RESPONSE OF *MEDICAGO TRUNCATULA* TO ABIOTIC STRESS

Response of *Medicago truncatula* to flooding stress

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Response of *Medicago truncatula* to drought stress

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Response of *Medicago truncatula* to Salt stress

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Acclimation of *Medicago truncatula* to cold stress

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INTRODUCTION

Environmental constraints represent the most limiting factors for agricultural productivity and play a major role in the distribution of plant species across different types of environments. Apart from biotic stress caused by plant pathogens, there is a variety of distinct abiotic stresses, such as availability of water (drought, flooding), extreme temperature (chilling, freezing, heat), salinity, heavy metals (ion toxicity), photon irradiance (UV-B), nutrients availability, and soil structure. Abiotic stresses result, singly or in combination, in both general and specific detrimental effects on plant growth and development, and finally crop yield.

Major abiotic stresses (drought, salinity, freezing) lead to reduced availability of water for vital cellular functions and maintenance of turgor pressure. Dehydration or osmotic stress induces stomatal closure and, consequently, a reduction of the biochemical capacity for carbon assimilation and use. This leads to limitations of photosynthetic capacity and thereby plant growth. One characteristic cellular feature activated by abiotic stresses is the high production of reactive oxygen species (ROS) in the chloroplasts, mitochondria or in peroxisomes, causing irreversible cellular and tissue damages. However, most plants have developed various adaptation and detoxification mechanisms to deal with stress conditions, and considerable knowledge has been gained over the last decade on the activation of plant stress signal transduction pathways as well as physiological and molecular stress responses. Some of the most common responses for abiotic stress tolerance in plants are overproduction of several compatible organic solutes termed osmoprotectants or osmolytes (such as sucrose, betaines, proline) for osmotic adjustment and protection of subcellular structures, cellular metabolic changes (defense-related secondary metabolite production, proteolytic activity, adenylate energy charge, ionic homeostasis, redox state regulation, antioxidant enzymes activation…), anatomical and morphological changes in plant tissues, and induction of stress-responsive gene expression, Among them, activation of genes involved in signal transduction pathways (such as genes encoding protein kinases or transcription factors) may lead to complex changes in gene expression resulting in plant adaptation to abiotic stresses.

The undertaken basic research in the model plant *Medicago truncatula* is to elucidate the physiological and molecular mechanisms by which these legume plants sense and respond to abiotic stresses. In this chapter, main research advances gained in *M. truncatula* concerning major abiotic stresses (e.g. water availability, salt and cold stresses) will be specifically addressed.

RESPONSE OF *MEDICAGO TRUNCATULA* TO FLOODING STRESS

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1. Introduction

Flooding is one of the adverse environmental factors that harm severely germination, seedling establishment and plant development (Subbaiah and Sachs, 2003). Gases diffuse approximately 10 000 times slower in water than in air (Jackson, 1985), as a consequence when soils are saturated with water, oxygen fluxes into plants become too slow to support respiration, resulting in energy deficits and, eventually, death of cells and tissues in non-adapted plants (Jackson and Armstrong, 1999; Gout et al., 2001). The slow diffusion rate of gases through water also causes accumulation of endogenously produced gases, such as ethylene. Plants have evolved inducible developmental and metabolic mechanisms to adapt to low oxygen stress conditions that determine their sensitivity / tolerance to flooding. Adaptation to long-term submergence is frequently associated with developmental changes such as root aerenchyma formation, internode and petiole elongation, adventitious root development and alteration of root porosity, morphology and depth. Ethylene has been shown to be involved in these submergence-induced adaptations (Hoffmann-Benning and Kende, 1992; Van Der Straeten et al., 2001; Voesenek et al., 2003a; Voesenek et al., 2003b). However, the initial cellular response to decreased oxygen availability is promotion of anaerobic metabolism of pyruvate in both tolerant and sensitive species or different genotypes in the same specie. In hypoxic/anoxic tissues pyruvate content increased and glycolytic (glyceraldehydes 3phosphate dehydrogenase) and fermentative enzymes (pyruvate decarboxylase (*PDC*), alcohol dehydrogenase (*ADH*) and lactate dehydrogenase (*LDH*) are induced as a consequence of the need for increased glycolysis to compensate for the lower ATP yield due to the inactivation of oxidative phosphorylation (Saglio et al., 1999; Sato et al., 2002). Fermentative products *i. e.* acetaldehyde, ethanol and lactate accumulate allowing for the regeneration of NAD^+ from NADH. Regeneration of NAD by fermentative enzymes ADH and LDH is vital for hypoxia/anoxia tolerance because in the absence of NAD^+ glycolysis ceases (Ismond et al., 2003; Kursteiner et al., 2003). Induction of alanine amino transferase (AlaAT) fermentative pathway has been shown to contribute to the strategy that confer tolerance to hypoxia and anoxia in several plant species in which it played a significant role in

limitation of acetaldehyde synthesis and lactate accumulation for better cytoplasmic pH regulation (Reggiani et al., 2000; Ismond et al., 2003) and in rescue of C3 skeletons, that would otherwise go through ethanolic fermentative pathway causing a shortage in carbon availability (Ricoult et al., 2005).

Seed germination and seedling establishment are crucial phases of plant life cycle during which any stress would jeopardize adult plant vigor and performance by damaging the capacity of seedlings to colonize uniformly and rapidly the soil. Metabolic adaptive responses of *Medicago truncatula* seedlings to hypoxia stress were studied. Besides the activation of alcohol and lactic fermentative pathways, a concerted action of alanine aminotransferase (AlaAT) and glutamate dehydrogenase (GDH) contribute to hypoxia stress tolerance in *Medicago truncatula* seedlings. AlaAT and GDH pathways were dissected at the molecular (gene expression) and biochemical (^{15}N labelling) levels.

2. Involvement of alanine metabolism in metabolic adaptation of *Medicago truncatula* seedlings to hypoxia stress

Alanine aminotransferases, a pyridoxal phosphate multigene family has been characterized in *Medicago truncatula* (Ricoult et al., 2006). The enzymes catalyze transamination reactions using several amino donor/acceptor combinations (i) mitochondrial (mAlaAT) and cytosolic (cAlaAT) isoforms catalyze reversible transamination of glutamate (alanine : 2-oxoglutarate, glutamate : pyruvate), (ii) mitochondrial alanine/glyoxylate transaminase (AGT) isoform catalyzes reversible transamination of glycine (alanine : glyoxylate, glycine : pyruvate) and (iii) alanine/branched-chain amino acids transaminase isoform catalyzes reversible transamination of Valine and Leucine (alanine : 3-methyl-2-oxobutanoate, valine : pyruvate). Only *m-AlaAT* and *AGT* isogenes were expressed in embryo axes of germinating *Medicago truncatula* and were differentially regulated by hypoxia at transcriptional level. While the levels of transcript and protein of m-AlaAT increased under hypoxia, levels of transcript and protein of AGT decreased supporting the idea that m-AlaAT is the isoform involved in the adaptive response of embryo axis to hypoxia.

Activities of both enzymes have been measured *in vivo* by ^{15}N-labelling experiments under normoxia and hypoxia stress. Feeding embryo axis ^{15}N-glutamate or ^{15}N-alanine under normoxia showed that m-AlaAT catalyzed a reversible reaction allowing for synthesis of alanine with glutamate as amino donor and synthesis of glutamate with alanine as amino donor. The same experiment showed that glycine synthesis occurred at the expense of either glutamate or alanine indicating that beside alanine – glyoxylate transaminase (AGT) a

glutamate – glyoxylate transaminase (GGT) was also operating. Under hypoxia, it appeared that m-AlaAT was still allowed to synthesize alanine using glutamate as amino donor while its glutamate synthesis activity using alanine as amino donor was inhibited. This finding indicates that m-AlaAT might be regulated at post-translational level to have its activity directed towards alanine synthesis only under hypoxia stress. As a result, labeled alanine is a major amino acid accumulated in hypoxic embryo axes. By competing with ethanolic fermentation for pyruvate, alanine synthesis saves C3 skeletons avoiding a shortage in carbon availability and limits accumulation of acetaldehyde a toxic compound. Also, an increase in alanine synthesis, by competing with lactic fermentation for pyruvate, intervenes in cytosolic pH regulation. Furthermore synthesis of alanine arising from the decarboxylation of malate to pyruvate as a result of malic enzyme activation by hypoxia along with decarboxylation of glutamate to γ-amino butyric acid (GABA) are proton consuming reactions (Bouché and Fromm, 2004; Carroll et al., 1994; Edwards et al., 1998; Ricoult et al., 2005).

