	39.82	(0.27)	a	4.19	(0.09)	b	9.51	(0.16)	b	-32.24	(0.04)	a
NOD(i)	39.25	(2.16)	a	2.31	(0.14)	c	17.31	(0.45)	c	-31.12	(0.11)	b

Significant differences between the three N regimes are indicated by distinct letters (n=3-5, ANOVA, Fisher's test, $P < 0.05$). Values in parentheses are standard errors.

With respect to plant water relations, comparable leaf areas were observed (Table 2.2) resulting in similar evapotranspirational demands at the start of the experiment (Figure 2.2b). During the stress treatment (day seven) older leaves of both NOD plants tended to have slightly lower stomatal conductance than similarly aged leaves of NN plants (Figure 2.3), n= 4, $P < 0.1$, LSD). However, when water was withheld, the rate and extent of substrate desiccation was virtually identical in all three treatments (Figure 2.2a). After eight days of water withholding, *c.* 50% of the pot water holding capacity were reached. In addition, leaf predawn water potential was comparable in all three N regimes (Figure 2.2c).

Both groups of NOD plants, NOD(e) and NOD(i), lost their leaves at similar rates and proportions, while the rate of leaf abscission was increased by almost 50% in NN plants during the first 13 days of the experiment (Figure 2.4a and A.3).Day 13 was also the time point, when NN plants had already lost 100 % of the leaves that

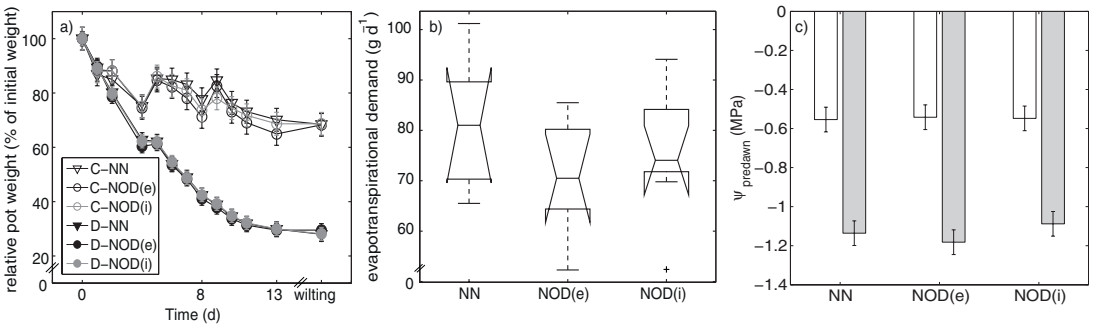

Figure 2.2: Drought stress imposition during a lethal drought stress experiment in *Medicago truncatula*: (a) estimated substrate water content measured gravimetrically, the day of wilting was *c.* day 15 in all stressed conditions. (b) Plant evapotranspirational demand from day zero to day one. Notches indicate 95% confidence intervals. (c) Predawn leaf xylem water potential, measured on day seven. Values are means (and medians in the boxplot); error bars indicate 95% LSD confidence intervals; n = 5-7. C: control, D: drought treated, NN: non-nodulated, NOD(e): *Sinorhizobium medicae* nodulated and NOD(i): *S. meliloti* nodulated.

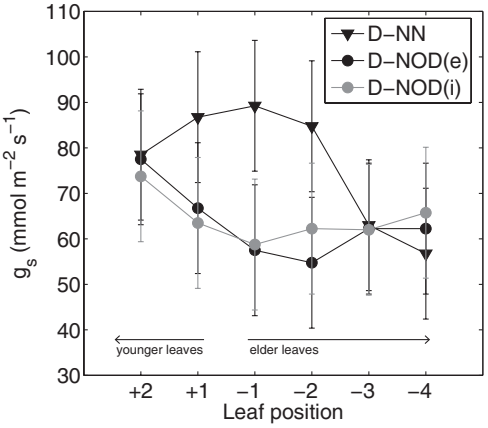

Figure 2.3: Stomatal conductance along an ontogenetic gradient in drought stressed *Medicago truncatula* plants measured on day 7 of a lethal drought stress experiment. The vertical bar indicates the last visible internode developed at day 0. Values are means; error bars indicate 90% LSD confidence intervals; n = 4. D: drought treated, NN: non-nodulated, NOD(e): *Sinorhizobium medicae* nodulated, NOD(i): *S. meliloti* nodulated.

were fully developed (i.e. autrotrophic) at the start of the drought stress treatment. The youngest leaves emerging at the last two internodes of the side axes remained vital. On day 8 the chlorophyll index of NN plants was decreased by 18%, while the index of NOD plants was unaffected or increased (Figure 2.4b). The mean SWC at wilting was similar in all treatments ranging from 0.11 to 0.14 g g^{-1} dried substrate (n = 5-7, $P < 0.05$, LSD, see Figure A.2). Additionally, the three groups of plants did not show a significant divergence in the respective time of death (*c.* 15 days, n = 5-7, $P < 0.05$, LSD).

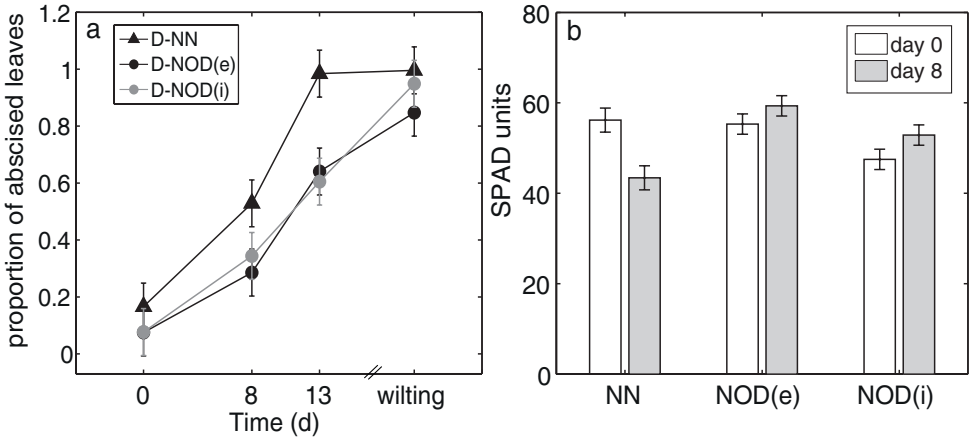

Figure 2.4: Leaf senescence symptoms in *Medicago truncatula* induced by water withholding. (a) Leaf abscission rate. The day of wilting was c. day 15 in all conditions. (b) Leaf chlorophyll index at the start of the desiccation period (day zero, white bars) and after eight days of water withholding (grey bars). Values are means; error bars indicate 95% LSD confidence intervals; n = 5. D: drought treated, NN: non-nodulated, NOD(e): *Sinorhizobium medicae* nodulated. and NOD(i): *S. meliloti* nodulated

2.3.2 Stable isotopic signatures and photosynthesis

To assess whether NOD and NN plants differed substantially in stomatal or photosynthetic behavior during the seven weeks of normal growth, the stable isotopic signatures of bulk leaf material was analysed. The δ^{13}C of NOD(i) plants was on average 1 ‰ higher than the δ^{13}C of NN and NOD(e) leaf material, indicating that metabolic processes of NOD(i) plants were less discriminative against ^{13}C relative to ^{12}C. However, no significant difference was observed when the rate of photosynthesis was measured punctually using an IR-gas analyser (Table 2.2).

2.4 Discussion

The interaction with various microorganisms is known to alter plant drought stress tolerance as for example the interaction with plant growth promoting bacteria (Bres-

Table 2.2: Photosynthesis (A), leaf area (LA) and specific leaf area (SLA) in seven *wk* old non-nodulated (NN) and nodulated (NOD) *Medicago truncatula*, inoculated with *Sinorhizobium medicae* NOD(e) or *S. meliloti* NOD(i) under well-watered conditions.

	A (µmol cm^{-2} s^{-1})			LA (cm^2)			SLA (cm^2 g^{-1}DW)		
NN	53.103	(3.04)	a	142.48	(8.8)	a	262.86	(4.6)	a
NOD(e)	53.103	(1.02)	a	160.27	(30.1)	a	281.99	(22.0)	a
NOD(i)	56.392	(1.82)	a	128.99	(18.2)	a	251.51	(16.0)	a

Significant differences between the three N regimes are indicated by distinct letters (n = 3, ANOVA, Fisher's test, $P < 0.05$). Values in parentheses are standard errors.

son et al., 2013), or with vesicular-arbuscular mycorrhiza as reviewed by (Augé, 2001). However, the impact of root nodule bacteria on legume drought stress tolerance is still poorly understood. This study demonstrates that nodulation with *Sinorhizobium meliloti* or *S. medicae* has the potential to delay drought induced leaf senescence in *M. truncatula* relative to non-nodulated plants solely relying on mineral N fertilizer.

2.4.1 Water limitation and evapotranspiration

The comparison of drought response differences between distinctly sized plants is complicated by the fact that plant size tends to interact with water demand (Poorter et al., 2012). Smaller plants often seem less sensitive to drought, just because they may deplete substrate water reserves more slowly due to reduced total transpiration and not because of differences in stress responsiveness (Jones et al., 2007). Enhanced N availability generally promotes plant biomass accumulation. Thus, studies dealing with the comparison of drought responses in nodulated (NOD) and non-nodulated (NN) plants have to address this subject (Antolín et al., 1995; Lodeiro et al., 2000; Frechilla et al., 2000). When the direct effect of leaf water status on physiological and morphological parameters is to be examined, it is appropriate to evaluate the results relative to the leaf water status (e.g. Antolín et al., 1995). Another alternative way to overcome this issue is to provide the plants with a fraction of their daily evapotranspirational demand, as employed by Frechilla et al. (2000). In the study presented here, differential N nutrition did not result in altered evapotranspirational demand at the start of the experiment. When exposed to water limitation, it is well recognized that younger leaves tend to be more drought tolerant than older leaves (Pinheiro and Chaves, 2011). During the stress period, slightly enhanced stomatal conductance of older leaves was observed in NN relative to NOD plants. This finding indicates, that older leaves of NOD plants may exhibit tighter stomatal

control. However, the difference reported here, was not important enough to be reflected in distinct total evapotranspiration per day (Figure2.2a), which could be due to the relatively high evapotranspirational demand of both groups of plants. It was sufficient to withhold water from the plants in order to obtain similar external stress intensities in terms of SWC. In this case, it is therefore appropriate to compare the stress responsiveness of plants treated with distinct N regimes over time.

2.4.2 Leaf senescence is influenced by symbiotic interaction during drought stress

Leaf abscission is one of the strategies that plants may employ in order to reduce transpiring surfaces and postpone the depletion of substrate water reserves. This process is preceded by leaf senescence during which nutrients are remobilized and exported to other plant tissues (Wingler et al., 2004). Previous studies described a delay of leaf senescence during drought in nodulated alfalfa and common bean relative to their non-symbiotic counterparts (Antolín et al., 1995; Lodeiro et al., 2000). In the lethal drought stress experiment presented here, nodulation with *S. medicae* or *S. meliloti* was associated with a delay in stress-induced leaf senescence and abscission when compared to NN plants, suggesting that tolerance strategies might be favoured by NOD plants, whereas avoidance strategies may be more important in NN plants.

However, it is important to mention that interactions between initial N nutritional levels and drought stress have been reported. For example, suboptimal N nutrition increases stomatal sensitivity to ABA (Radin and Boyer, 1982), and reduces the root hydraulic conductivity under well-watered conditions (Clarkson, 2000). Several previous studies restricted their comparative analysis to only two N nutritional regimes (one NOD and one NN) not allowing for the distinction of the aforementioned factors (Borsani et al., 1999; Frechilla et al., 2000; Kirova et al., 2008). Therefore, several N regimes were chosen in order to dissect NN specific from NOD specific drought responses that are not directly in relation to initial leaf N concentration. The results presented here indicate that the symbiotic interaction overlays the effects of N nutritional levels described above. In fact, the rhizobial symbiosis exerts a strong effect on the plant's strategy utilized when exposed to drought (leaf maintenance or shedding).

This investigation showed that *M. truncatula* employs distinct drought response strategies at the whole-plant level, depending on whether the plant is nodulated or not. In addition, the initial leaf N content and C/N ratio are empirically not directly involved in the stay-green phenotype of NOD plants. To gain more insights into the metabolic changes involved, the following chapter will focus on the comparative

analysis of systems responses to drought and rehydration in NN and symbiotic plants inoculated with *S. medicae*.

Chapter 3

The molecular basis of differential drought response strategies in *Medicago truncatula*

3.1 Introduction

Delayed leaf senescence is a valuable trait for crop improvement (Sakuraba et al., 2012; Thomas and Ougham, 2014). Plants that, relative to others, show delayed leaf yellowing during aging or in response to environmental cues are called 'stay-greens'. Several studies showed that the stay-green character was related to higher yield during drought, especially under moderate water limitation (e.g Borrell et al., 2000; Rolando et al., 2015). In forage plants the stay-green trait could in addition be beneficial during the entire growth phase (Gregersen et al., 2013). This was shown for alfalfa (*Medicago sativa*) resulting in enhanced hay nutritional value (Zhou et al., 2011). Hormonal signalling pathways interacting with sugar signalling are involved in the regulation of leaf senescence (Roitsch and González, 2004; Thomas, 2013). For instance, cytokinin is an important inhibitor of leaf senescence (Zwack and Rashotte, 2013). There is indication that this antagonizing effect is related to source-sink relationships (Thomas, 2013) which in part are mediated by increased cell wall invertase activity (Lara et al., 2004; Jin et al., 2009), an enzyme involved in the phloem unloading of sucrose to sinks. Finally, there are reports on stay-green phenotypes mediated by altered levels of ethylene (Grbic and Bleecker, 1995), jasmonic acid and salicylic acid (Miao and Zentgraf, 2007).

The previous chapter showed that nodulated *M. truncatula* presented an enhanced ability to maintain green leaf area during severe water deprivation, relative to NN plants. To study the molecular mechanisms involved, NOD(e) and NN plants were exposed to transient drought followed by rehydration. Elemental analysis, sol-

uble ions and major metabolites were assessed to decipher the effects of nodulation on plant metabolism under well-watered conditions and on plant stress responsiveness. In addition, a proteomic approach was used to gain a more holistic view of relevant mechanisms.