Alanine glyoxylate transaminase (AGT) was inhibited at transcriptional level by hypoxia resulting in lower amounts of the protein in hypoxic embryo axes than in control and inhibition of *in vivo* enzyme activity as revealed by ^{15}N-labelling. Glycine content was dramatically lower in hypoxic embryo axes than that in the control indicating that GGT did not compensate for the lack of AGT activity probably because glutamate was competitively recruited in alanine and GABA synthesis pathways catalyzed by m-AlaAT and GDC (GABA decarboxylase).

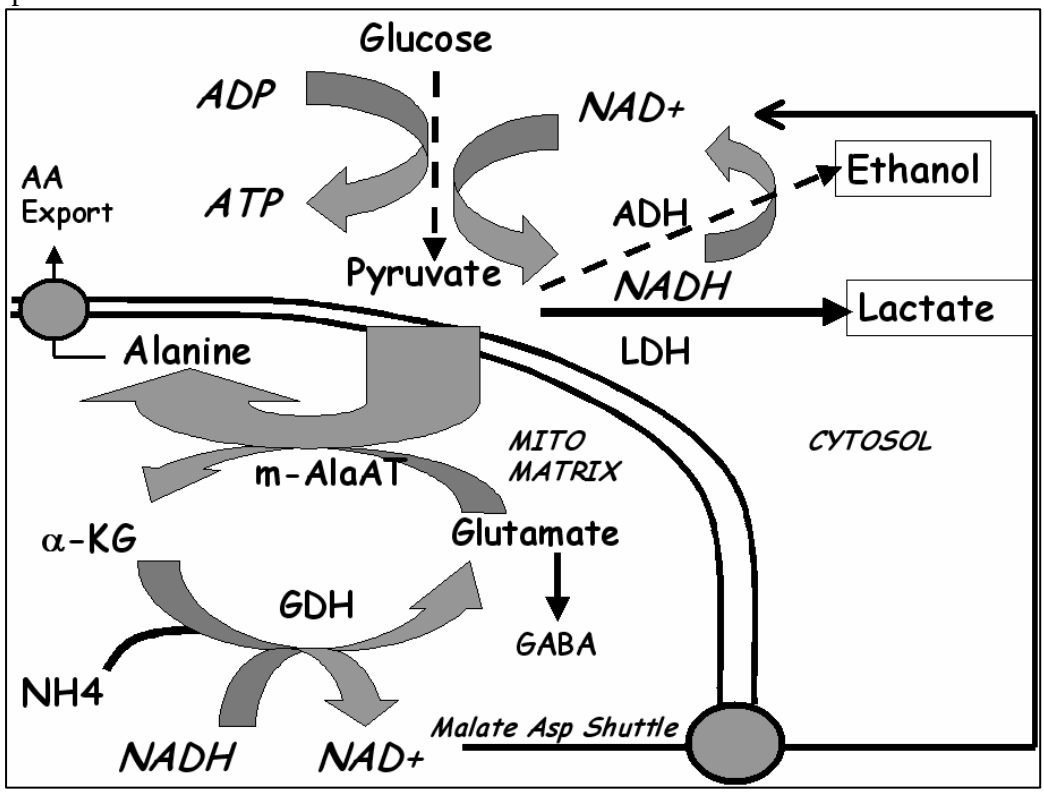
3. Regeneration of glutamate, the amino donor for alanine synthesis

Regeneration of glutamate by m-AlaAT is an important issue in the adaptive reaction of plants to hypoxia. In *Medicago truncatula* we have shown that under hypoxia ammonium assimilation by glutamine synthesis (GS) was inhibited due probably to the lack of ATP while its assimilation by (GDH) glutamate dehydrogenase was promoted (Limami et al., unpublished data). Although anabolic role of GDH *i.e.* assimilation of ammonium is still matter of debate (Glevarec et al., 2004) evidences in favor of this role were shown under specific situations such as stress caused by excess ammonium in Arabidopsis (Melo-Oliveira et al., 1996), and various abiotic stresses (Skopelitis et al., 2006).

Taken altogether these findings allowed us to propose that in *Medicago truncatula* seedlings under hypoxia stress mitochondrial AlaAT may contribute in coordination with the mitochondrial enzyme GDH to the maintenance of the redox balance during fermentative growth, with pyruvate used as a catabolic electron sink. Glutamate synthesis by GDH in

hypoxic tissues presents the advantage to not use ATP, furthermore in the anabolic direction the enzyme regenerate oxidized NAD that is crucial to the maintenance of glycolysis in hypoxic tissues (see below, Fig. 1). Similar adaptive reactions have been described in the hyperthermophilic archaeon *Pyrococcus furiosus* (Ward et al., 2000). This strict anaerobic utilizes a modified Embden-Meyerhof pathway for the catabolism of sugars which involves a unique glyceraldehydes-3-phosphate:ferredoxin oxidoreductase. The main products of sugar fermentation are acetate, CO2, H2 and alanine. Regeneration of oxidized ferredoxin is assumed to be accomplished by the formation of alanine when *P. furiosus* is grown in the absence of S. Alanine is synthesized by AlaAT transamination of pyruvate, the amino donor glutamate is proposed to be replenished through the action of NADP-dependent GDH. The necessary NADPH can be generated by the transfer of reducing equivalents from reduced ferredoxin to NADP by the ferredoxin:NADP oxidoreductase activity of the sulfide dehydrogenase regenerating then oxidized ferredoxin. AlaAT and GDH coordinated activities result in a change in the relative flux of pyruvate to acetate formation toward alanine formation. Pyruvate is therefore used as a catabolic electron sink (Ward et al., 2000).

Figure 1 : Representation of the coordinated contributions of mitochondrial AlaAT and GDH



to the maintenance of the redox balance during fermentative growth in *Medicago truncatula* seedlings under hypoxia stress.

RESPONSE OF *MEDICAGO TRUNCATULA* TO DROUGHT

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1. Introduction

Drought causes extensive crop losses worldwide (Boyer, 1982; Bray et al., 2000). Under field conditions, crops are routinely subjected to a combination of different abiotic stresses. Plant responses under these conditions cannot be directly extrapolated from responses of plants to individual stresses (Mittler, 2006). However, while the holistic approach is used in ecological terms, the study of individual stresses is more convenient in order to broad our knowledge of the mechanisms involved in plant responses to environmental constraints. When analysing molecular and biochemical processes, the need to create conditions similar to those experimented by plants in the field is required to extrapolate laboratory results to agronomic conclusions. This is especially relevant for the model legume *M. truncatula*, which is currently a focus of research worldwide. Its genetic proximity to important legume crops provides the rationale for potential application of the basic knowledge gained on this model. Hence, regarding abiotic stress studies, there is a clear need to analyse stress research under a physiological context.

2. Applying water stress under controlled conditions

Drought stress is particularly difficult to impose as water deficit is a gradual process. Inert polymers of high molecular weight, such as polyethyleneglycol, have been applied to simulate a programmed level of drought stress by reducing the water potential of the nutrient solution (Kaufmann et al., 1971). However, these polymers have been shown to enter the plant and exert a toxic effect, regardless the water uptake limitation (Emmert, 1974; Mexal et al., 1975; Munns et al., 1979). The physiological responses to drought and osmotically-applied stress are largely different in terms of carbon shoot:root allocation and the metabolic pathways involved (Frechilla et al., 1993). Despite its proven negative effect on plants, these polymers

are still employed nowadays for the analysis of drought response in plants (Foolad et al., 2003)

Alternative systems like pure hydroponic or aeroponics, although very useful for basic research under controlled conditions, are not suitable for drought studies. Plants grown in pure hydroponic systems with unlimited water supply until vegetative stage would dry promptly as soon as the nutrient solution is removed. Hence, these plants would be unable to respond to water stress in the way they would do under field conditions, where water deficit occurs gradually.

Altogether, the most recommended plant growth system to provoke a physiological water stress is that which allows a gradual depletion of water. This is easily achieved using a growth system based on soil or inert substrate with water retention capacity such as vermiculite. It should be reminded that growth conditions, plant density, plant developmental stage and size, together with the soil/substrate water retention capacity, have also a major effect on the magnitude and rate of water depletion.

Aiming to obtain physiological responses to drought stress on *M. truncatula* plants, we use the following protocol to create a gradual water deficit stress:

Plants are grown in 1 L pots (1 plant per pot), containing a 2:5 (v:v) perlite:vermiculite mixture under controlled environment conditions (14-h photoperiod, 600 µmol photons m^{-2} s^{-1}, 22°C/18°C day/night regime, 70% relative humidity). Drought is imposed when plants are 8-10 weeks old. In order to allow progressive water depletion in the pots, plants are supplied with water/nutrient solution to field capacity (substrate water content after excess water has drained) and then drought is imposed by withdrawing the watering solution. Control plants are daily supplied with nutrient solution to field capacity.

Some considerations should be made to this protocol: It is possible to use a higher plant density, but water stress will progress faster. It is also possible to use larger pots to grow more than one plant per pot. In this case, special attention should be paid to the homogenous water stress response of all the plants growing in the pot, since water within the substrate/soil will be consumed outside in.

Finally, drought regarded as limited water availability can be achieved in different ways. Generally, when evapotranspirational water loss is high, some water/nutrient can be added in order to allow gradual water depletion within the substrate and thus, a gradual water stress treatment. Obviously, the added amount should be also lower than the daily evapotranspirational water loss. An interesting approach to mimic real field conditions, is to generate temporary drought, created by cycles of water deprivation and re-watering (Antolin et al., 1995).

3. Monitoring water stress in *Medicago truncatula*

There are two main methods for estimating the water status of a plant: by measuring the amount of water or by measuring its energy status. Measurements based on plant water content are sometimes not reliable indicators, as they do not reflect plant water availability. Therefore, measuring plant water status in energetic terms is generally preferred. The concept of water potential (Ψ_w) was first introduced as a measure of the free energy of water. Ψ_w is expressed in pressure units (MPa or bar). Ψ_w is the sum of a number of components: in general, osmotic potential due to dissolved solutes (Ψ_s), pressure potential (Ψ_P) and gravitational potential (Ψ_g). Water potential is a measure of how hydrated a plant is, providing a relative index of the water stress the plant is experiencing. Although there are several methods available for its measurement, the most extensively used are pressure chambers and thermocouple psychrometers.

Pressure chambers are often used for estimating the water potential of leaves or small shoots both in laboratory and under field conditions (Scholander et al., 1965). A piece of tissue is excised from the plant and sealed into a chamber, by leaving the shoot end outside through a rubber gasket. Then, the chamber is closed and pressurised using a compressed gas. The pressure is gradually increased until the xylem sap appears on the cut surface, indicating that the leaf water potential has been reached. Drought stress can be carefully monitored by this method as it is shown in Figure 1 for three different *M. truncatula* genotypes.

Thermocouple psychrometers measure the water vapour pressure of a plant tissue, based on the principle that when water evaporates from a surface, this surface is cooled down. For the measurement, a piece of plant tissue is sealed inside a small chamber containing a thermocouple. Then, the air contained in this chamber is allowed to equilibrate with the plant sample. As vapour pressure equilibrates in the chamber airspace, the thermocouple senses the ambient temperature of the air, thus establishing the reference point for the measurement.

Under electronic control, the thermocouple seeks the dew point temperature within the enclosed space, giving an output proportional to the differential temperature. Due to the extreme sensitivity of the measurement to temperature fluctuations, temperature is measured and corrected before every use. This method it has been shown to be useful to determine local water potential in tissues as root or nodules (Gálvez et al., 2005) for which pressure chambers are not suitable. In contrast to those obtained by using the pressure chamber, psychrometer measurements are not instantaneous due to the time needed for sample equilibration inside the chamber.

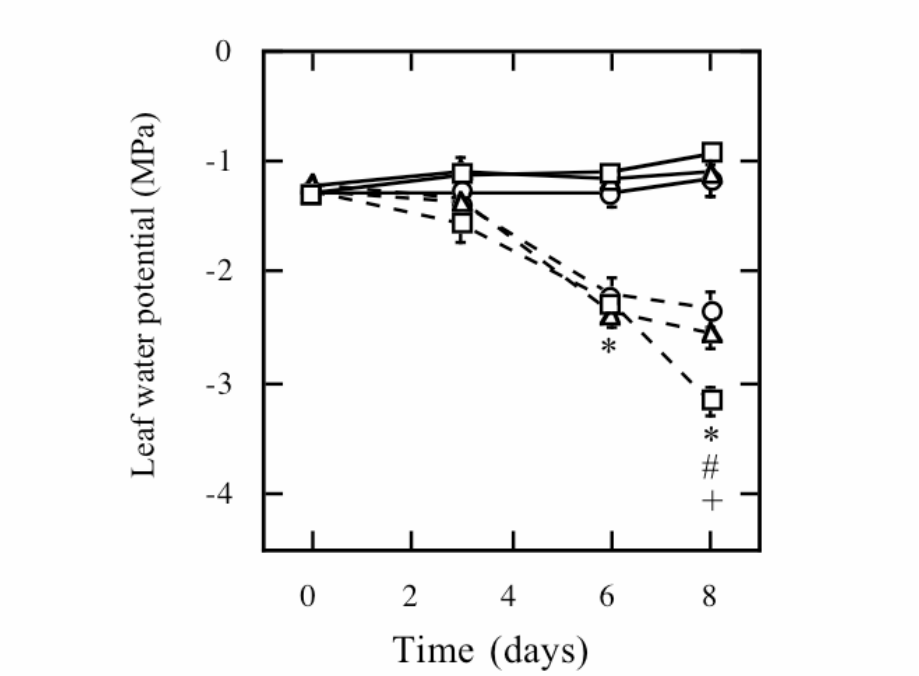

Figure 1. Effect of water stress on leaf water potential of three different genotypes of *Medicago truncatula,* F83.005-5 (square), Jemalong A17 (triangle) and TN1.11 (circle). Control plant values are represented with a continuous line and values from drought plants are represented with a discontinuous line. Asterisk (*), hash (#) and plus (+) symbols mean significant differences for F83.005-5, Jemalong A17 and TN1.11 with their corresponding controls, respectively. Values represent mean ± standard errors (n=12).

4. Effect of drought on *Medicago truncatula*

M. truncatula is a quite drought-tolerant plant species compared to other legumes like pea (González et al., 1998; Gálvez et al., 2005) or soybean (González et al., 1995). Considering plant biomass as an indicator of drought tolerance, water deficit has not a clear effect on *Medicago truncatula* plant biomass at a leaf water potential value around -1.4 ± 0.06 MPa

(D1, Figure 2), which causes a significant decline in pea (González et al., 1998; Gálvez et al., 2005) and soybean (González et al., 1995).

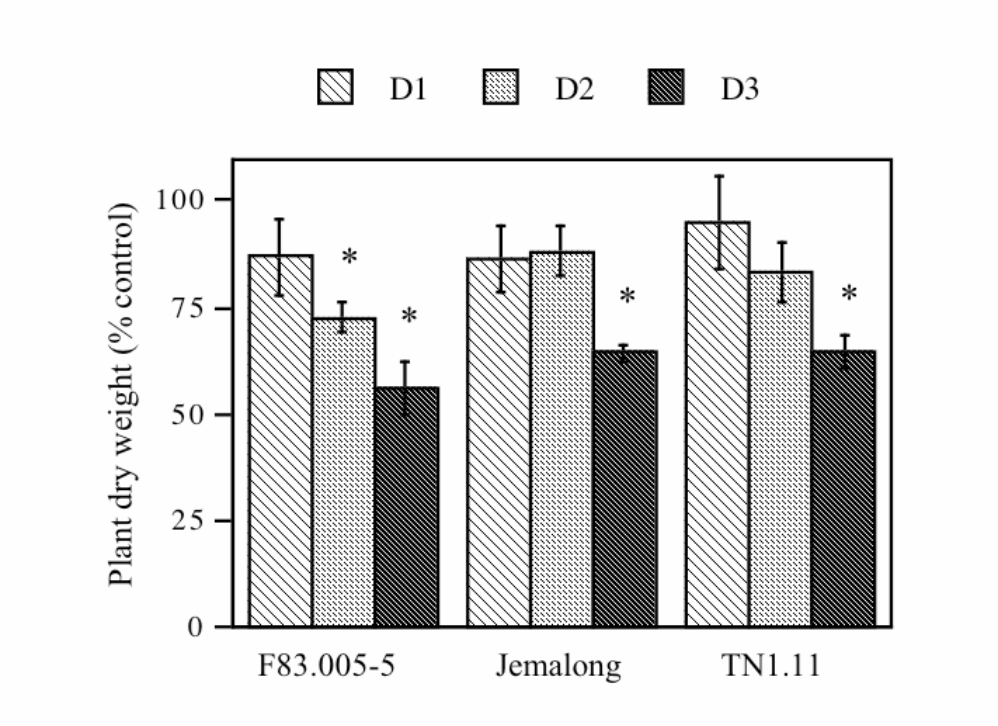

Figure 2. Effect of water stress on plant biomass of three different genotypes of *Medicago truncatula*, F83.005-5, Jemalong A17 and TN1.11. D1, D2 and D3 represent values from plants after 3, 6 and 8 days of water stress, respectively (see Figure 1 for water potential values). Values represent mean ± standard errors (n=12).