3.2 Materials and methods

3.2.1 Plant and rhizobial growth conditions

The temperate model legume *Medicago truncatula* Gaertn. cv Jemalong 17 was chosen to study the effect of plant-rhizobia interactions on drought acclimation. Seeds were incubated for 7 min in 98 % sulfuric acid and surface sterilized for 3 min in 5 % sodium hypochloride. After extensive washing with sterile water, the seeds were placed on 0.8 % agar plates and left 3 days for germination in darkness: one day at 4 °C and the following two days at room temperature. Seedlings were then transferred to 1 L pots containing a sterile vermiculite-perlite mixture (2:5, v:v). The plants grew in a climatic chamber equipped with metal halide lamps (Radium HRI-TS 250W/NDL) under controlled environmental conditions (500 µmol m^{-2} s^{-1} for 14 h photoperiod, 22 ± 2.8 °C and 16 ± 0.5 °C day and night temperatures; 59 ± 10 % relative humidity). Plants were watered daily with nutrient solution (Evans, 1974, see Table A.1). During the first week a solution containing 0.5 mmol NH_4NO_3 (pH 6.2) was used, during the subsequent two weeks plants received 2.5 mmol NH_4NO_3 (pH 7.4). Three week-old plants were then separated into two groups: non-nodulated (NN) plants receiving 2.5 mM NH_4NO_3 and nodulated (NOD(e)) plants, which were inoculated with *Sinorhizobium medicae* WSM419 receiving 0.5 mmol NH_4NO_3 during the growth period in order to ensure more similar growth performances. *S. medicae* shows a better symbiotic efficiency when associated with *M. truncatula* (Terpolilli et al., 2008; Larrainzar et al., 2014). Thus, *S. medicae* was here chosen over *S. meliloti* due to the resulting plant growth performance which was more comparable to NN plants. The NN-fertilization was chosen on the basis of preliminary experiments.

Sinorhizobium medicae WSM419 was grown at 27 °C for 72 h on a rotary shaker in yeast extract-mannitol medium containing 10 g L^{-1} mannitol, 0.5 g L^{-1} K_2HPO_4, 0.4 g L^{-1} yeast extract, 0.2 g L^{-1} $MgSo_4 \cdot 7\,H_2O$, 0.1 g L^{-1} NaCl, pH adjusted to 6.8. Each plant designated for nodulation was inoculated with 2 mL of the culture.

3.2.2 Experimental design

A drought and rehydration experiment was set up for molecular analyses consisting of four sample sets: droughted NN and NOD(e) plants and their respective con-

trols. After seven weeks of growth, plants were subjected to drought by withholding irrigation. Five sample time points were chosen: five (D5) and nine (D9) days of water withholding and two hours (R10), one day (R11) and three days (R13) of rehydration using nutrient solution.

3.2.3 Drought stress treatment and water status measurements

Water deficit was imposed by water withholding for several days. Before the onset of the drying period, pots were watered to pot capacity in order to minimize heterogeneity in substrate water content (SWC). After one day, the pot weight was recorded and served as a reference point for the following experiment. Drought stress was then imposed by withholding irrigation. To estimate the daily water requirement of control plants, the pots were weighed before the photoperiod on days 0 and 1. The changes in substrate water content (SWC) of stressed plants were also estimated gravimetrically, presuming that the contribution of plant growth to pot weight was negligible compared to the total amount of water lost. Predawn leaf xylem water potential was measured when the SWC reached *c.* 50% of the pot capacity (i.e. day nine). For each plant, the measurements were performed on mature leaves which were cut and directly sealed in the pressure chamber, as described previously (Scholander et al., 1965).

3.2.4 Thiobarbituric acid reactive substances assay

Malondialdehyde (MDA) is a reactive carbonyl species originating from lipid peroxidation. The Thiobarbituric acid reactive substances (TBARS) assay (Hodges et al., 1999) was used to measure MDA levels in *M. truncatula* leaves by spectrofluorometry. In this assay, MDA and other TBARS react with TBA to give a fluorescent red product. Briefly, 500 µL of 0.1 M HCl and 40 µL of 0.8 % BHT were added to 100 mg of finely ground leaf material. The suspension was then sonicated for 5 min. After centrifugation (12000 g, 10 *min*), 100 µL of the extract were transferred to glass vials and incubated with 100 µL of 1 % TBA for 30 min at 70 °C. To determine the blank fluorescence emission, TBA was replaced by 5 % TCA. After cooling, 500 µL n-butanol were added. The samples were then thoroughly vortexed and centrifuged (4000 g, 5 *min*). The fluorescence emission of the upper phase at 551 nm was measured after excitation at 515 nm. The concentration of TBARS was then determined by using MDA as an external standard, which was produced by hydrolysis of 17 µL 1,1,3,3-Tetrahydroxypropan in 10 mL 0.1 M HCl at 40 °C for 1 h.

3.2.5 Anion and cation chromatography

Ultrapure water was added to lyophilized and finely ground leaf material in order to prepare a 4 % solution (v:w). The mixture was subsequently incubated for 30 min at 90 °C. After centrifugation at 13000 rpm for 10 min, the supernatant was collected and again centrifuged (13000 rpm, 5 min). For determinations of soluble anion concentrations a 1:20 dilution was analyzed. HPLC was used to separate inorganic anions on an anion exchange column (AS11, 2 x 250 mm) with pre-guard column (AG11, 2 x 50 mm) using a linear KOH gradient (1-10 mM, total run time 30 min), chemical suppression (ASRS 300-2 mm) and conductivity detection (CD-20) on an ion-chromatography system (ICS 3000, Dionex, Vienna, Austria). Data analysis was performed using Chromeleon Version 6.80 SR7 Build 2528. Soluble cation concentrations were measured using an ion chromatography instrument (Metrohm 881 Compact IC pro) equipped with a conductivity detector. An acidified 1:200 dilution was prepared and 0.2 mL were loaded on a Metrosep C 4 column (150 x 4 mm) and eluted with 34 mM nitric acid/14 mM dipicolinic acid. The results were analyzed using Mag IC Net 2.4 software package.

3.2.6 Starch content

Leaf starch content was determined enzymatically. To remove glucose 100 mg of fresh leaf material was extracted twice with 80 % ethanol (80 °C, 30 min). After centrifugation for 10 min at 13000 rpm the supernatant was discarded and the pellet was dissolved in 0.5 N NaOH and incubated at 95 °C for 30 min. After acidifying the suspension by adding 1 mL 1 N CH_3COOH, 100 µL were incubated with 100 µL amyloglucosidase reagent (0.1 % (w/v) amyloglucosidase, 0.2 N CH_3COOH, 0.1 N NaOH). The glucose content of the resulting digest was determined by adding glucose oxidase/peroxidase reagent (0.03 % (w/v) glucose-oxidase, 0.003 % peroxidase, 0.1 % o-dianisidine-HCl in tris-glycerin buffer, pH7). During this reaction glucose is oxidized to gluconic acid and H_2O_2 which is in turn used by peroxidase to oxidize o-dianisidine, functioning as chromogenic oxygen acceptor. The solution was then acidified with 200 µL 5 N HCl. The absorption was determined at 540 nm. The absolute concentration was then determined via a glucose calibration curve.

3.2.7 Elemental Analysis - Isotope Ratio Mass Spectrometry (EA-IR/MS)

Natural abundance of C stable isotopes was measured by Elemental Analyzer coupled to Isotopic Ratio Mass Spectrometer (EA1110, CE Instruments; mass spectrometer DELTAplus Finnigan MAT, Thermo Fisher Scientific). For the analysis

of bulk leaf, 1.5-2 mg of pulverized material were weighed into tin capsules and introduced into the EA-IR/MS. Tin capsules with sample were combusted at 1000 °C and oxidized by a chromium oxide column. Molecular oxygen was excluded. Water vapor was trapped by a column of Mg-perchlorate. Helium as carrier gas transports the constituents of the sample through a packed column at 120 µL min^{-1} to separate CO_2 and N_2. The mobile phase is ionized by electron-impact and detected in the mass spectrometer.

3.2.8 Metabolite extraction

The metabolites of 20 ± 2 mg ground and lyophilized leaf material were extracted using 1 mL ice-cold methanol/chloroform/water (5:2:1, v:v:v). The mixture was vortexed several seconds and incubated on ice for 8 min. After centrifugation (4 min, 14000 g, 4 °C), the supernatant was collected and the extraction process was repeated once again. The pellet was stored at -20 °C for subsequent protein extraction. For phase separation, 750 µL of ultra-pure water were added to the supernatant and centrifuged (6 min, 4400 g, 4 °C). The upper polar phase was then collected and separated into aliquots with a volume corresponding to 2 mg of dry plant material. As an internal standard ^{13}C-Sorbitol was added (80 ng per aliquot). The samples were then dried in a vacuum concentrator. For derivatization of metabolites, the dried pellet was dissolved in 20 µL pyridine containing 0.5 mg methoxyamine hydrochloride and incubated on a thermoshaker at 30 °C for 90 min. After addition of 80 µL MSTFA the mixture was agitated at 37 °C for 30 min. A mixture of even *n*-alkanes (C_{12}-C_{40}) was spiked into the sample prior to measurement allowing for retention time indexing.

3.2.9 Protein extraction

Proteins were extracted from the remaining pellet of the metabolite extraction described above using urea buffer (8 M Urea, 50 mM Hepes, pH 7.8) and a glass homogenizer as described previously (Gil-Quintana et al., 2013). After centrifugation at 10000 g for 10 min, the leaf soluble proteins were precipitated over night at -20 °C after adding 5 volumes of ice-cold acetone containing 0.5 % β-mercaptoethanol. Proteins were pelleted (4000 g, 10 min, 4 °C) and washed once with ice-cold methanol. The air-dried pellet was then resuspended in urea buffer. Protein concentration was measured using the Bradford assay with bovine serum albumin as a standard. For subsequent digestion, 0.1 µg sequencing-grade endoproteinase LysC (Roche) was added to 100 µg of protein and incubated at 30 °C for 5 h. Trypsin digestion was performed over-night using Porosozyme immobilized trypsin beads according to the manufacturer's instructions. The protein digest was desalted using SPEC C18

columns (Agilent Technologies, Santa Clara, CA, USA). Peptides were eluted with methanol, vacuum dried and stored at -20 °C until use.

3.2.10 Gas Chromatography-Time-of-flight mass spectrometry (GC-TOF/MS)

Measurements were performed on an Agilent 6890 gas chromatograph coupled to a LECO Pegasus 4D GC-TOF mass spectrometer. Samples were injected using a 4 mm inner diameter liner at 230 °C and the components were separated on an Agilent HP-5MS column (30 m length, 0.25 mm inner diameter). Temperature was held for 1 min at 70 °C and then increased with 9 °C per minute until 330 °C were reached. Detector voltage was set to 1600 V and the mass range from 40 to 600 m/z was analyzed with a scan rate of 20 spectra per second. Spectrum deconvolution, base line correction, peak searching, retention time indexing and peak annotation was performed using the LECO-Chromatof software in combination with the Golm Metabolome Database spectral library (Kopka et al., 2005). After visual inspection of the hits with a minimum match factor of 850 and comparison to standard runs, unique fragment ion masses of 47 compounds originating from 32 metabolites were chosen for relative quantification (see Table A.2). By matching the chromatograms of all runs against this reference list, peak areas were obtained. Sucrose was excluded from further statistical analysis due to overloading of the detector.

3.2.11 Liquid Chromatography-Linear Trap Quadrupole Orbitrap Mass spectrometry (LC-LTQ-Orbitrap/MS)

Tryptic peptide mixtures were analyzed by LC-MS/MS using a nanoLC-Ultra 1D+ (Eksigent, Dublin, CA) system coupled to a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). Peptides were dissolved in 0.1 % formic acid and 5 % acetonitrile and an amount corresponding to 1 µg of protein was loaded onto a Peptide ES-18 column (Supelco 15 15 cm/0.1 mm, 2.7 µm; Sigma-Aldrich). Analytes were eluted from the column during a 95 min gradient ranging from 98 % solvent A (0.1 % formic acid) to 55 % solvent B (90 % acetonitrile, 0.1 % formic acid) at a flow rate of 400 nL min^{-1}. The mass spectrometer was operated in a data-dependent manner. Full scans (300-1700 m/z) were acquired in the Orbitrap with a maximum injection time of 500 ms and a target automated gain control (AGC) of 1e6 charges. Atmospheric polydimethylcyclosiloxane (m/z 371.101230) was used as a lock mass for internal calibration. Up to seven peaks per cycle were selected for CID fragmentation in the linear ion trap (1e4 target AGC charges, maximum injection time 100 ms). Dynamic exclusion settings were 30 s repeat duration and

60 s exclusion time.

3.2.12 Protein identification and computational data analysis

For peptide identification, ProteomeDiscoverer 1.3 was used with the Sequest algorithm. Fragment spectra of peptide precursors were searched against in-silico spectra generated from the UniRef100 database of the *M. truncatula* reference proteome (as of 30th October 2014). Methionine oxidation was chosen as variable modification, a maximum of two missed cleavages was allowed. Precursor mass tolerance was set to 5 ppm and fragment ion mass tolerance to 0.8 Da. The Mercator application was used to add functional annotations to the Uniprot protein accessions (http://mapman.gabipd.org/web/guest/app/mercator).

Quality control, filtering and statistical analysis was performed in MATLAB (R2010b). The data matrices exported from ProteomeDiscoverer were processed as follows: first, a protein filter was applied. To pass this filter, a protein had to present values in more than half of the observations in at least one treatment. Missing value estimation was then performed in two distinct ways. When a protein was detected in more than half of the observations per treatment, the k-nearest neighborhood method was employed. When a protein was detected only in half or less of the observations per treatment, then a minimum value was imputed. The statistical significance of protein abundance changes among treatments were evaluated by converting the P-values from the Kruskal-Wallis test to q-values in order to correct for multiple testing (Benjamini and Hochberg, 1995). The abundance of a protein was judged significantly altered if it met two criteria: (1) adjusted Kruskal-Wallis test q-value < 0.05 and (2) minimum twofold change.

3.2.13 Statistical analysis

Unless stated otherwise, statistical analysis was conducted using one-way ANOVA. The homogeneity of variances was tested employing Levene's test. All analyses were performed using MATLAB(R2010b) and STATGRAPHICS 16.1.11.

3.3 Results

3.3.1 Drought and rehydration experiment

In Chapter 2, we saw that during water deprivation NOD plants exhibit a stay-green phenotype when compared to NN plants. To obtain a better understanding of the mechanisms involved, this study focused on (i) assessing the effect of nodulation

on molecular parameters relevant to plant water relations under control conditions, (ii) studying the dynamic system responses to non-lethal drought and rehydration within vital leaves in NN and NOD(e) *M. truncatula*. Plants were subjected to progressively increasing drought stress during nine days followed by a rehydration period of four days (Figure 3.1a). Plants were harvested at five time points: after five days (D5) and nine days (D9) of drought, two hours (R10), one day (R11) and three days (R13) of rehydration. Water withholding resulted in a 58 % decrease in SWC on day 9. At this timepoint, NN and NOD(e) plants had similar predawn leaf xylem water potentials (Figure 3.1c). No differences in SWC between pots accommodating well-watered control NN and NOD(e) or drought-rehydration treated plants were observed throughout the study (Figure 3.1b).

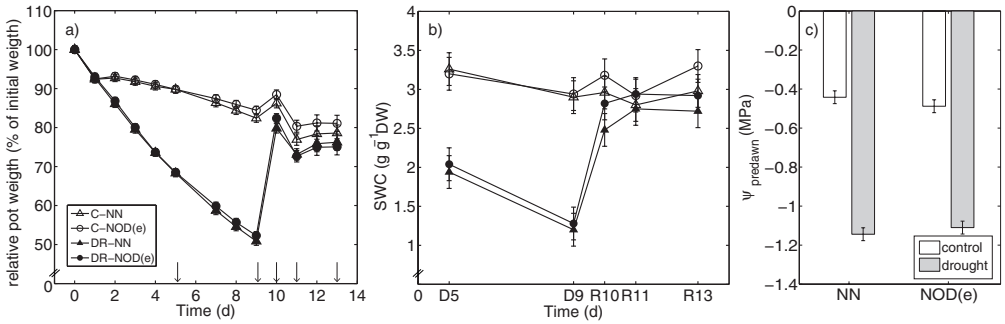

Figure 3.1: Water status during drought and rehydration. Estimated (a) and absolute (b) SWC and D9 predawn leaf xylem water potential (c). Well-watered (open symbols, C) and treated *Medicago truncatula* (closed symbols, DR) was grown in a vermiculite/perlite mixture. Arrows in (a) designate the sampling time points shown in (b), ψ was measured on day nine. Values are means; error bars indicate 95% LSD confidence intervals; n = 5-10 in (a), n = 5 in (b) and (c). NN: non-nodulated, NOD(e): *Sinorhizobium medicae* nodulated.

3.3.2 Inorganic anion and cation concentrations

The N regime (NN or NOD(e)) had significant effects on the leaf concentrations of soluble anions and cations under well-watered conditions (Table 3.1). Symbiotically grown plants had significantly higher concentrations of NO_3^-, NH_4^+, K^+, Na^+ and PO_4^{3-}, whereof the absolute mean difference in K^+ was highest (226 µmol g^{-1} DW). Drought induced decreases in the leaf Cl^-, PO_4^{3-} and K^+ concentrations in plants grown under both N regimes (Figure 3.2). NN plants showed significant differences at the first sampling time point (D5), in NOD(e) plants significant differences from D9 on were apparent, except for SO_4^{2-}. The decreased concentrations were then completely recovered on the last rehydration time point R13. During the rehydration phase, NO_3^- and NH_4^+ concentrations substantially increased on R11 and R13 in NN plants relative to controls (the mean (± SE) was 17.9 ± 4.2 and 35 ±

23.05 µmol g^{-1} DW, respectively; n = 5; $P < 0.05$).

Table 3.1: Mean soluble anion and cation concentrations in *Medicago truncatula* leaves under well-watered conditions and effects of N regime (NOD(e) or NN), time (D5, D9, R10, R11, R13) and the interaction term (N x T) as calculated by two-way ANOVA. The percentage shows the difference in pool size of NN plants relative to NOD plants.

	µmol g^{-1} DW		N regime			Time		N x T	
	NOD(e)	NN	(%)	F(1)	*P*	F(2)	*P*	F(1,2)	*P*
Cl^-	194.9	192.0	-1.5	0.05	ns	18.12	***	0.02	ns
NO_3^-	3.2	0.4	**-85.8**	22.77	***	0.01	ns	0.77	ns
SO_4^{2-}	227.3	256.2	12.7	2.22	ns	9.42	**	0.02	ns
PO_4^{3-}	128.3	104.7	**-18.9**	17.16	***	16.21	***	7.66	*
Na^+	24.4	17.8	**-27.2**	32.32	***	1.28	ns	1.20	ns
NH_4^+	14.8	4.7	**-68.1**	27.10	***	0.14	ns	3.78	ns
K^+	729.1	502.6	**-31.1**	277.00	***	8.05	*	9.65	**
Ca^{2+}	261.0	337.0	**29.1**	25.51	***	3.33	ns	0.04	ns
Mg^{2+}	345.7	347.1	0.4	0.01	ns	0.54	ns	0.44	ns

NN: non-nodulated, NOD(e): *Sinorhizobium medicae* nodulated; n = 5. Values in bold show singificant effects of the N regime. Asterisks indicate levels of significance (ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

3.3.3 Changes in stable C isotopic signature, starch, N and protein content

In response to substrate drying, the bulk leaf material of both N regimes was enriched in ^{13}C by D9 relative to controls. The enrichment effect tended to be strongest in NOD(e) plants (Figure 3.3a). Upon rehydration the $\delta^{13}C$ progressively decreased at similar rates in both N regimes.

Compared to NN plants, *M. truncatula* symbiotically grown with *S. medicae* had significantly higher leaf elemental N and protein contents, which were both not affected by the irrigation treatment. By contrast, the N and protein contents of NN plants decreased significantly during drought and progressively increased during rehydration (Figure 3.3b and c).

Leaf starch concentrations were assessed enzymatically. Interestingly, symbiotic plants had on average 52 % lower starch levels than NN plants (n = 25, $P < 0.05$). The starch content was not significantly altered by drought or rehydration in both groups of plants, with the exception of drought treated NN plants on R11 (Figure 3.3d).

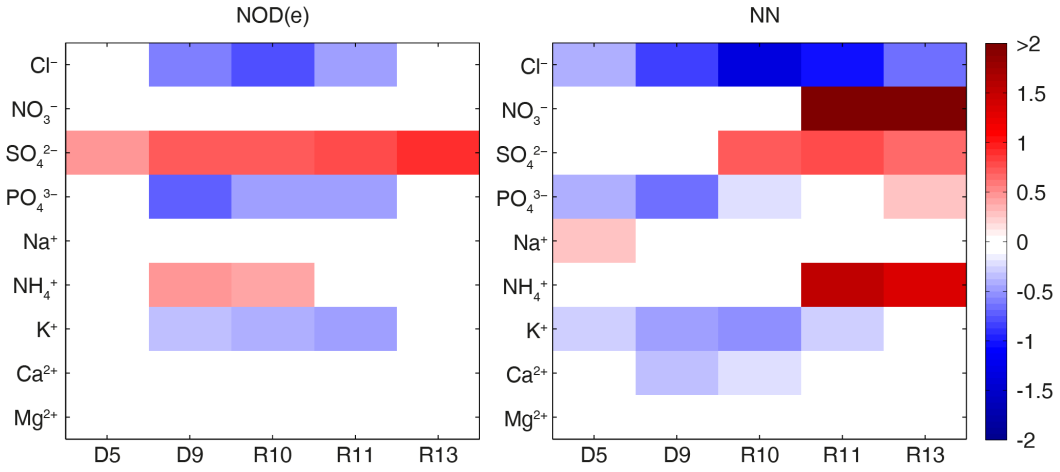

Figure 3.2: Changes in leaf soluble anion and cation concentrations during drought (D5, D9) and rehydration (R10, R11 and R13) in nodulated (NOD(e)) and non-nodulated (NN) *Medicago truncatula*. Values are the log2-fold change of treated relative to control plants; statistically significant changes are shown in color and were determined using Fisher's least significant difference after one-way ANOVA (n = 5, $P < 0.05$).

3.3.4 TBARS assay

The concentration of thiobarbituric acid reactive substances (TBARS) was assessed to determine the extent of lipid peroxidation in *M. truncatula* leaves. In this assay electrophilic species react with the electron donor thiobarbituric acid to yield a chromogen. Interestingly, under well-watered conditions, the TBARS levels of symbiotically grown *M. truncatula* were on average increased by 246 nmol g^{-1} DW (n = 25, $P < 0.001$, Kruskal-Wallis) relative to non-nodulated (NN) plants, which corresponds to an increment by 86 % (Figure 3.4). NOD(e) plants also showed a time-dependent increase in TBARS under control conditions at the rehydration time points R11 and R13. There was no such time-dependent effect in NN plants.

During drought and rehydration, TBARS levels were found to be stable in NN plants when compared to the respective controls. In leaves of NOD(e) plants, however, the TBARS content increased to levels 2-fold higher than in well-watered plants (n = 5, $P < 0.001$) and remained elevated during the rehydration period.

3.3.5 Relative changes in metabolite pool size

In this study, 32 metabolites of the primary C and N metabolism were relatively quantified. In total, 24 metabolites (the majority of amino acids and organic acids) were significantly enriched in NOD(e) leaves under control conditions (Table 3.2). Only threitol and galactinol had higher concentrations in NN leaves.

The effects of drought and rehydration on metabolite concentrations are shown in

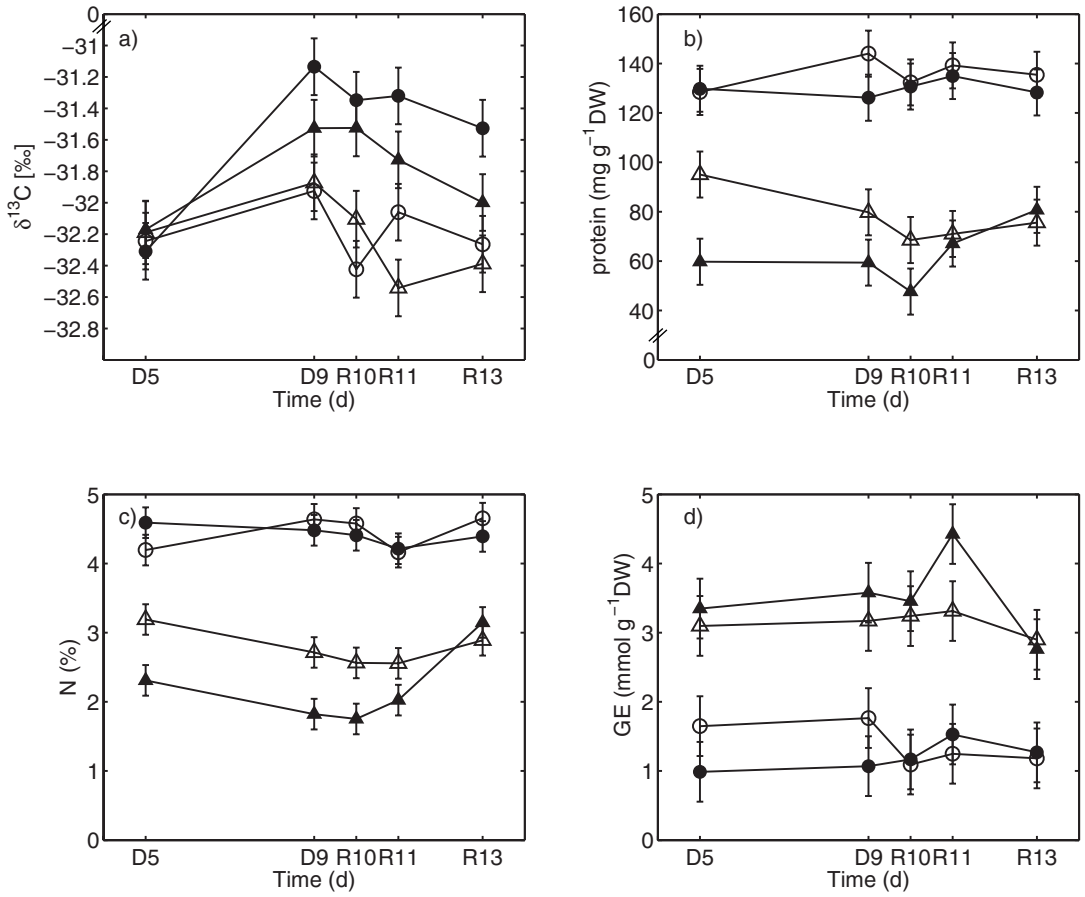

Figure 3.3: Leaf biochemical composition of *Medicago truncatula* during a drought (D5, D9) and rehydration (R10, R11, R13) experiment in control (open symbols) and treated plants (closed symbols). Triangles represent non-nodulated plants, circles represent nodulated plants. The graphs show bulk leaf stable isotopic signature (a), soluble protein content (b), N (c) and starch content in terms of glucose equivalents (GE). Values are means; error bars indicate 95 % LSD confidence intervals; n = 5.

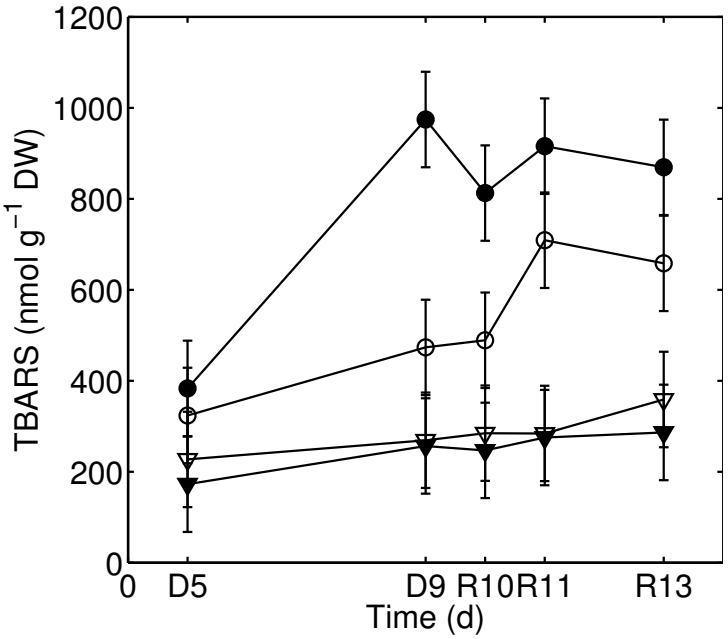

Figure 3.4: Thiobarbituric acid reactive substances in *Medicago truncatula* leaf extracts during a drought (D5 and D9) and rehydration (R10, R11 and R13) experiment in control (open symbols) and treated plants (closed symbols). Triangles represent non-nodulated plants, circles represent *Sinorhizobium medicae* nodulated plants. Values are means; error bars indicate 95 % LSD confidence intervals; n = 5.