Moreover, water stress tolerance may differ among cultivars/ecotypes. The response to drought of three different *Medicago truncatula* genotypes, F83.005-5 (French origin), Jemalong A17 (Australian origin) and TN1.11 (Tunisian origin) has been recently analysed in our research group (Ladrera et al., unpublished data). These three lines were initially described as sensitive (F83.005-5), tolerant (Jemalong A17) and very tolerant (TN1.11) to salinity (Dr. Thierry Huguet, INRA, France, personal communication). F83.005-5, Jemalong A17 and TN 1.11 were grown for 8, 9 and 10 weeks, respectively, to achieve a similar plant biomass. Although drought causes a similar decline in leaf water potential in the three lines, F83.005-5 showed the lowest water potential at the end of the drought period, in agreement with its described drought sensitivity (Figure 1). Furthermore, F83.005-5 experienced the largest biomass reduction, being statistically significant after 6 days of water deprivation (D2, Figure 2) and showing a reduction of 55% of biomass when compared to control plants at D3 (Figure 2). In contrast, Jemalong A17 and TN1.11 did not experience any significant biomass

reduction at these stages. This decline was only statistically significant at day 8 after the onset of drought, when their leaf water potential was around -2.3 MPa (Figure 2).

Based on physiological and biochemical studies, *M. truncatula* responses to drought are similar to those described in other *Medicago* species, like alfalfa (Rubio et al., 2002), which have been shown to be more drought tolerant than pea (Moran et al., 1994) or ureide-producing grain legumes (Serraj et al., 1996). In these studies, photosynthesis rate was inhibited by 77% in pea leaves at a water potential value of -1.3 MPa, but only decreased 28% in alfalfa leaves at a water potential of -1.8 MPa. This inhibition was consistent with a decline in antioxidant activities and soluble protein content in pea leaves, but only slight changes were observed in alfalfa leaves. In relation to the nitrogen fixation process in root nodules of legume plants, Naya et al. (2007) suggest that alfalfa present a higher tolerance to drought stress compared to other species like pea and soybean. This higher drought tolerance appears to be similar to the one exhibited by nitrogen-fixing *Medicago truncatula* plants (Ladrera et al., unpublished results). Although an enhanced tolerance has been aimed using transgenic approaches (i. e. Zhang et al., 2007), the molecular basis of drought tolerance in the model legume *Medicago truncatula* has not been yet unravelled.

RESPONSE OF *MEDICAGO TRUNCATULA* TO SALT STRESS

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1. Introduction

Salinity in the arid and semi-arid regions of the world as well as in irrigated lands is a serious threat to agriculture, affecting plant growth and crop yields (Duzan *et al.*, 2004; Zahran, 1999). Current estimates indicate that 10 - 35% of the world's agricultural land is now affected, with very significant areas becoming unusable each year. Soil salinization significantly limits crop production and consequently has negative effects on food security. The consequences are damaging in both socioeconomic and environmental terms. It is a world-wide problem, but most acute in North and Central Asia, South America, Australasia and Mediterranean area. Management of salt-affected soils requires a combination of agronomic practices and socioeconomic considerations. However, where salinity is increasing as a problem on an irrigated farm, it may be necessary to select crop varieties that have a greater tolerance to salt.

An ecologically and agriculturally relevant aspect of legume biology is their specific ability to interact with soil bacteria to form root nodules which fix atmospheric nitrogen. The symbiotic bacteria, differentiated into bacteroids and surrounded by a peribacteroid membrane (that isolates them from the host cytoplasm), fix nitrogen inside the plant cells of this organ (Crespi and Galvez, 2000). However, this symbiotic interaction is affected by salt stress. Legumes are usually more sensitive to salinity than rhizobia which can be tolerant up to 700 mM NaCl (Singleton and Bohlool, 1984, Arrese-Igor *et al.*, 1999, del Papa *et al.*, 1999). Different steps of the symbiotic interaction as well as nodule development and metabolism are affected by salt stress, leading to a reduction in nodule number and limited nitrogen fixation (Singleton and Bohlool, 1984). Salt stress notably affects rhizobial colonization of roots and early infection events (McKay and Djordjevic, 1993, Tu, 1981). In addition, the nitrogen fixation process is very sensitive to salt stress, affecting peribacteroid membrane structure and bacteroid number (Bolanos *et al.*, 2003).

Medicago truncatula has been used as a model for understanding growth and development in legumes. Glycophyte *M. truncatula* show a large diversity of genotypes adapted to varying environmental conditions, including saline soils

(http://www.noble.org/medicago/ecotypes.html), and may therefore be a good model for understanding salt response also in other legumes (Bonin *et al.*, 1996). Shoot biomass production among accessions of *M. truncatula* exposed to NaCl have been analysed by Veatch *et al*. (2004). This study revealed that irrigation with a 115 m*M* NaCl solution decreased mean shoot biomass by over 46% relative to that with non-saline irrigation.

2. Physiological and metabolic adaptations to salt stress

In general, high NaCl concentrations affect plant physiology and metabolism at different levels (water deficit, ion toxicity, nutrient imbalance, and oxidative stress; Vinocur and Altman, 2005), and at least two main responses can be expected: a rapid protective response together with a long term adaptation response. This salt tolerance is generally associated with modifications of morphological and physiological traits, such as changes in plant architecture and growth (shoots and roots), variations in leaf cuticle thickness, stomatal regulation, germination, and photosynthesis rate (Edmeades *et al.*, 2001). These changes are linked to diverse cellular modifications, including, changes in membrane and protein stability, increased antioxidant capacity and activation of hormonal signaling pathways, notably those depending on the "stress hormone" abscissic acid (Vinocur and Altman, 2005).

A large number of genes from a variety of biochemical pathways participate in responses conferring salt tolerance. These pathways include notably those involved in signal transduction; in carbon metabolism and energy production; in oxidative stress protection; in uptake, exclusion, transport and compartmentalization of sodium ions; and in modifications of structural components of cell walls and membranes. Moreover, as regulation of metabolism to optimise growth in the changing environment is an essential trait, studies have been performed to identify metabolites whose accumulation may increase tolerance of legume plants to salinity. For example, lipid changes in response to salt treatment have been characterised in soybean root membranes (Surjus and Durand, 1996) as well as the effect of salt stress on amino acid, organic acid, and carbohydrate composition of roots, bacteroids, and cytosol of alfalfa (*Medicago sativa;* Fougere *et al*., 1991). In response to salt stress, trehalose has also been proposed as an osmoprotectant in *Lotus japonicus* (Lopez *et al*, 2006) whereas an increase in proline and glycine betaine content, as well as in protease and ATPase activities has been described in peanut (*Arachis hypogaea* L.; Muthukumarasamy and Panneerselvan, 1997). Trinchant *et al*. (2004) have investigated the long-term responses of nodulated alfalfa plants to salt stress, with a particular interest for proline and betaine accumulation, compartmentalization, and metabolism. Trigonelline, a pyridine betaine, which also functions

as a cell cycle regulator, accumulates in salt-stressed leaves of soybeans and alfalfa (Tramontano and Jouve, 1997). However, few metabolic studies have been performed in *M. truncatula*. Proline accumulation has been shown to induce tolerance to salt stress, and *M. truncatula* transgenic plants over-expressing delta(1)-pyrroline-5-carboxylate synthetase (P5CS), and which consequently accumulates high levels of proline, display enhanced osmotolerance (Armengaud *et al*., 2004; Verdoy *et al*., 2006).