Figure 3.5. There was an accumulation of the sugars fructose, glucose and galactinol along with the progression of drought, independent of the N regime, which still persisted on the first rehydration time point, R10. An accumulation of maltose, myo-inositol, pinitol and threitol was observed in NOD(e) plants. During later stages of recovery, the initial increase of the sugar and polyol levels was followed by a decrease to (NN) or even below (NOD(e)) control levels.

With respect to changes in amino acid and organic acid pools NN and NOD(e) plants responded distinctly to changes in water availability: symbiotic plants showed increases in many amino acids during drought (especially in Pro, Asp and Gln). These changes were paralleled by decreases in organic acids. During rehydration, the organic acid pool recovered to control levels within one day, followed by amino acids. On the contrary in NN plants, drought induced little changes in the amino acid and organic acid pools. Gradual decreases were observed in Asp and Glu. The pools of Ala and Met were punctually decreased at D5 and D9, respectively. However, most amino acid pool sizes substantially increased during later stages of rehydration (R11 and R13). Taken together, these results suggest that NOD(e) and NN *M. truncatula* differentially regulated their amino acid and organic acid pools during drought and rehydration. Moreover, NOD(e) plants adjusted most metabolite concentrations to control levels, whereas in NN plants 18 metabolites were increased at the last rehydration time point.

Table 3.2: Effects of N regime (NOD(e) or NN), time (D5, D9, R10, R11, R13) and the interaction term (N x T) on metabolite pools under well watered conditions as calculated by two-way ANOVA. The percentage shows the difference in pool size of NN relative to NOD(e) *M. truncatula*.

	N regime			Time		N x T	
	(%)	F(1)	*P*	F(2)	*P*	F(1,2)	*P*
Amino acids							
Alanine	**-52.3**	63.00	***	1.78	ns	1.86	ns
Asparagine	**-83.7**	231.22	***	0.65	ns	0.53	ns
Aspartic acid	**-78.4**	326.53	***	3.68	ns	3.88	**
Cysteine	16.8	2.22	ns	3.44	ns	1.06	ns
Glutamic acid	**-64.8**	655.64	***	3.81	ns	0.98	ns
Glutamine	**-68.9**	247.44	***	1.13	ns	4.49	**
Glycine	**-36.1**	38.17	***	0.77	ns	0.83	ns
Isoleucine	-13.4	2.31	ns	1.57	ns	2.25	ns
Methionine	**-69.1**	367.43	***	8.80	***	10.28	***
Phenylalanine	**-34.5**	95.81	***	0.53	ns	2.59	ns
Proline	**-55.5**	74.21	***	1.15	ns	2.94	ns
Serine	**-49.7**	117.96	***	8.65	***	7.38	***
Threonine	**-43.9**	138.02	***	5.97	***	2.28	ns
Tyrosine	**-29.3**	46.41	***	1.37	ns	4.43	**
Valine	**-33.5**	58.78	***	2.61	ns	1.84	ns
Organic acids							
Citric acid	-16.7	6.57	ns	4.92	**	0.35	ns
Fumaric acid	**-30.5**	75.57	***	10.95	***	2.09	ns
Gluconic acid	**-28.6**	59.16	***	7.10	***	0.94	ns
2-ketoglutaric acid	**-60.3**	132.82	***	5.60	**	1.30	ns
Lactic acid	18.6	2.37	ns	6.89	***	1.80	ns
Malic acid	**-60.6**	194.99	***	2.22	ns	0.83	ns
Pyruvic acid	**-37.5**	39.45	***	3.94	**	0.98	ns
Succinic acid	**-32.3**	33.45	***	17.79	***	1.58	ns
Threonic acid	**-38.6**	131.79	***	4.78	**	2.29	ns
Sugars and polyols							
Fructose	**-47.8**	114.52	***	10.29	***	4.62	***
Galactinol	**53.5**	8.40	**	8.86	***	0.20	ns
Glucose	**-37.3**	67.59	***	7.62	***	3.79	**
Maltose	6.9	1.36	ns	2.42	ns	1.43	ns
myo-Inositol	3.3	0.95	ns	1.88	ns	5.13	**
Pinitol	1.1	0.53	ns	54.89	***	2.25	ns
Threitol	**25.8**	20.44	***	10.00	***	1.45	ns

NN: non-nodulated, NOD(e): *Sinorhizobium medicae* nodulated; n = 5. Values in bold show significant effects of the N regime. Asterisks indicate levels of significance (ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

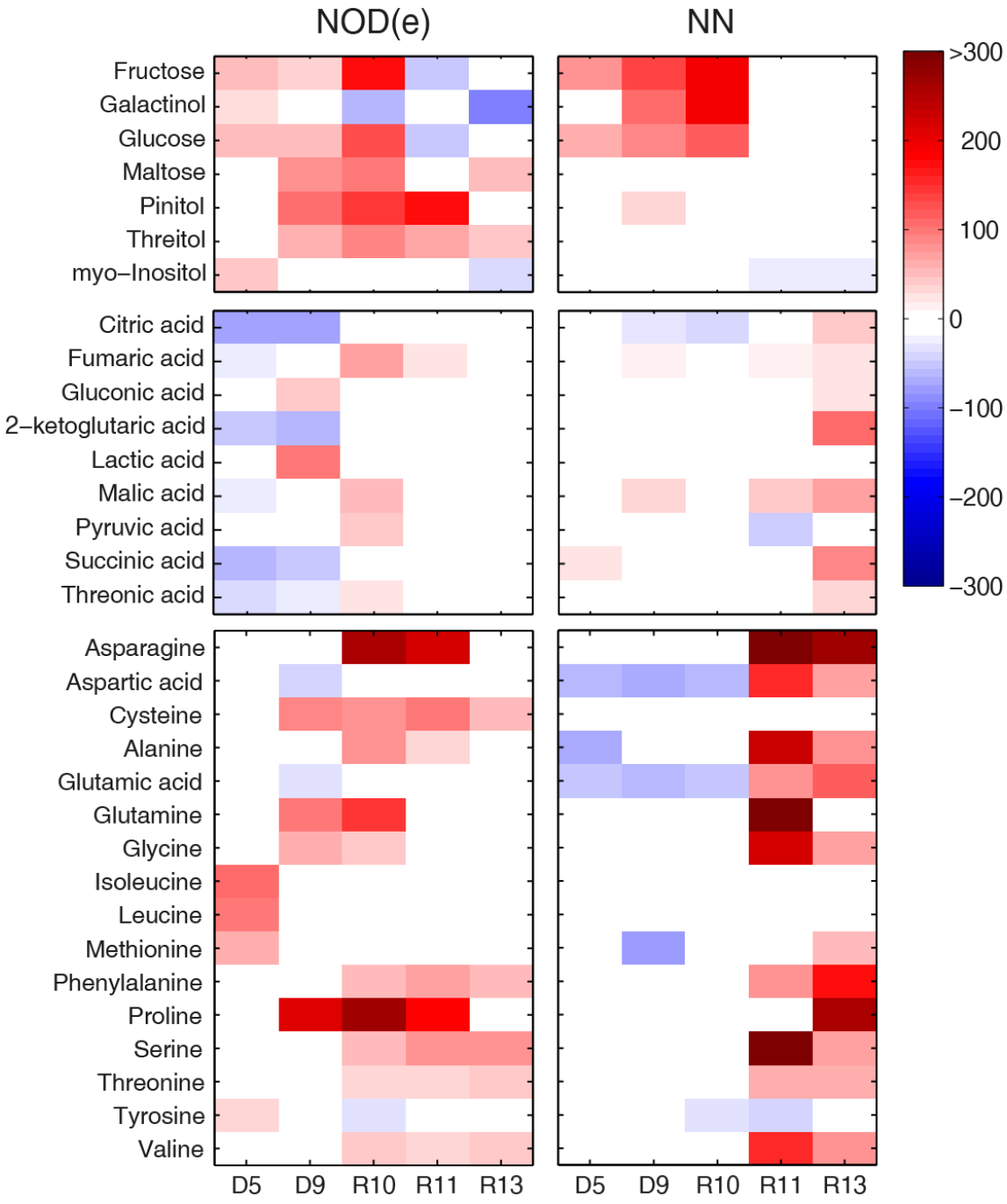

Figure 3.5: Changes in leaf metabolite content during drought (D5, D9) and rehydration (R10, R11 and R13) in nodulated (NOD(e)) and non-nodulated (NN) *Medicago truncatula*. Values are the percent change relative to controls; statistically significant changes are shown in color and were determined using Fisher's least significant difference after one-way ANOVA (n = 5, $P < 0.05$).

3.3.6 Relative changes in protein abundance

Using a shotgun proteomics approach to study dynamic changes of the leaf soluble proteome, 859 protein groups were identified. The rhizobial treatment (NOD(e) or NN) had a significant effect on the abundance of 128 protein groups under well-watered conditions (Figure 3.6 and Table A.3). On the one hand, NOD(e) plants had increased abundances of pyruvate kinase, ribosomal proteins and of enzymes involved in ethylene and jasmonate metabolism relative to their non-nodulated counterparts. On the other hand, NN plants had higher abundances of proteins assigned to amino acid and lipid metabolism, starch degradation, redox metabolism and protein degradation.

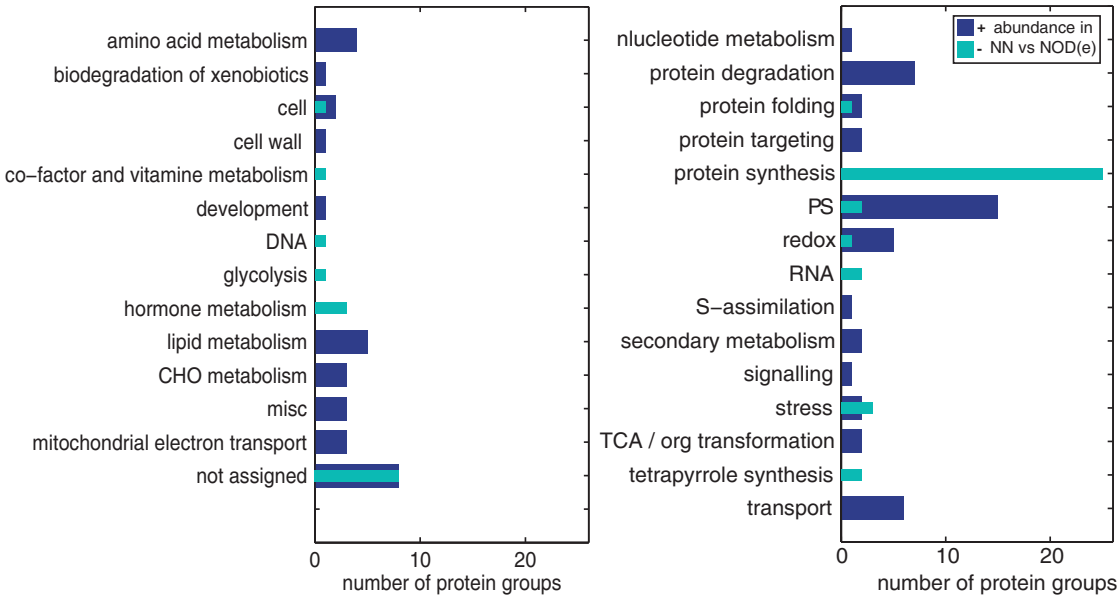

Figure 3.6: Mapman functional categories of differentially abundant protein groups, quantified by spectral counting in *Medicago truncatula* leaves of NN and NOD(e) plants under well-irrigated conditions. + and - designate proteins more and less abundant in NN relative to NOD(e) plants, respectively. Protein groups with a minimum two-fold change and $P < 0.05$ were considered (Kruskal-Wallis test after Benjamini-Hochberg adjustment, n = 21 or 23).

Table 3.3 shows the number of drought and rehydration responsive proteins in plants of both N regimes. NN and NOD(e) plants had similar drought responsive protein patterns as visualized by PC2 in Figure 3.7, although NOD(e) plant response was significantly delayed compared to NN plants. The abundances of ribosomal proteins, α-amylase and enzymes involved in tetrapyrrole biosynthesis exhibited significant decreased on D5 and D9 in both N regimes. The NN-specific changes in response to drought included decreased abundances in amino acid synthesizing enzymes and in two more starch degrading enzymes (glucane water dikinase and starch phosphorylase). The NOD(e)-specific drought responsive proteins were the

increase in several redox-related enzymes (Table 3.4). Interestingly, drought-induced changes in enzyme abundance of the senescence related jasmonic acid (JA) and ethylene metabolism showed opposite directionalities in NOD(e) and NN plants. Symbiotic plants had increased levels of JA biosynthesis enzymes (lipoxygenases, allene oxide synthase and allene oxide cyclase) and decreased levels of enzymes synthesizing ethylene (ACC oxidase) when compared to well-watered control plants. On the contrary, NN plants decreased the abundance of JA metabolism enzymes and increased the levels of ACC oxidase.

Table 3.3: Number of drought and rehydration responsive protein groups in non-nodulated and nodulated *Medicago truncatula*. Protein abundances of droughted plants (D5 or D9) were compared to controls and rehydrated plants (R11 and R13) were compared to drought stressed plants.

	NN			**NOD(e)**		
	D5/C	D9/C	R11,R13/D	D5/C	D9/C	R11,R13/D
up	12	10	38	3	32	15
down	14	30	14	5	21	2

n = 5-10; minimum two-fold change, $P < 0.05$; Kruskal-Wallis test after Benjamini-Hochberg adjustment.

During rehydration distinct responses were observed only at R13 between NN and NOD(e) plants (Figure 3.7). At R13, in NN plants, several PS related enzymes had lower abundances relative to the levels observed in drought stressed plants. In addition, the number of upregulated proteins in NN plants largely exceeded those observed in symbiotic plants (Table 3.3), mostly ribosomal proteins. On the contrary, protein abundances of NOD(e) plants remained at the levels that were observed at R11.

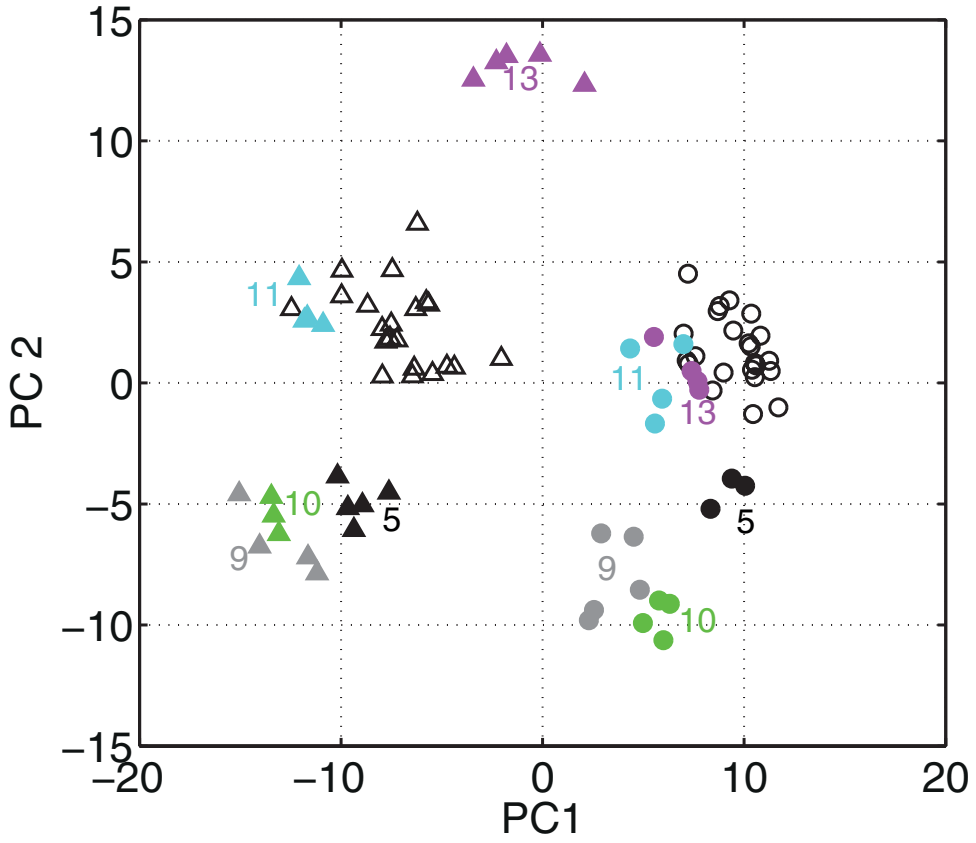

Figure 3.7: PCA scores plot of 620 protein abundances as affected by drought and rehydration in NN (triangles) and NOD(e) (circles) *Medicago truncatula*. Open symbols represent well-watered control samples of all five time points. Black and grey symbols indicate drought stressed plants (D5 and D9), colored symbols indicate rehydrated plants (R10, R11 and R13). PC 1 and PC 2 account for 19 % and 7 % of the total variance, respectively.

Table 3.4: Changes in protein abundance of selected protein groups during drought and rehydration in *Medicago truncatula* leaves. The log2-fold change of significantly altered proteins is shown (minimum two-fold change), and the P-values in parentheses ($P < 0.05$; Kruskal-Wallis test after Benjamini-Hochberg adjustment).

	NN			NOD(e)			
Protein group	D5	D9	R11, R13	D5	D9	R11, R13	**Mapman bin information**
amino acid metabolism							**amino acid metabolism**
G7JTY4: LL-diaminopimelate aminotransferase		-2.5 (0.047)	2.4 (0.023)			1.2 (0.039)	synthesis.aspartate family
G7IAZ2: 2-isopropylmalate synthase	-2.6 (0.030)						synthesis.branched chain group
G7J648: Cobalamin-independent methionine synthase		3.6 (0.008)					synthesis.aspartate family.methionine
A0A072UCM6: Glutamate decarboxylase						8.7 (0.039)	synthesis.central GABA.
hormone metabolism							**hormone metabolism**
G7K5B0: Cystathionine beta-synthase (CBS) family protein				-1.6 (0.005)	-1.3 (0.004)		ethylene
G7J9K5: 1-aminocyclopropane-1-carboxylate oxidase					-9.6 (0.007)		ethylene
G8A022: 1-aminocyclopropane-1-carboxylate oxidase-like protein	2.4 (0.029)						ethylene
Q711Q9: Allene oxide cyclase					3.6 (0.001)		jasmonate
A0A072UMH4: Linoleate 13S-lipoxygenase 2-1, related protein					4.9 (<0.001)		jasmonate
A0A072VAE0: Seed linoleate 9S-lipoxygenase		-2.6 (0.035)					jasmonate
mitochondrial electron transport							**mitochondrial electron transport**
G7KAG6: Cytochrome C oxidase, subunit VIb family protein		-10.1 (0.013)			3.9 (0.001)		cytochrome c oxidase
G7J8R4: NADH-ubiquinone oxidoreductase 75 kDa subunit			-1.8 (0.048)				NADH-DH.localisation not clear
N-metabolism							**N-metabolism**
G7JL79: Ferredoxin-nitrite reductase		10.7 (0.036)					nitrate metabolism
redox							**redox**
B7FGM0: Type II peroxiredoxin					1.8 (0.027)		peroxiredoxin
G7KPG2: Type II peroxiredoxin		-2 (0.006)	1.8 (0.033)				peroxiredoxin
I3SA84: Thioredoxin M-type protein					1.9 (0.014)		thioredoxin
I3SXR8: Ferredoxin-thioredoxin reductase, variable chain					2.4 (0.014)		thioredoxin
G7IE85: Thioredoxin-like protein		-2 (0.006)					thioredoxin
G7J9T0: Cytochrome b5-like heme/steroid-binding domain protein				3.4 (0.016)			ascorbate and glutathione
A0A072VNM9: GDP-D-mannose-3,5-epimerase			1 (0.033)				ascorbate and glutathione
starch degradation							**starch degradation**
A0A072TQP7: Alpha amylase domain protein				-8.3 (<0.001)	-8.3 (<0.001)		starch cleavage
A0A072UWD1: Glycogen/starch/alpha-glucan phosphorylase family protein						1 (0.039)	starch phosphorylase
A0A072TSJ2: Alpha amylase domain protein	-9.7 (0.024)	-9.7 (0.017)					starch cleavage
TCA / org transformation							**TCA / org transformation**
G7IPF6: 2-oxoacid dehydrogenase acyltransferase family protein					3.5 (0.001)	-7.6 (0.039)	TCA
G7K3L9: 2-oxoacid dehydrogenase acyltransferase family protein					3.5 (0.001)		TCA
tetrapyrrole synthesis							**tetrapyrrole synthesis**
A0A072VAY8: NADPH-protochlorophyllide oxidoreductase	-7.8 (0.029)	-7.8 (0.034)	4.2 (0.018)			1 (0.039)	protochlorophyllide reductase
A0A072TTZ3: Dicarboxylate carrier protein	-2.9 (0.029)						transport
G7IK85: Magnesium-chelatase subunit ChlI		-1.1 (0.035)					magnesium chelatase
G7JQM6: Porphobilinogen deaminase			1.6 (0.033)				porphobilinogen deaminase
G7K0I2: Glutamyl-tRNA(Gln) amidotransferase subunit B				-2.5 (0.014)			glu-tRNA synthetase

3.4 Discussion

3.4.1 Effects of nodulation on metabolism, soluble ion and protein abundance

Symbiotic N fixation is an energy costly process fuelled by assimilates which are transported from photosynthetic tissues to nodules. Thus, nodulation constitutes an additional sink to the plant. In a previous study, the enhanced demand for respiratory substrates was concomitant with an increased activity of sucrose phosphate synthetase and decreased starch levels in three out of four NOD soybean cultivars relative to their NN counterparts (Huber et al., 1982). In this study, NN plants had higher starch levels associated with lower sugar and organic acid levels than NOD(e) plants (Figure 3.3d and Table 3.2). However, there was no indication that nodulation leads to a substantial sink stimulation of photosynthetic rates (Table 2.2), as suggested by others (Kaschuk et al., 2009). Here, the treatment with rhizobia had little effect on photosynthesis but it exerted a strong effect on plant daytime C allocation between starch and soluble sugars.

Additional sink organs, such as nodules, might also increase the sucrose loading into the phloem. Interestingly, NOD(e) plants showed substantially higher K^+ concentrations compared to NN plants. By contributing to pH stabilization across membranes and to formation of osmotic potential, K^+ is essential for sucrose phloem loading and mass flow-driven solute transport in the vascular tissues (Marschner et al., 1996). Previous studies demonstrated the importance of K in the functioning of root nodule symbiosis. Indeed, NOD legumes receiving adequate K nutrition supplied more sugars to nodules, thereby increasing the rates of N fixation, when compared to K-deficient plants (Mengel et al., 1974; Collins and Duke, 1981). This suggests that NOD(e) *M. truncatula* accumulate more K^+ in order to optimize assimilate transport to below-ground organs.

Root infection by rhizobia induces signaling pathways reminiscent of those induced during plant defense, which are both driven by phytohormonal changes. Ethylene (ET) and jasmonic acid (JA) are negative regulators of nodulation by inhibiting the plant's response to rhizobial Nod factors (Sun et al., 2006; Oldroyd and Downie, 2008). Recently, Plett et al. (2014) showed that ET and JA pathways in poplar roots associated with ectomycorrhiza were induced to limit fungal growth during later stages of colonization. In addition, infection with rhizobia results in significant increases in plant ET levels (Ligero et al., 1986; Lee and LaRue, 1992). There is evidence that ET-dependent pathways regulate nodule numbers in temperate legumes such as the genera *Medicago*, *Pisum* and *Vicia*, whereas the ET-mediated suppression of nodulation is less pronounced in tropical legumes (Schmidt et al., 1999). In

line with this reports, the proteomic analysis presented here of *M. truncatula* leaves provides some indication that ET and JA pathways were systemically induced by nodulation. ET and JA are known to induce leaf senescence (Guo and Gan, 2005), however, no implication to the well-watered NOD(e) plants was observed. Furthermore, increased levels of proteins involved in JA-synthesis did not induce leaf senescence during drought in NOD compared to NN plants, but rather the opposite effect was observed. Although, enhanced levels of enzymes involved in hormone synthesis *per se* do not necessarily translate into enhanced hormone levels they give further evidence of playing a key role in leaf senescence regulation during drought.

Taken together, higher concentrations in leaf sugars, amino acids and ions such as potassium may contribute to turgor and leaf maintenance during drought. Nodulation may thus mediate the stay-green phenotype through nutritional priming.

3.4.2 Rhizobial effects on hormone metabolism and leaf osmolyte production during drought

The observed delay in leaf senescence of NOD plants during drought suggest that senescence inhibiting hormones such as cytokinin might play a more important role than JA. There are some reports on rhizobia derived cytokinin in the xylem of legumes (e.g. Upadhyaya et al., 2012). Furthermore, decreased abundances of proteins involved in ethylene synthesis support the observation of a nodulation-specific stay-green phenotype in *M. truncatula*.

Water deficits have been shown to induce increases in C rich compounds such as soluble carbohydrates, several amino acids and organic acids, many of which are osmolytes and considered to counteract the deleterious effects of cellular water deficits (reviewed by Muller et al., 2011). In both, NN and NOD(e) plants the drought stress treatment led to the accumulation of glucose and fructose, as observed by others (Frechilla et al., 2000; Sanchez et al., 2012; Zhang et al., 2014). The concentration of the sugar alcohol pinitol however, only increased in NOD(e) plants. In another study, pinitol increased only at very low water potentials (*c.* -3 MPa, day seven) in NN *M. truncatula* (Zhang et al., 2014). Pinitol is a major carbohydrate in legumes which may also act as an osmolyte (Streeter et al., 2001; Reddy et al., 2004).

A massive investment of reserves into the synthesis of osmolytes, such as proline, was previously observed in NOD alfalfa exposed to drought (Aranjuelo et al., 2011). Here, the N regime exerted a strong effect on amino acid levels during drought. The concentrations of Asp and Glu were significantly decreased in NN plants. Glu is a precursor of proline biosynthesis and Asp is formed by transamination involving 2-oxoglutarate and Glu. The depletion of Asp and Glu together with the decrease in

leaf N and soluble protein content suggests that protein derived N was remobilized and exported to other actively growing tissues. Indeed, a previous study reported that proline concentrations decreased in shoots of NN *M. truncatula* within six days of water withholding, while roots accumulated substantial amounts of proline (Staudinger et al., 2012). Here, increases in leaf amino acid concentrations (including proline) were restricted to NOD(e) plants. At the same time, drought did not affect the protein content of these plants. However, we observed increased NH_4^+ levels during drought. Thus mobilization of other N reserves rather than protein degradation might have accounted for the increases in amino acid pool size. Moreover, the elevated K^+ levels observed in NOD(e) relative to NN plants under well-watered conditions might have contributed to turgor maintenance during drought.

3.4.3 Evidence for a more effective drought stress recovery in nodulated plants

As expected, plants resumed leaf biomass accumulation upon rehydration as evidenced by rapidly decreasing sugar concentrations and the depletion of ^{13}C in the bulk leaf tissue (Figure 3.5 and Figure 3.3). Others also reported that sugars are among the first rehydration-responsive metabolites (Larrainzar et al., 2009; Kang et al., 2011).

Apart from sugars, the N regime had a strong effect on the plant's molecular responses to rehydration. N-fixation as well as NO_3-assimilation are drought sensitive processes, which are rapidly inhibited by water deficits (Fresneau et al., 2007; Larrainzar et al., 2009). The considerable increase in Asp suggests that N-fixation in NOD(e) plant was already induced after 2 h of rehydration, since Asp is one of the major N transport forms in *Medicago* (Lea et al., 2007). The onset of NO_3-assimilation might have been delayed in NN plants, elevated NO_3^- and NH_4^+ concentrations were observed one day after rehydration concomitant with an increase in amino acids relative to controls. Finally, proteomics data show a stronger increase in up-regulated proteins belonging to protein synthesis (ribosomal proteins) and N-metabolism such as nitrite reductase.