3. Activation of transcriptional pathways in response to salt stress

Molecular studies have been used to identify candidate genes to be regulators of osmotolerant and salinity responses, particularly putative regulatory genes such as transcription factors (Hasegawa *et al*, 2000). In alfalfa, *MsAlfin1* has been identified as a salt-inducible transcript that encodes a zinc-finger protein predominantly expressed in roots (Winicov, 1993). Overexpression of this putative transcription factor enhances root growth under control and saline conditions (Winicov and Bastola, 1999; Winicov, 2000). The lack of root formation and root growth of alfalfa transgenic lines expressing *MsAlfin1* in an antisense orientation also supports a crucial role for this gene in root development (Winicov, 1999). Another C_2H_2 zinc-finger transcription factor (*ZPT2-1*) was identified in alfalfa and characterized in *M. truncatula* (Frugier *et al*., 1998, Frugier *et al*., 2000). This gene, expressed in vascular tissues of roots and nodules, is also induced by salt stress (Merchan *et al*., 2003) and *M. truncatula* antisense transgenic lines developed nodules unable to fix nitrogen due to a block in bacteroid differentiation (Frugier *et al*., 2000). These *MtZpt2-1* antisense lines were also less able to recover from a salt stress compared to a wild-type plant (Merchan *et al*., 2003), suggesting that this transcription factor may be involved in nodule and root adaptive responses to osmotic and salt stresses. Moreover, using either antisense Mt*Zpt2-1* plants or overexpression of this transcription factor, three putative target genes could be identified, one of which corresponded to a known abiotic stress-related marker the cold regulated A (CorA) gene (Merchan *et al*., 2007). These results further suggest a role for a TFIIIA-like transcription factors and their regulated target networks in the adaptation of legume roots to salt stress.

A few genomic analyses have been performed to examine salt stress responses in legumes. One of these studies aimed to identify regulatory genes involved in root adaptations during salt-stress recovery in *M. truncatula* (Merchan *et al*., 2007). Two SSH libraries were made and analyzed in order to construct a dedicated macroarray, which allowed refined expression studies in plants submitted to salt stress and allowed to reassume growth. Novel regulatory

genes associated to root recovery from salt stress where identified, potentially affecting various transcriptional and postranscriptonal regulatory mechanisms.

4. Methods for salt stress treatments

Different methods to apply salt stress in *M. truncatula* plants.

- *In vitro* growth conditions:

Seeds are scarified by rubbing between medium grain and fine-grain sandpaper, and are sterilized by immersion for 20 min in Inov'chlore (8.64 g/L; Inov'chem SA, Tanneries, France), followed by thorough washing with sterile water. Seeds are then germinated overnight in water-agar plates. Seedlings were grown vertically in growth chamber at 24 °C under a 16-h light period on a porous growth paper (from CYG seed germination pouches; Mega International, Minneapolis, MN, USA) for 3 days. Then the seedlings grown on the paper support are transferred to a new medium containing various NaCl concentrations. A 100 or 150mM NaCl concentration strongly reduce root and shoot growth of *M. truncatula* Jemalong. Salt recovery experiments can be also performed, where *M. truncatula* seedlings are grown as previously described and then re-transferred after 4- 7 days (depending on genotypes) to a fresh medium without salt (Merchan *et al*., 2003 and 2007).

- Greenhouse conditions:

Germinated seeds are transferred on a mix of perlite and sand (3:1 V: V ratio). After 1 week, plants are irrigated with 50 m*M* NaCl solution (acclimatation period) and then 2 days later with the assigned saline solution (50, 75, 100, or 150 mM NaCl). Alternatively, plants can be directly irrigated with the assigned saline solution. Similarly as for *in vitro* experiments, a concentration of 100-150 mM NaCl typically inhibits root growth and strongly delays plant development. In all cases, a plastic cover is placed on the plants to limit evaporation, and if prolonged salt treatments are tested, limited amounts of water (without salt) can be added to avoid increase in salt concentration.

ACCLIMATION OF *MEDICAGO TRUNCATULA* TO COLD STRESS

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1. Introduction

Low temperature is one of the most important factors limiting growth (Boyer, 1982), development and distribution of plants in the world. It disturbs metabolic activity, inhibits normal function of physiological processes and can lead to death by causing permanent injuries. Freezing temperatures can particularly account for significant losses in plant productivity and then limit farmers' income. Plants encountering negative temperatures can follow two main strategies to survive this stress: freezing avoidance or freezing tolerance (Sakai and Larcher, 1987). Freezing avoidance is mainly obtained by supercooling of the tissue water. This mechanism is however limited to specific organs such as seeds or overwintering buds (Sakai and Larcher, 1987). The acquisition of freezing tolerance is therefore the most common mechanism developed by plants to survive freezing stress.

In natural conditions, the response to low non-freezing temperatures is the primary factor which allows plants to increase their tolerance, a phenomenon known as cold acclimation (Levitt, 1980; Sakai and Larcher, 1987; Thomashow, 1999). Thus, the most logical way to improve plant freezing tolerance is to exploit their natural ability of acclimation. Determining the nature of the genes and mechanisms responsible for freezing tolerance and regulatory mechanisms that activate the cold acclimation response would provide the potential for new strategies to improve the freezing tolerance of agronomic plants (Thomashow, 1999). Such strategies would be highly significant as traditional plant breeding approaches have had limited success in improving freezing tolerance (Sarhan and Danyluk, 1998).

Cold acclimation is a quantitative trait (Thomashow, 1990) involving a large number of genes and is associated with physiological and biochemical changes as well as alterations in gene expression (Hughes and Dunn, 1996; Palva and Heino, 1998; Thomashow, 1999; Lee et *al*., 2002; Heino and Palva, 2003). Cold regulated genes can be divided into two main groups. The first group holds genes encoding enzymes or structural components that participate in direct protection of cells against freezing damage. This group includes genes encoding for example late embryogenesis-abundant (LEA) proteins, enzymes required for osmolyte biosynthesis, antifreeze proteins, chaperones and detoxification enzymes (Bray, 1993; Ingram

and Bartels, 1996; Palva and Heino, 1998; Thomashow, 1999, Puhakainen et *al*., 2004). The second group includes genes encoding transcription factors and other regulatory proteins controlling the low temperature response either transcriptionally or posttranscriptionally (Thomashow, 1998, 1999; Heino and Palva, 2003).

2. Studying *Medicago truncatula*

Plant scientists often use a model plant to investigate basic plant biology. As legumes are a particular group of plants especially for their symbiotic ability to fix the atmospheric nitrogen, it is funded to use a legume model species in a first step to understand the behaviour of agronomical legume species.

2.1. *Medicago truncatula* and cold acclimation studies

The model species *M. truncatula* has been chosen to help elucidating the genetic determinism of freezing resistance in pea (*Pisum sativum* L.), this crop being important for both food and feed. The goal of our study is to search candidate genes that control cold acclimation in *Mt*, using a 216 RIL population from the cross between F83-005 (tolerant to frost) and DZA-045 (sensitive). This work has been undertaken following two experimental stages:

- mapping QTLs for frost damage and for biochemical and physiological parameters related to cold acclimation (such as biomass, soluble sugar content, percentage of ions leakage and chlorophyll content) explaining the acclimation ability and checking colocalisation between them;
- searching for candidate genes involved in the cold acclimation process by a transcriptomic study and exploiting *in silico* mapping informations for the candidate sequences.

The analytical part of this study will rely on the study of the colocalizations between the different QTLs on one hand and between QTLs and potential candidate genes on the other hand.

2.2. Experimental protocol

a- Germination

After scarification, seeds were put in 2ml water-containing Eppendorf tubes for 6 hours imbibition. Seeds were then spread in wet paper-containing Petri dishes. To break dormancy and allow rapid and synchronized germination, the Petri plates were maintained at 5°C in the

dark during 3 days in a SANYO climatic chamber. They were then transferred to 20°C for germination and were ready for planting after approximately 2 days.

b- Sowing

The cold acclimation period and subsequent freezing test were performed in a climatic chamber (DAGARD; size: 3.2 x 3.2 x 2.4m; type: MA 100, class M1). The plantlets were placed in home-made polystyrene blocks of 100 or 50 holes (figure 1). Each hole was half filled with perlite (for optimal root development) and we inserted in each of them a 38mm well-moistened (water-saturated for up to 3 hours) Jiffy-7 pellet made of sphagnum peat (http://www.jiffypot.com/Products.asp). Lime and a special fertilizer with low ammonium content are pre-added to the peat in order to stimulate growth. The pellets have a pH of approximately 5.3.