From a metabolic and proteomic perspective, these results suggest that at the one hand NOD plants exhibited a faster response to rehydration enabling the plants to adjust metabolite and protein abundances to control levels; on the other hand NN plants responded primarily with more energy demanding re-sprouting efforts and de-novo synthesis of proteins which may have been limited by ribosomal availability during the first three days of rehydration.

3.4.4 TBARS as signs of lipid peroxidation or rhizobial priming to stress

One of the swiftest responses of plants to limited water availability is stomatal closure. Restriction of stomatal apertures regulates the amount of water lost through transpiration in the short term. At the same time, the availability of CO_2 within the leaf is restricted and light energy is continuously absorbed by the light harvesting complexes. When non-photochemical quenching processes are insufficient to remove excess excitation energy from the photosynthetic electron transport chain, reactive oxygen species (ROS) accumulate (Takahashi and Badger, 2011). ROS can oxidize a broad range of cellular compounds such as nucleic acids, proteins, lipids and other small molecules. Under aerobic conditions, ROS are constantly produced as byproducts in chloroplasts, mitochondria and peroxisomes. Consequently, plants have evolved antioxidant and scavenging systems to carefully control ROS levels and thus minimize their damaging impact.

Apart from the cytotoxic effect of ROS, transient accumulations in ROS can also fulfill a signaling function. For example, ephemeral ROS increases are required to elicit cytoprotective responses during pathogen attack (Apel and Hirt, 2004). Similarly, they can induce acclimatory responses when plants experience unfavorable abiotic conditions (Pintó-Marijuan and Munné-Bosch, 2014). This duality is also encountered during leaf senescence. On the one side, a transient increase of ROS levels is necessary to trigger the senescence program involving nutrient remobilization; alternatively, prolonged elevated levels of ROS seem to be restricted to the latest stage of leaf senescence (Zimmermann and Zentgraf, 2005).

Lipids, notably polyunsaturated fatty acids (PUFAs), are readily oxidized by ROS. During the non-enzymatic oxidation of PUFAs many reactive electrophilic species are formed among which malondialdehyde (MDA) is a major breakdown product in plants (Weber et al., 2004). In the vast majority of literature, MDA levels (indirectly estimated via the TBARS assay) have been correlated to cellular oxidative stress. In this study, it was thus surprising to see that the level of TBARS was unaffected by drought and rehydration in the NN group of plants which showed pronounced signs of leaf senescence. In this respect it is important to note, that only vital leaves were collected during the sampling procedure. Together with previous observations indicating that sustained ROS accumulation occurs at the last stage of senescence (Zimmermann and Zentgraf, 2005), this result suggest that photo-oxidative damage was restricted to older leaves. In addition, several studies show that MDA levels do not respond in a stress-dose dependent manner (Pintó-Marijuan and Munné-Bosch, 2014; Farmer and Davoine, 2007).

Another interesting finding was the elevated TBARS concentration in leaves of

NOD(e) plants under well-watered conditions when compared to NN controls presenting comparable photosynthetic rates (see Chapter 2). Higher abundances of ROS scavenging enzymes which were observed in NN plants could account for this finding. Traditionally, higher TBARS concentrations in NOD(e) samples indicates that the extent of lipid peroxidation was more elevated in symbiotically grown plants during homeostatic conditions. However, this observation could also be explained by two additional lines of argumentation. First, the TBARS assay has its limitations when it comes to comparing lipid systems differing in their PUFA composition: different PUFAs give rise to different levels of reactive electrophilic species (Gutteridge and Halliwell, 2006). Cell membrane stability during drought has been shown to correlate positively with increasing N fertilizer applications (Saneoka et al., 2004). Hence, different N nutritional levels may also alter the membrane lipid composition, which in turn could influence the amount of TBARS generated during lipid peroxidation. Secondly, MDA could also play a role as 'latent RES', as plants have relatively high levels of MDA during normal growth (Farmer and Davoine, 2007). Since MDA is rather unreactive at neutral pH and shows a high reactivity when pH is more acidic, it could be readily mobilized in response to environmental stimuli and trigger specific acclimatory responses. In this respect, plants with higher MDA concentrations potentially could react faster to environmental conditions that alter the pH in various cellular compartments. This proposed role of MDA as a latent signal can be underpinned by the results presented here: when compared to their NN counterparts, NOD(e)plants had higher TBARS concentrations during control conditions, but simultaneously did not show enhanced abundances of ROS scavenging enzymes, nor signs of senescence. However, a variety of osmotically active compounds accumulated in NOD(e) plants already after five days of stress treatment, while the majority of metabolites remained unaltered in NN plants. These results could thus indicate that nodulation induced priming in terms of TBARS (including MDA) concentrations enhanced the plant's capacity to unleash tolerance responses.

Chapter 4

Summary

Water availability is one of the most important factors shaping plant life and species distribution. Consequently, plants have evolved a plethora of strategies to cope with water limitation. From an agricultural perspective, it is a major goal to stabilize plant growth and yield under transient drought conditions. In this respect, legumes play an important role as they form a root nodule symbiosis with nitrogen (N)-fixing soil bacteria, which makes them a major source of N for the human diet. However, it is largely unknown, how a symbiotic or non-symbiotic lifestyle alters plant responsiveness to water scarcity.

In this thesis, the model legume *Medicago truncatula* was used to test the hypothesis that nodulation induces changes in the plant metabolism that in turn are relevant under drought stress conditions. A positive relationship between leaf N concentration and plant drought tolerance has been observed previously. Therefore, an additional aspect of this thesis aimed at the distinction of nodulation specific responses from responses that are influenced by the leaf N level at the onset of a drought period.

Two types of experiments were performed: First, a lethal drought stress experiment, where the overall performance of three groups of plants was assessed (a group nodulated with *Sinorhizobium medicae* (NOD(e)), a group nodulated with *S. meiloti* (NOD(i)) and a group was non-nodulated (NN) and provided with N-fertilizer); and second, NOD(e) and NN plants were exposed to drought and subsequent rehydration to understand the molecular mechanisms involved in drought stress response. In addition to physiological analyses, molecular parameters were assessed using proteomic, metabolomic and ionomic approaches.

At the whole organism level two distinct strategies became apparent, when plants were exposed to comparable decreases in substrate water content. In NN plants older leaves underwent leaf senescence followed by leaf abscission. NOD plants maintained green leaves and showed a substantial delay in leaf abscission. This behavior is also referred to as stay-green phenotype. In NOD plants, the stomata of

older leaves also tended to respond faster to water deprivation, although no distinct total evapotranspiration was observed when compared to NN plants. This stay-green phenotype was empirically independent of the initial leaf N concentration and C/N ratio.

However, differing nutritional balances played a role in the observed phenomenon, as revealed by an in-depth study of the NOD(e) and NN response to drought and rehydration. At the molecular level, the responses to drought and rehydration were similar in terms of soluble protein abundance and ion concentration changes. However, nodulation had an important effect on the plant metabolism under well-watered conditions. Nodulation was associated with a shift in daytime C allocation. Relatively more sugars and less starch were present in the leaves of NOD(e) *M. truncatula* when compared to NN counterparts. NOD(e) plants also presented higher potassium concentrations and amino acid pools. These compounds all together act as osmolytes and may thus play an important role in leaf turgor and canopy maintenance during drought. Interestingly, NOD(e) plants had higher concentrations of TBARS under control conditions. The suitability of this method for assaying lipid peroxidation and a potential role of TBARS as latent signals is discussed. The differences in protein abundance provide indication that nodulation altered the plant's hormonal balance. During rehydration, NOD(e) plants responded rapidly at the metabolite level and were at control levels at the end of the experimental period. In NN plants, however, rehydration induced resprouting which is more energy costly.

This study highlights the versatility of a single species when exposed to water limitation and it shows that nodulation induces shifts within the plant's metabolism that have the potential to favour leaf maintenance during water deprivation (nutritional priming). This stay-green phenotype is of interest for transient drought scenarios, where it may have a positive effect on plant growth and yield.

Chapter 5

Zusammenfassung

Wasserverfügbarkeit ist einer der wichtigsten abiotischen Faktoren, die pflanzliches Leben und die Verbreitung von Arten beeinflussen. Deshalb entwickelten Pflanzen eine Vielzahl von Strategien um mit Wasserlimitierung umzugehen. In Regionen mit vorübergehenden Trockenperioden, sind stabiler Ertrag und Wachstum wünschenswerte Merkmale landwirtschaftlich genutzter Arten. In diesem Zusammenhang sind Leguminosen von besonderer Bedeutung, da sie durch ihre Symbiose mit Stickstoff (N)-fixierenden Bodenbakterien wesentlich zur menschlichen N-Versorgung beitragen. Bisher ist jedoch wenig darüber bekannt, wie ein symbiontischer bzw. ein nicht-symbiontischer Lebensstil die pflanzliche Stressantwort auf Wasserknappheit beeinflusst.

Für die vorliegende Arbeit kam die Modellleguminose *Medicago truncatula* zum Einsatz, mit dem Ziel folgende Hypothese zu überprüfen: Nodulierung bringt Veränderungen im pflanzlichen Metabolismus mit sich, welche für das Verhalten der Pflanze bei Trockenstress ausschlaggebend sind. Frühere Untersuchungen zeigten ein positives Verhältnis zwischen N-Konzentration im Blatt und Trockentoleranz. Ein weiterer Aspekt dieser Arbeit zielte deshalb darauf ab, Nodulierungs-spezifische Stressantworten von jenen Antworten zu unterscheiden, die durch das N-Level im Blatt zu Beginn einer Trockenperiode beeinflusst werden.

Zwei Experimenttypen wurden durchgeführt: ein letales Trockenstressexperiment, bei dem die Leistung von drei Pflanzengruppen untersucht wurde (eine noduliert mit *Sinorhizobium medicae* (NOD(e)), eine noduliert mit *S. meilioti* (NOD(i)) und eine nicht-nodulierte (NN) Gruppe, die mit N-Dünger versorgt wurde). Im zweiten Experimenttyp wurden NOD(e) und NN Pflanzen einer Trocken- und Rehydrierungsperiode ausgesetzt. Einerseits wurden hierbei die relevanten molekularen Mechanismen während der Stressantwort untersucht; andererseits wurde der Effekt der Nodulierung auf den pflanzlichen Metabolismus während ausreichender Wasserverfügbarkeit ermittelt. Neben physiologischen Messungen wurden hierzu proteomische, melabolomische und ionomische Analysen angewandt.

Waren die Pflanzen ähnlich sinkendem Substratwassergehalt ausgesetzt, so verwendeten die Pflanzen auf organismischer Ebene zwei unterschiedliche Strategien. Die alten Blätter von NN Pflanzen erfuhren Blattseneszenz und Laubfall. NOD Pflanzen behielten grüne Blätter und zeigten eine verzögerten Laubfall. Dieses Verhalten wird auch als Stay-green Phänotyp bezeichnet. In den NOD Pflanzen reagierten die Stomata tendenziell schneller auf Wasserlimitierung. Trotzdem wurden keine signifikanten Unterschiede in der Gesamttranspiration beobachtet. Dieser Stay-green Phänotyp war, empirisch betrachtet, unabhängig von der anfänglichen N - Konzentration und dem C/N Verhältnis im Blatt.

Die eingehende Untersuchung von NOD(e) und NN Pflanzen während Trockenheit und Rehydrierung deutet darauf hin, dass Nodulierung einen Ernährungszustand in den Pflanzen hervorruft, der maßgeblich am Stay-green Phänotyp der NOD Pflanzen beteiligt ist. Auf molekularer Ebene zeigten sich ähnliche Reaktionen in der Proteinzusammensetzung sowie Veränderungen der Ionenkonzentrationen im Blatt. Die Nodulierung hatte jedoch einen großen Einfluss auf den pflanzlichen Metabolismus unter gut bewässerten Kontrollbedingungen. Nodulierung ging mit einer Verschiebung in der C-Allokation einher. Verglichen mit NN Pflanzen, wurden mehr Zucker und weniger Stärke in Blättern von NOD(e) *M. truncatula* beobachtet. NOD(e) Pflanzen hatten auch eine höhere Kaliumkonzentration und größere Aminosäure-Pools. Diese Verbindungen fungieren auch als osmotisch aktive Substanzen und könnten deshalb einen wesentlichen Beitrag zum Erhalt des Blattturgors und des Blattwerks während Trockenstress leisten. NOD(e) hatten interessanterweise höhere TBARS-Konzentrationen unter Kontrollbedingungen. Die Eignung des TBARS-Assays um Lipidperoxidation in Pflanzen zu bestimmen und die mögliche Rolle von TBARS als latente Signale wird diskutiert. Unterschiede in der Proteinzusammensetzung deuten darauf hin, dass Nodulierung für Veränderungen im Hormonhaushalt der Pflanze verantwortlich ist, die auch in der verzögerten Blattseneszenz eine Rolle spielen können. Während der Rehydrierungphase konnten sich NOD(e) Pflanzen schneller wieder erholen. Ihre Metabolit- und Proteinlevels glichen sich an die Kontrolllevels an. Die molekulare Antwort der NN Pflanzen weist darauf hin, dass die Ressourcen zur Initialisierung von neuen Blättern verwendet wurden.

Die Ergebnisse dieser Arbeit zeigen, wie vielseitig eine einzelne Art auf Wasserlimitierung reagieren kann und, dass Nutritional Priming, induziert durch Nodulierung, den Blatterhalt während Stress fördern kann. Dieser Stay-green Phänotyp kann im Besonderen in Regionen mit vorübergehenden Trockenperioden einen positiven Einfluss auf Ertrag und Wachstum haben.

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Appendix A

Supporting information

Table A.1: Composition of the nutrient solution used in this study (Evans, 1974). Nitrate was added in the form of 0.5 mmol or 2.5 mmol NH_4NO_3 for nodulated and non-nodulated plants, respectively.

Macronutrients	(g/l)	Micronutrients	(mg/l)
$MgSO_4 \cdot 7\,H_2O$	0.493	H_3BO_3	1.43
K_2SO_4	0.279	$MnSo_4 \cdot 7\,H_2O$	0.772
KH_2PO_4	0.023	$ZnSO_4 \cdot 7\,H_2O$	0.22
$CaCl_2$	0.056	$CuSO_4 \cdot 5\,H_2O$	0.08
EDTA-Fe	0.017	$CoCl_2 \cdot 6\,H_2O$	0.117
K_2HPO_4	0.145	$NaMoO_4 \cdot 2\,H_2O$	0.05
$CaSO_4$	1.033		

Table A.2: Reference list showing the retention time index and the unique ion mass/charge ratio of compounds used for relative quantification by GC-TOF/MS.