One seedling was inserted in each pellet and was gently watered to allow a firm anchoring in the substrate. During the nursery stage (figure 2), the seedlings were gently watered everyday (de-ionized water) to avoid the desiccation of the pellets which could have been induced by the lighting and the temperature applied during this stage (20°C, see below).

c- Fertilization

In order to prevent premature yellowing of leaves, fertilizer was added in the pellets (about 10ml), from the end of the nursery stage until the beginning of the frost period. This is performed about once a week by using the commercial NPK (6:3:6) mixture supplemented with micronutrients (Substral, KB): 6 % total N (2.7 % ammoniacal N, 3.3 % nitric N), 3 % P_2O_5, 6 % K_2O; micronutrients: 0.01 % B, 0.001 % Mo, 0.002 % Cu-EDTA, 0.01 % Mn-EDTA, 0.002 % Zn-EDTA, 0.03 % Fe-DTPA (taken from European Molecular Biology Organization (EMBO) practical course at http://www.isv.cnrs-gif.fr/embo01/manuels/index.html).

d- Temperature, light intensity and photoperiod settings

We used two complementary protocols for our studies including or not an acclimation period (Figure 2). The successive stages, i.e. nursery, homogenization, acclimation, frost and warming, differ essentially for their temperature conditions as described below. The photoperiod was set to 14 (day)/10 (night) hours during all the stages except the frost period (10(day)/14 (night) hours). Light was provided by 2 types of PHILIPS lamps: HPI-T400W

(metal iodide) and SON-TP400W (sodium), with a mean photosynthetic active radiation (PAR) of 550 µmol.m^{-2}.s^{-1}:

- nursery stage: during this 15 days stage, the plants had optimal conditions for growth; temperature was set to 20°C (day) and 14°C the night. .
- homogenization: in this stage the temperature was set to 12°C day and night during 8 or 15 days for the protocol with and without acclimation respectively. Its goal is to assure a relative homogenization of plants' growth, by allowing young emitted leaves to develop without emitting new ones.
- acclimation: the temperature was set to 8°C (day) and 2°C (night); for the protocol with acclimation, this period allows plants to cold acclimate in order to better resist to the freezing temperatures of the following stage.
- frost: the plants were submitted to a freezing night temperature (-6°C) and a subzero day temperature (4°C) during 8 days. We chose, instead of applying a high negative temperature (e.g. -10°C) for a few days, to apply a minimum of -6°C for 8 days, in order to follow gradually the effects of the freezing temperature, and at the same time, to avoid killing some lines of the RIL population by applying suddenly a severe negative temperature.
- warming: at the end of the frost period, the plants were placed back in warmer conditions by applying 16°C (day)/5°C (night). Alternatively, the plants were placed in a greenhouse with optimal temperature conditions with the same objective to analyze re-growth capacity of surviving plants.

2.3. Frost damage QTLs mapping

We recorded the levels of frost damage everyday during the frost period. The plants were individually given a mark from 0 (no damage; figure 3) to 5 (dead). The warming period allowed us to confirm really dead plants, which did not resume their growth during this period. We then compare the strength of QTLs of each daily scoring in order to identify the most suitable day of scoring. At present, the scoring of the 4^{th} day seems to be the most suitable one for QTL mapping.

2.4. QTLs of biochemical and physiological parameters

Some parameters (biomass, soluble sugar content, percentage of ions leakage and chlorophyll content) that can explain the acclimation ability were also analyzed through QTLs mapping. Samples were taken on successive days during the acclimation and the non acclimation

periods according to the protocol. The dates of sampling were chosen to allow a comparison between the acclimation *vs* the non acclimation period; each sampling date corresponds to the same sum of degree days (base = 0°C) in both protocols (see Figure 2). In order to make sure that the same type of leaves was sampled at each date, we used the identification method described by Moreau et *al*. (2006). We checked the colocalization between the QTLs detected for the above cited parameters and those detected for the freezing damage at the plant level.

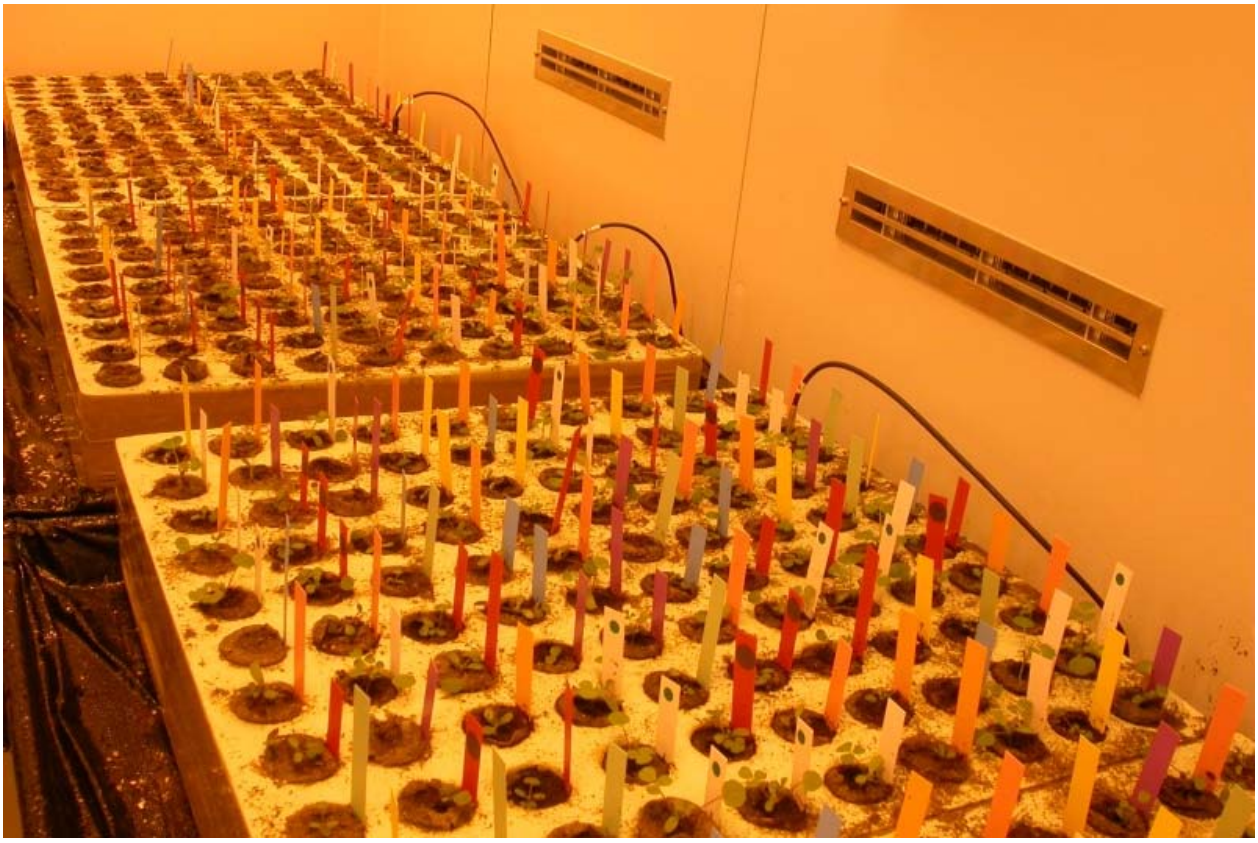

Figure 1: Home-made experimental blocks used for cold acclimation studies and freezing tests the in climatic chamber