Compound	**Retention Index**	**Unique Mass (m/z)**
Pyruvic acid (1MEOX) (1TMS)	1062.4	174
Lactic acid (2TMS)	1073.3	117
Valine (1TMS)	1097.3	72
D-Alanine (2TMS)	1113.6	116
Leucine (1TMS)	1163.9	86
Proline (1TMS)	1183.2	70
Valine (2TMS)	1227.5	144
Serine (2TMS)	1267.4	132
Leucine (2TMS)	1283.5	158
Threonine, allo- (2TMS)	1305.5	117
Isoleucine (2TMS)	1305.8	158
Continued on next page		

Compound	Retention Index	Unique Mass (m/z)
Proline (2TMS)	1309.5	142
Glycine (3TMS)	1319.2	174
Succinic acid (2TMS)	1321	75
Fumaric acid (2TMS)	1351.9	245
Serine [MPI-MDN35]	1372.8	204
Threonine, allo- (3TMS)	1399.7	57
Methionine (1TMS)	1416.6	104
Aspartic acid (2TMS)	1431.2	160
Glutamine [-H2O] (2TMS) MP	1486.3	155
Malic acid (3TMS)	1504	233
Threitol (4TMS)	1520.5	217
Methionine (2TMS)	1532.4	176
Aspartic acid (3TMS)	1535.7	232
Glutamic acid (2TMS)	1540.4	84
Phenylalanine (1TMS)	1556.7	120
Cysteine (3TMS)	1569.9	220
Threonic acid (4TMS)	1581.2	292
Glutaric acid, 2-oxo- (1MEOX) (2TMS) MP	1587.1	198
Proline [+CO2] (2TMS)	1592.8	142
Asparagine (2TMS)	1604.7	159
Glutamic acid (3TMS)	1633.2	246
Phenylalanine (2TMS)	1641.9	218
Asparagine (3TMS)	1687.6	116
Glutamine (3TMS)	1787.7	156
Citric acid (4TMS)	1843.9	273
Pinitol (5TMS)	1867	174
Fructose (1MEOX) (5TMS) MP	1909	103
Fructose (1MEOX) (5TMS) BP	1918.9	103
Glucose (1MEOX) (5TMS) MP	1935.9	319
Glucose (1MEOX) (5TMS) BP	1954	319
Tyrosine (3TMS)	1958.7	218
Gluconic acid (6TMS)	2036.7	333
myo-Inositol (6TMS)	2132	217
Sucrose	2713.5	361
Maltose (1MEOX) (8TMS) BP	2841.1	361
Galactinol (9TMS)	3072.7	204
End of Table		

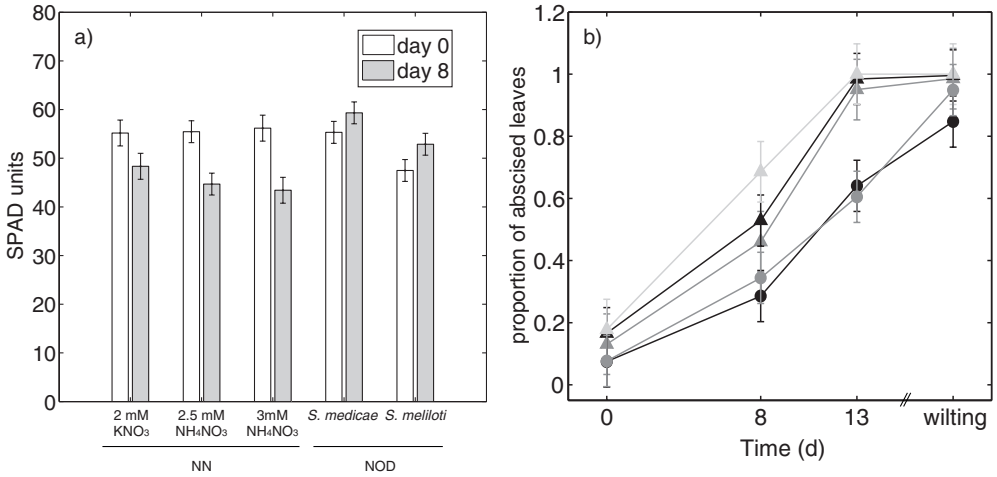

Figure A.1: Leaf senescence during a preliminary lethal drought stress experiment: (a) chlorophyll index of *M. truncatula* leaves at the start of a desiccation period (day zero) and after eight days of water withholding. Plants were grown for seven weeks with distinct nitrogen sources. (b) Leaf abscission rate during a lethal drought stress experiment in non-nodulated (triangles) and nodulated (circles) *Medicago truncatula*, provided with different N levels: 3 mmol NH_4NO_3 (dark grey triangles), 2.5 mmol NH_4NO_3 (black triangles), 2 mmol KNO_3 (light grey triangles), inoculated with *S. medicae* (black circles) or *S. meliloti* (grey circles). The day of wilting was *c.* day 15 in all conditions. Plants experienced similar decreases in substrate water content (Figure A.2). Values are means; error bars indicate 95%-LSD confidence intervals; n = 5-7.

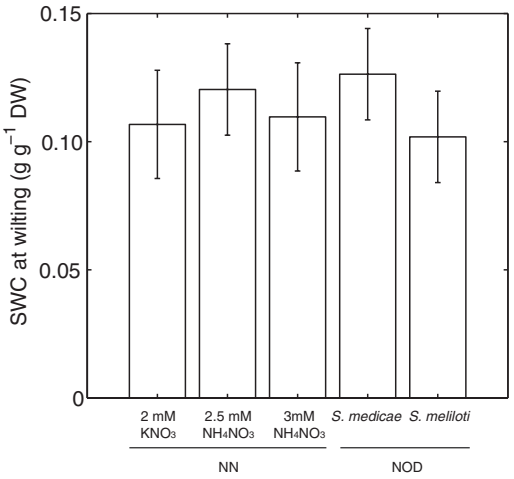

Figure A.2: The absoulte SWC at wilting. *Medicago truncatula* plants, treated with distinct N regimes, were grown individually and exposed to water-withholding. Values are means; error bars indicate 95%-LSD confidence intervals; n = 5-6.

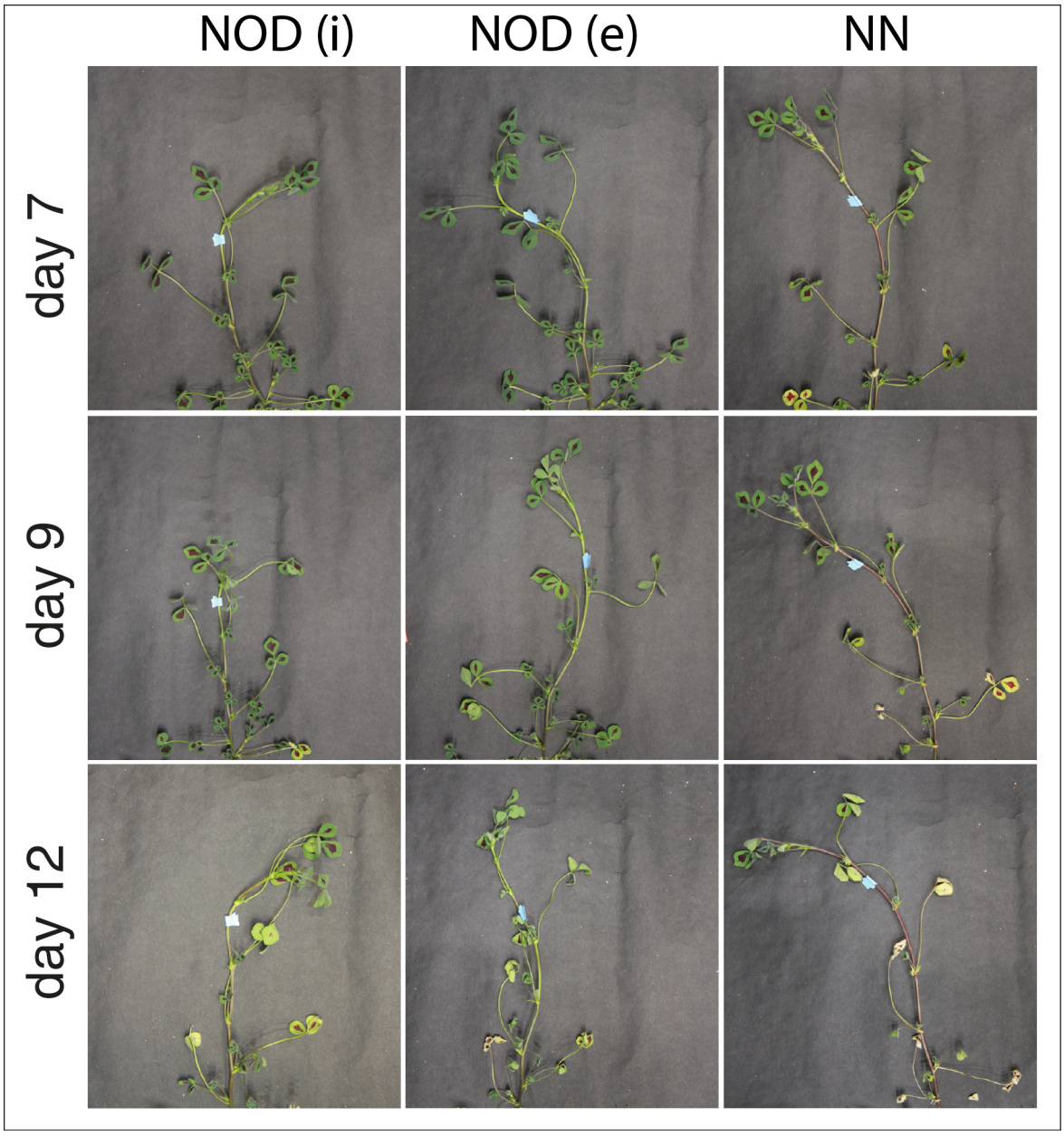

Figure A.3: Phenotype of the *Medicago truncatula* first side axis during a lethal drought stress experiment. The blue marks indicate the last visible internode developed at day 0. Non-nodultated (NN), nodulated (NOD) plants were inoculated with *Sinorhizobium medicae* (NOD(e)) or *S. meliloti* (NOD(i)).

Table A.3: Differentially abundant protein groups in non-nodulated (NN) and nodulated (NOD(e)) *Medicago truncatula* under well-watered conditions sorted according to MapMan bins. + and - designate proteins more and less abundant in NN relative to NOD(e) plants, respectively. Proteins with a minimum two-fold change and $P < 0.05$ are shown (Kruskal-Wallis test after Benjamini-Hochberg adjustment, n = 21 or 23).

Mapman bin		**Protein group**
amino acid metabolism		
synthesis.aspartate family.methionine	+	A4PU48:S-adenosylmethionine synthase
synthesis.branched chain group.leucine specific.2-isopropylmalate synthase	+	G7IAZ2:2-isopropylmalate synthase
synthesis.serine-glycine-cysteine group.cysteine.OASTL	+	A0A072U2J9:Cysteine synthase/L-3-cyanoalanine synthase
synthesis.serine-glycine-cysteine group.serine.phosphoglycerate dehydrogenase	+	A0A072VR00:D-3-phosphoglycerate dehydrogenase family protein
biodegradation of xenobiotics		
lactoylglutathione lyase	+	A0A072V507:Lactoylglutathione lyase-like protein
cell		
cycle.peptidylprolyl isomerase	+	I3S7Z5:Peptidyl-prolyl cis-trans isomerase
cycle.peptidylprolyl isomerase	+	A0A072U108:Peptidyl-prolyl cis-trans isomerase
organisation.cytoskeleton.actin.actin depolymerizing factors	-	G7IFU0:Cofilin/actin-depolymerizing factor-like protein
cell wall		
degradation.mannan-xylose-arabinose-fucose	+	D7RIC7:Alpha-L-arabinofuranosidase
co-factor and vitamine metabolism		
riboflavin.riboflavin synthase	-	A0A072UP30:6,7-dimethyl-8-ribityllumazine synthase
development		
storage proteins	+	A0A072TM25:Patatin-like phospholipase
DNA		
synthesis/chromatin structure.histone.core.H2A	-	A0A072UKD3:Core histone H2A/H2B/H3/H4

Continued on next page

Mapman bin		Protein group
glycolysis		
cytosolic branch.pyruvate kinase (PK)	-	G7I9C1:Pyruvate kinase
hormone metabolism		
ethylene.synthesis-degradation.1-aminocyclopropane-1-carboxylate oxidase	-	G7J9K5:1-aminocyclopropane-1-carboxylate oxidase
jasmonate.synthesis-degradation.lipoxygenase	-	G7LIZ2:Lipoxygenase
jasmonate.synthesis-degradation.lipoxygenase	-	A0A072VAE0:Seed linoleate 9S-lipoxygenase
lipid metabolism		
exotics(steroids, squalene etc)	+	A0A072UN88:NAD(P)H:quinone oxidoreductase, type IV protein
FA synthesis and FA elongation.Acetyl CoA Carboxylation.heteromeric Complex.Biotin Carboxylase	+	G7LIV6:Acetyl-CoA carboxylase biotin carboxylase subunit
FA synthesis and FA elongation.acyl-CoA binding protein	+	G7K6T1:Acyl-CoA-binding domain protein
FA synthesis and FA elongation.beta hydroxyacyl ACP dehydratase	+	A0A072UPD2:Beta-hydroxyacyl-ACP dehydratase
FA synthesis and FA elongation.enoyl ACP reductase	+	G7JNJ7:Enoyl-acyl-carrier reductase
major CHO metabolism		
degradation.starch.glucan water dikinase	+	A0A072TTK9:Alpha-glucan water dikinase
minor CHO metabolism		
myo-inositol.InsP Synthases	+	G7LAD5:Myo-inositol 1-phosphate synthase
others	+	G7JTE4:Aldo/keto reductase family oxidoreductase
misc		
alcohol dehydrogenases	+	G7KYI4:Zn-dependent alcohol dehydrogenase family, class III protein
misc2	+	A0A072UQU5:Endo-1,3-1,4-beta-D-glucanase-like protein
peroxidases	+	A0A072V5Z4:Peroxidase family protein
mitochondrial electron transport / ATP synthesis		
cytochrome c oxidase	+	G7KAG6:Cytochrome C oxidase, subunit VIb family protein

Continued on next page

Mapman bin		Protein group
F1-ATPase	+	A0A072UU27:F0F1 ATP synthase subunit gamma
F1-ATPase	+	G7I9M9:ATP synthase D chain
not assigned		
no ontology.pentatricopeptide (PPR) repeat-containing protein	+	G7JVZ3:Thylakoid lumenal protein
no ontology	+	A0A072U4Q2:Haloacid dehalogenase-like hydrolase
no ontology	+	G7J637:Smad/FHA domain protein
no ontology	+	G7I7H7:Thylakoid lumen 18.3 kDa protein
unknown	+	G8A207:Carboxy-terminal region remorin
unknown	+	A0A072VI54:Plastid lipid-associated protein
unknown	+	G7J8F1:Uncharacterized protein
unknown	+	B7FH20:ATP synthase
no ontology	-	A0A072VCZ9:Bark storage-like protein
no ontology	-	G7JEG1:DS12 from 2D-PAGE of leaf protein, putative
unknown	-	I3S066:60S acidic ribosomal protein
unknown	-	A0A072TND8:DUF2996 family protein
unknown	-	G7KG86:Uncharacterized protein
unknown	-	B7FM98:Plant/F25P12-18 protein
unknown	-	G7JFM1:YbaB/EbfC DNA-binding family protein
unknown	-	G7IBZ5:ABA/WDS induced protein
nucleotide metabolism		
phosphotransfer and pyrophosphatases.adenylate kinase	+	B7FN18:Adenylate kinase
protein degradation		
cysteine protease	+	G7I5V9:Papain family cysteine protease
metalloprotease	+	A0A072V376:ATP-dependent zinc metalloprotease FTSH protein
serine protease	+	G7KIR6:Trypsin-like serine protease
ubiquitin.proteasom	+	I3SSX1:Proteasome subunit alpha type

Continued on next page

Mapman bin		Protein group
protein.degradation	+	A0A072V8W3:Cytosol aminopeptidase family protein
protein.degradation	+	G7JQA0:ATP-dependent Clp protease proteolytic subunit
protein.degradation	+	G7JGP9:Cytosol aminopeptidase family protein
protein folding		
protein.folding	+	Q1RSH4:GroEL-like chaperone, ATPase
protein.folding	+	G7JEW8:Peptidyl-prolyl cis-trans isomerase
protein.folding	-	G7JS70:GroES chaperonin
protein targeting		
mitochondria	+	A0A072TYG3:Processing peptidase
secretory pathway.unspecified	+	A0A072UTX7:Patellin-like protein
protein synthesis		
ribosomal protein.eukaryotic.40S subunit.S18	-	G7IEW7:Ribosomal protein S13P/S18e
ribosomal protein.eukaryotic.40S subunit.S18	-	B7FMJ8:Ribosomal protein S13P/S18e
ribosomal protein.eukaryotic.40S subunit.S24	-	B7FMK4:40S ribosomal protein S24
ribosomal protein.eukaryotic.40S subunit.S25	-	A0A072UX65:Ribosomal protein S25
ribosomal protein.eukaryotic.40S subunit.S3A	-	G7K3P1:40S ribosomal protein S3a
ribosomal protein.eukaryotic.40S subunit.S8	-	I3T3T7:40S ribosomal protein S8
ribosomal protein.eukaryotic.60S subunit.L13	-	G7KWF7:60S ribosomal protein L13
ribosomal protein.eukaryotic.60S subunit.L15	-	I3T9M3:Ribosomal protein L15
ribosomal protein.eukaryotic.60S subunit.L18A	-	B7FJC3:60S ribosomal protein L18a
ribosomal protein.eukaryotic.60S subunit.L23A	-	G7JTN9:60S ribosomal protein L23a-2
ribosomal protein.eukaryotic.60S subunit.L27A	-	G7J4S6:60S ribosomal protein L27a-3
ribosomal protein.eukaryotic.60S subunit.L28	-	G7IUL4:60S ribosomal L28-like protein
ribosomal protein.eukaryotic.60S subunit.L28	-	I3TAF1:60S ribosomal L28-like protein
ribosomal protein.eukaryotic.60S subunit.L3	-	G7IBY1:60S ribosomal protein L3B
ribosomal protein.eukaryotic.60S subunit.L6	-	G7LBA5:60S ribosomal L6-like protein

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Mapman bin		Protein group
ribosomal protein.eukaryotic.60S subunit.L6	-	B7FH28:60S ribosomal L6-like protein
ribosomal protein.eukaryotic.60S subunit.L8	-	G7L3L0:60S ribosomal L8-like protein
ribosomal protein.eukaryotic.60S subunit.P0	-	G7JC94:60S acidic ribosomal protein P0-1
ribosomal protein.prokaryotic.chloroplast.30S subunit.S3	-	G7JH60:30S ribosomal protein S3
ribosomal protein.prokaryotic.chloroplast.30S subunit.S4	-	G7ZW80:30S ribosomal protein S4
ribosomal protein.prokaryotic.chloroplast.30S subunit.S6	-	A0A072TUR4:Ribosomal protein S6 family protein
ribosomal protein.prokaryotic.chloroplast.50S subunit.L10	-	B7FHY0:Ribosomal protein L10 family protein
ribosomal protein.prokaryotic.chloroplast.50S subunit.L2	-	S4T029:Ribosomal protein L2
ribosomal protein.prokaryotic.chloroplast.50S subunit.L3	-	G7J8L6:50S ribosomal protein L3P
ribosomal protein.prokaryotic.chloroplast.50S subunit.L31	-	G8A1R6:50S ribosomal protein L31
PS		
lightreaction.other electron carrier (ox/red).plastocyanin	+	G7J5X6:Plastocyanin
lightreaction.photosystem I.LHC-I	+	A0A072U7I8:Light-harvesting complex I chlorophyll A/B-binding protein
lightreaction.photosystem I.LHC-I	+	G7ZZ39:Chlorophyll a-b binding protein
lightreaction.photosystem I.PSI polypeptide subunits	+	Q2HW07:Photosystem I reaction center subunit XI
lightreaction.photosystem I.PSI polypeptide subunits	+	G7JAX6:Photosystem I reaction center subunit
lightreaction.photosystem I.PSI polypeptide subunits	+	G7KZJ5:Photosystem I reaction center subunit IV A
lightreaction.photosystem I.PSI polypeptide subunits	+	I3SN70:Photosystem I reaction center subunit III
lightreaction.photosystem I.PSI polypeptide subunits	+	B7FN63:Photosystem I reaction center subunit VI
lightreaction.photosystem II.LHC-II	+	G7JB75:Light-harvesting complex I chlorophyll A/B-binding protein
lightreaction.photosystem II.PSII polypeptide subunits	+	A0A072U5R2:Photosystem II reaction center PsbP family protein
lightreaction.photosystem II.PSII polypeptide subunits	+	S4T093:Photosystem II 44 kDa reaction center protein
lightreaction.photosystem II.PSII polypeptide subunits	+	G7J6G6:Photosystem II reaction center PsbP family protein
lightreaction.photosystem II.PSII polypeptide subunits	+	S4T074:Photosystem II CP47 chlorophyll apoprotein
lightreaction.photosystem II.PSII polypeptide subunits	+	A2Q5A0:Photosystem Q(B) protein
lightreaction.photosystem II.PSII polypeptide subunits	+	G7KGQ5:Oxygen-evolving enhancer protein

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Mapman bin		Protein group
calvin cycle.rubisco interacting	-	G7JTD2:Ribulose bisphosphate carboxylase/oxygenase activase
calvin cycle.rubisco small subunit	-	A0A072TWG1:Ribulose bisphosphate carboxylase small chain
redox		
ascorbate and glutathione.glutathione	+	A0A072VL94:Glutathione reductase
dismutases and catalases	+	B7FHQ5:Superoxide dismutase [Cu-Zn]
peroxiredoxin	+	B7FGM0:Type II peroxiredoxin
thioredoxin	+	G7IBZ4:Thioredoxin
thioredoxin	+	A0A072V2Y4:Thioredoxin H-type 1 protein
thioredoxin.PDIL	-	G7IDU4:Protein disulfide isomerase-like protein
RNA		
RNA binding	-	G7KDR6:RNA-binding (RRM/RBD/RNP motif) family protein
RNA binding	-	G7I6P1:RNA-binding (RRM/RBD/RNP motif) family protein
S-assimilation		
ATPS	+	G7ZV13:ATP sulfurylase
secondary metabolism		
isoprenoids.carotenoids.carotenoid cleavage dioxygenase	+	B5BLW2:Carotenoid cleavage dioxygenase
phenylpropanoids.lignin biosynthesis.CCoAOMT	+	B7FHM1:Caffeoyl-CoA 3-O-methyltransferase
signalling		
phosphinositides	+	B7FH09:DREPP plasma membrane protein
stress		
abiotic.heat	+	A2Q199:Chaperone DnaK
biotic.PR-proteins	+	G7IYL0:LRR receptor-like kinase
abiotic.heat	-	G7K4R2:Heat shock protein 81-2
abiotic.heat	-	G7I7Q4:Heat shock protein 81-2
biotic	-	G7LA76:Chitinase (Class Ib) / Hevein
TCA / org transformation		

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Mapman bin		Protein group
TCA / org transformation.TCA.aconitase	+	G7JYQ8:Cytoplasmic-like aconitate hydratase
TCA / org transformation.TCA.pyruvate DH.E1	+	B7FJJ4:Pyruvate dehydrogenase E1 beta subunit
tetrapyrrole synthesis		
ALA dehydratase	-	G7J506:Delta-aminolevulinic acid dehydratase
glu-tRNA synthetase	-	G7K0I2:Glutamyl-tRNA(Gln) amidotransferase subunit B, chloroplastic/mitochondrial
transport		
metabolite transporters at the mitochondrial membrane	+	A0A072TS91:Carnitine/acylcarnitine carrier-like protein
p- and v-ATPases	+	A0A072TTZ3:Dicarboxylate carrier protein
p- and v-ATPases	+	G7KW90:Vacuolar H+-ATPase subunit C
porins	+	A0A072URM9:Archaeal/vacuolar-type H+-ATPase subunit A
porins	+	I3S1P8:Porin/voltage-dependent anion-selective channel protein
porins	+	G7K2J4:Porin/voltage-dependent anion-selective channel protein

End of Table A.3

Appendix B

Curriculum vitae

Christiana Staudinger

Education

1995 - 2003	Stiftsgymnasium Kremsmünster, Austria Matura
2003 - 2005	Lycée Polyvalent Elisa Lemonnier Paris, France BTS Brevet de Technicien Supérieur Ethétique Cosmétique
2005 - 2010	University of Vienna, Austria Department Molecular Systems Biology Diploma study of Biology, specialized in botany, focus physiology and phytochemistry
since 2010	University of Vienna, Austria Department Ecogenomics and Systems Biology PhD studies in biology

Appendix C

List of publications

The work presented here was in part published within the following publications:

Staudinger, C., Gil-Quintana, E., Gonzalez, E. M., Hofhansl, F., Bachmann, G. and Wienkoop, S. (2015). Linking drought response strategies to symbiotic interaction with rhizobia in *Medicago truncatula*. *Journal of Experimental Botany*, (manuscript submitted for publication).

Staudinger, C., Mehmeti, V., Turetschek, R., Lyon, D., Egelhofer, V. and Wienkoop, S. Possible role of nutritional priming for early salt and drought stress responses in *Medicago truncatula*. *Frontiers in Plant Science*, 285, 2012.

Other publications:

Aranjuelo, I., Erice, G., Sanz-Sáez, Á., Abadie, C., Gilard, F., Gil-Quintana, E., Avice, J. C., **Staudinger, C.**, Wienkoop, S., Araus, J. L., Irigoyen, J. J., Tcherkez, G. (2015). Differential CO_2 effect on primary carbon metabolism of flag leaves in durum wheat (*Triticum durum* Desf.). *Plant, Cell and Environment*, doi: 10.1111/pce.12587.

Castillejo, M. A., **Staudinger, C.**, Egelhofer, V., and Wienkoop, S. *Medicago truncatula* proteomics for systems biology: novel rapid shotgun LC-MS approach for relative quantification based on Full-Scan Selective Peptide Extraction (Selpex). In Plant Proteomics (pp. 303–313). Springer, 2014.

Lyon, D., Castillejo, M. A., **Staudinger, C.**, Weckwerth, W., Wienkoop, S., and Egelhofer, V. Automated Protein Turnover Calculations from ^{15}N Partial metabolic labeling LC/MS shotgun proteomics data. *PloS One*, 9(4), e94692, 2014.

Lyon, D., Castillejo-Sanchez, M.-A., Mehmeti, V., **Staudinger, C.**, Kleemaier, C., and Wienkoop, S. (2015). Molecular elasticity and adjustment of drought recovery dynamics of ^{14}N- and ^{15}N-fertilized legume *Medicago truncatula*. *Molecular and cellular proteomics*, (manuscript submitted for publication).

Navascués, J., Pérez-Rontomé, C., Sánchez, D. H., **Staudinger, C.**, Wienkoop,

S., Rellán-Álvarez, R., and Becana, M. Oxidative stress is a consequence, not a cause, of aluminum toxicity in the forage legume *Lotus corniculatus*. *New Phytologist*, 193(3), 625–36, 2012.

Sainz, M., Calvo-Begueria, L., Pérez-Rontomé, C., Wienkoop, S., Abián, J., **Staudinger, C.**, Bartesaghi, S., Radi, R., Becana, M. Leghemoglobin is nitrated in functional legume nodules in a tyrosine residue within the heme cavity by a nitrite/ peroxide-dependent mechanism. *The Plant Journal*, 2015. doi:10.1111/tpj.12762

Staudinger, C., and Wienkoop, S. Plant Systems Biology. In: *Encyclopedia of Systems Biology* (pp. 1716–1717). Springer, 2013.

Wienkoop, S., and **Staudinger, C.**. Proteomics, Quantification-Unbiased and Target Approach. In: *Encyclopedia of Systems Biology* (pp. 1799–1800). Springer, 2013.

Wienkoop, S., **Staudinger, C.**, Hoehenwarter, W., Weckwerth, W., and Egelhofer, V. ProMEX - a mass spectral reference database for plant proteomics. Frontiers in Plant Science, 125, 2012. doi:10.3389/fpls.2012.00125