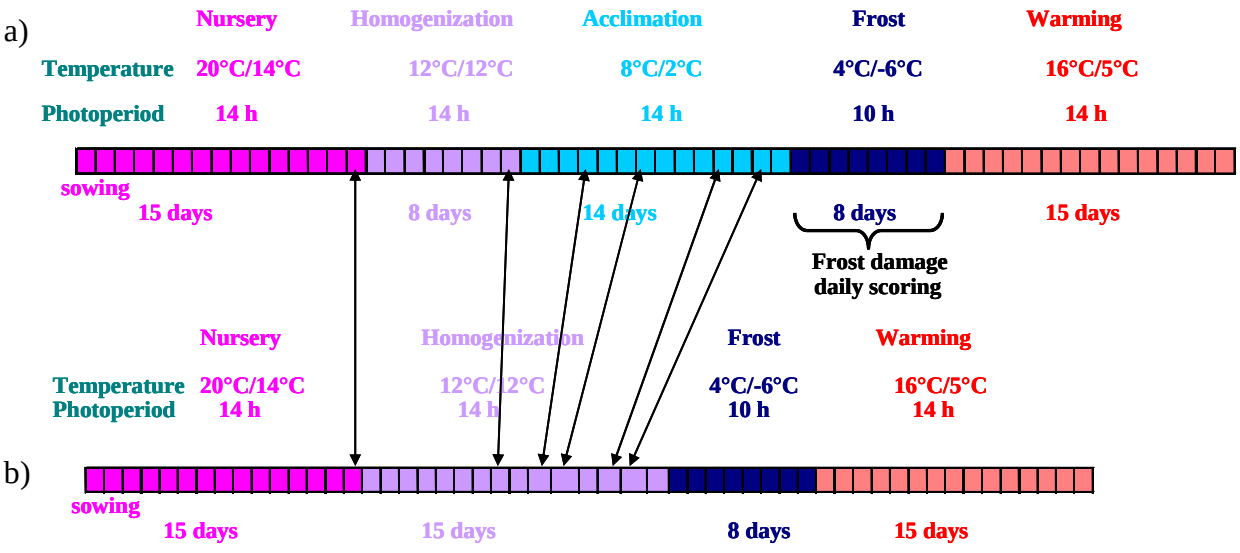

Figure 2: Experimental design with (a) and without (b) acclimation period. Arrows show sampling dates in both cases, corresponding to the same sum of °C - days.

a) b)

Figure 3: Examples of plants state at the end of the frost period: for the frost damage scoring here are two examples of different marks, 0 (a) and 4 (b).

References

Antolín MC, Yoller J and Sánchez-Díaz M (1995) Effects of temporary drought on nitrate-fed and nitrogen-fixing alfalfa plants. *Plant Sci* **107,** 159-165

Armengaud P, Thiery L, Buhot N, Grenier-De March G, Savoure A (2004) Transcriptional regulation of proline biosynthesis in *Medicago truncatula* reveals developmental and environmental specific features. *Physiol Plant*. **120:** 442-450

Arrese-Igor C, Gonzalez EM, Gordon AJ, Minchin FR, Galvez L, Royuela M, Cabrerizo PM and Aparicio-Tejo PM (1999) Sucrose synthase and nodule nitrogen fixation under drought and other environmental stresses. *Symbiosis* **27:** 189-212

Bolaños L, El Hamdaoui A and Bonilla I (2003) Recovery of development and functionality of nodules and plant growth in salt-stressed *Pisum sativum--Rhizobium leguminosarum* symbiosis by boron and calcium. *J Plant Physiol* **160:** 1493-1497

Bonin I, Huguet T, Gherardi M, Prosperi J and Olivieri I (1996) High level of polymorphism and spatial structure in a selfing plant species, *Medicago truncatula* (*Leguminosae*), shown using RAPD markers. *Am. J. Bot*. **83:** 843-855

Bouché, N. and Fromm, H. (2004). GABA in plants: Just a metabolite? *Trends in Plant Science* 9, 110-115

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

Bray EA (1993) Molecular responses to water deficit. *Plant Physiol*. **103:** 1035-1040

Carroll, A. D., Fox, G. G., Laurie, S., Phillips, R., Ratcliffe, R. G. and Stewart, G. R. (1994). Ammonium Assimilation and the Role of [gamma]-Aminobutyric Acid in pH Homeostasis in Carrot Cell Suspensions. *Plant Physiol.* **106**, 513-520.

Crespi M and Gálvez S. (2000). Molecular Mechanisms in Root Nodule Development. *J. Plant Growth Reg*. **19:**155-166.

Del Papa MF, Balague LJ, Sowinski SC, Wegener C, Segundo E, Abarca FM, Toro N, Niehaus K, Pühler P, Aguilar OM, Martinez-Drets G and Lagares A (1999) Isolation and characterization of alfalfa-nodulating rhizobia present in acidic soils of central Argentina and Uruguay. *Appl Environ Microbiol* **65:** 1420-1427

Duzan HM, Zhou X, Souleimanov A and Smith DL (2004) Perception of *Bradyrhizobium japonicum* Nod factor by soybean [*Glycine max* (L.) Merr.] root hairs under abiotic stress conditions. *J Exp Bot* **55:** 2641-2646

Edmeades GO, Cooper M, Lafitte R, Zinselmeier C, Ribaut JM, Habben JE, Löffler C and Bänziger M (2001) Abiotic stresses and staple crops. In: Crop Science: Progress and Prospects. Proceeding of the Third International Crops Science Congress, pp. 17-21. Nosberger J, Geiger HH and Struik PC (eds). Wallingford, UK

Edwards, S., Nguyen, B.-T., Do, B. and Roberts, J. K. M. (1998). Contribution of Malic Enzyme, Pyruvate Kinase, Phosphoenolpyruvate Carboxylase, and the Krebs Cycle to Respiration and Biosynthesis and to Intracellular pH Regulation during Hypoxia in Maize Root Tips Observed by Nuclear Magnetic Resonance Imaging and Gas Chromatography-Mass Spectrometry. *Plant Physiol.* **116**, 1073-1081.

Emmert FH (1974) Inhibition of phosphorus and water passage across intact roots by polyethylene glycol and phenylmercuric acetate. *Plant Physiol* **53**, 663-665

Foolad MR, Zhang LP and Subbiah, P (2003) Genetics of drought tolerance during seed germination in tomato: inheritance and QTP mapping. *Genome* **46**, 536-545

Fougere F, Le Rudulier D and Streeter JG (1991) Effects of salt stress on amino acid, organic acid, and carbohydrate composition of roots, bacteroids, and cytosol of alfalfa (*Medicago sativa* L.). *Plant Physiol*. **96:** 1228-1236

Frechilla S, Royuela M, Arrese-Igor C, Aparicio-Tejo PM (1993) Effect of osmotically-applied water stress or drought on photosynthesis in Pisum sativum *Plant Physiol* **102**, 177 Suppl. S

Frugier F, Kondorosi A and Crespi M (1998) Identification of novel putative regulatory genes induced during alfalfa nodule development with a cold-plaque screening procedure. *MPMI* **11:** 358-366

Frugier F, Poirier S, Satiat-Jeunemaitre B, Kondorosi A and Crespi M (2000) A Krüppel-like zinc finger protein is involved in nitrogen-fixing root nodule organogenesis. Genes & Dev **14:** 475-482

Gálvez L, González EM and Arrese-Igor C (2005) Evidence for carbon flux shortage and strong carbon/nitrogen interactions in pea nodules at early stages of water stress. *J Exp Bot* **56,** 2551-2561

Glevarec, G., Bouton, S., Jaspard, E., Riou, M. T., Cliquet, J. B., Suzuki, A. and Limami, A. M. (2004). Respective roles of the glutamine synthetase/glutamate synthase cycle and glutamate dehydrogenase in ammonium and amino acid metabolism during germination and post-germinative growth in the model legume Medicago truncatula. *Planta* **219**, 286-97.

González EM, Aparicio-Tejo PM, Gordon AJ, Minchin FR, Royuela M and Arrese-Igor C (1998) Water-deficit effects on carbon and nitrogen metabolism of pea nodules. *J Exp Bot* **49,** 1705-1714

González EM, Gordon AJ, James CL and Arrese-Igor C (1995) The role of sucrose synthase in the response of soybean nodules to drought. *J Exp Bot* **46,** 1515-1523

Gout, E., Boisson, A., Aubert, S., Douce, R. and Bligny, R. (2001). Origin of the cytoplasmic pH changes during anaerobic stress in higher plant cells. Carbon-13 and phosphorous-31 nuclear magnetic resonance studies. *Plant Physiol* **125**, 912-25.

Hasegawa PM, Bressan RA, Zhu JK and Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. *Annu Rev Plant Physiol Plant Mol Biol* **51:** 463-499

Heino P, Palva ET (2003) Signal transduction in plant cold acclimation. In: Topics in Current Genetics 4, pp. 151-186. Springer-Verlag, Berlin

Hoffmann-Benning, S. and Kende, H. (1992). On the role of abscisic - acid and gibberellin in the regulation of growth in rice. *Plant Physiol* **99**, 1156-1161.

Hughes MA, Dunn MA (1996) The molecular biology of plant acclimation to low temperature. *J. Exp Bot* **47**: 291-305

Ingram J, Bartels D (1996) The molecular basis of dehydration tolerance in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol* **47:** 377-403

Ismond, K. P., Dolferus, R., De Pauw, M., Dennis, E. S. and Good, A. G. (2003). Enhanced Low Oxygen Survival in Arabidopsis through Increased Metabolic Flux in the Fermentative Pathway. *Plant Physiol.* **132**, 1292-1302.

Jackson, M. (1985). Ethylene and responses of plants to soil waterlogging and submergence. *Annu. Rev. Plant Physiol. Plant Mol. Biol* 36, 145-174.

Jackson, M. and Armstrong, W. (1999). Formation of aerenchyma and the processes of plant ventilation in relation to soil flooding and submergence. *Plant Biology* **1**, 274-287.

Kaufmann MR and Eckard AN (1971) Evaluation of water stress control with polyethilene glycols by analysis of guttation. *Plant Physiol* **47,** 453-456

Lee B, Kin Y, Zhu JK (2002) Molecular genetics of plant responses to low temperatures. In: Plant Cold Hardiness, Gene Regulation and Genetic Engineering. Proceedings of the 6th International Plant Cold Hardiness Seminar, 1-5 July 2001, Helsinki, Finland, p. 294. Kluwer Academic/Plenum Publishers, New York

Levitt J (1980) Responses of plants to environmental stresses. New York: Academic. 2nd ed
Moreau D, Salon C, Munier-Jolain N (2006) Using a standard framework for the phenotypic analysis of *Medicago truncatula*: an effective method for characterising the plant material used for functional genomics approaches. *Plant Cell Env* **29:** 1087-1098

Lopez M, Herrera-Cervera JA and Lluch C (2006) Trehalose metabolism in root nodules of the model legume *Lotus japonicus* in response to salt stress. *Physiol Plant* **128:** 701–709

McKay IA, and Djordjevic MA (1993) Production and Excretion of Nod Metabolites by *Rhizobium leguminosarum bv. trifolii* Are Disrupted by the Same Environmental Factors That Reduce Nodulation in the Field. *Appl Environ Microbiol*. **59:** 3385-3392

Merchan F, de Lorenzo L, González-Rizzo S, Niebel A, Megías M, Frugier F, Sousa C and Crespi M. Identification of regulatory pathways involved in the reacquisition of root growth after salt stress in *Medicago truncatula*. *Plant J. in press*

Merchan F, Breda C, Hormaeche JP, Sousa C, Kondorosi A, Aguilar OM, Megías M and Crespi M (2003) A Krüppel-like transcription factor gene is involved in salt stress responses in *Medicago* spp. *Plant Soil* **257:** 1-9

Melo-Oliveira R, Oliveira IC, Coruzzi GM (1996) *Arabidopsis* mutant analysis and gene regulation define a nonredundant role for glutamate dehydrogenase in nitrogen assimilation. *PNAS* **93**: 4718-4723

Mexal J, Fisher JT, Osteryoung J and Reid CPP (1975) Oxygen availability in polyethylene glycol solutions and its implications in plant water relations *Plant Physiol* **55,** 20-24

Moran JF, Becana M, Iturbeormaetxe I, Frechilla S, Klucas RV and Aparicio Tejo P (1994) Drought induces oxidative stress in pea-plants. *Planta* **194,** 346-352

Mittler R (2006) Abiotic stress, the field environment and stress combination. *Trends Plant Sci* **11,** 15-19

Munns R, Brady CJ and Barlow EWR (1979) Solute accumulation in the apex and leaves of wheat during water-stress. *Aust J Plant Physiol* **6**, 379-389

Muthukumarasamy, M and Panneerselvam R (1997) Amelioration of NaCl stress by triadimefon in peanut seedlings. *Plant Growth Reg* **22:** 157-162

Naya L, Ladrera R, Ramos J, González EM, Arrese-Igor C, Minchin FR, and Becana M. (2007) The response of carbon metabolism and antioxidant defenses of alfalfa nodules to drought stress and to the subsequent recovery of plants. *Plant Physiol* **144**, 1104-1114

Palva ET, Heino P (1998) Molecular mechanism of plant cold acclimation and freezing tolerance. Plant Cold Hardiness, pp. 3-14. Plenum Press, New York

Puhakainen T, Li C, Boije-Malm M, Kangasjärvi J, Heino P, Palva ET (2004) Short-day potentiation of low temperature-induced gene expression of a C-repeat-binding factor-controlled gene during cold acclimation in silver birch. *Plant Physiol* **136**: 4299-4307

Reggiani, R., Nebuloni, M., Mattana, M. and Brambilla, I. (2000). Anaerobic accumulation of amino acids in rice roots: role of the glutamine synthetase/glutamate synthase cycle. *Amino Acids* **18**, 207-217.

Ricoult, C., Cliquet, J.-B. and Limami, A. M. (2005). Stimulation of alanine amino transferase (AlaAT) gene expression and alanine accumulation in embryo axis of the model legume Medicago truncatula contribute to anoxia stress tolerance. *Physiologia Plant* 123, 30-39**.**

Ricoult C, Echeverria LO, Cliquet JB, Limami AM (2006) Characterization of alanine aminotransferase (AlaAT) multigene family and hypoxic response in young seedlings of the model legume Medicago truncatula. *J Exp Bot*. 57, 3079-89**.**

Rubio MC, Gonzalez EM, Minchin FR, Webb KJ, Arrese-Igor C, Ramos J and Becana M (2002) Effects of water stress on antioxidant enzymes of leaves and nodules of transgenic alfalfa overexpressing superoxide dismutases. *Physiol Plant* **115,** 531-540

Saglio, P., Germain, V. and Richard, B. (1999). The response of plants to oxygen deprivation : role of enzyme induction in the improvement of tolerance to anoxia. In *Plant Responses to Environmental Stresses*, (ed. H. Lerner), pp. 373-393. New York: Marcel Dekker.

Sato, T., Harada, T. and Ishizawa, K. (2002). Stimulation of glycolysis in anaerobic elongation of pondweed (Potamogeton distinctus) turions. *J. Exp. Bot.* 53, 1847-1856

Sakai A, Larcher W (1987) Frost survival of plants: responses and adaptation to freezing stress. Springer-Verlag, Berlin

Scholander PF, Hammel HT, Dradstreet ED and Hemmingsen EA (1965) Sap pressure in vascular plants. *Science* **148**, 339-346

Serraj R and Sinclair TR (1996) Inhibition of nitrogenase activity and nodule oxygen permeability by water deficit. *J Exp Bot* **47**, 1067-1073

Singleton PW and Bohlool B (1984) Effect of salinity on nodule formation by soybean. *Plant Physiol* **74:** 72-76

Skopelitis DS, Paranychianakis NV, Paschalidis KA, Pliakonis ED, Delis ID, Yakoumakis DI, Kouvarakis A, Papadakis AK, Stephanou EG, Roubelakis-Angelakis KA. (2006) Abiotic stress generates ROS that signal expression of anionic glutamate dehydrogenases to form glutamate for proline synthesis in tobacco and grapevine. *Plant Cell*.18, :2767-81

Subbaiah, C. C. and Sachs, M. M. (2003). Molecular and cellular adaptations of maize to flooding stress. *Ann Bot)* 91 Spec No, 119-27

Surjus A and Durand M (1996) Lipid changes in soybean root membranes in response to salt treatment. *J. Exp. Bot* **47:** 17-23

Thomashow MF (1990) Molecular genetics of cold acclimation in higher plants. *Adv Genet* **28:** 99-131

Thomashow MF (1998) Role of cold-responsive genes in plant freezing tolerance. *Plant Physiol* **118:** 1-7

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu Rev Plant Physiol Plant Mol Biol* **50:** 571-599

Trinchant JC, Boscari A, Spennato G, Van de Sype G and Le Rudulier D (2004) Proline betaine accumulation and metabolism in alfalfa plants under sodium chloride stress. Exploring its compartmentalization in nodules. *Plant Physiol*. **135:** 1583-1594

Tramontano WA and Jouve D (1997) Trigonelline accumulation in salt stressed legumes and the role if other osmoregulators as cell cycle control agents. *Phytochemistry*. **44:** 1037-1104

Tu JC (1981) Effect of salinity on *Rhizobium*-root hair interaction, nodulation and growth of soybean. *Can J Plant Sci* **61:** 231-239

Van Der Straeten, D., Zhou, Z., Prinsen, E., Van Onckelen, H. A. and Van Montagu, M. C. (2001). A Comparative Molecular-Physiological Study of Submergence Response in Lowland and Deepwater Rice. *Plant Physiol.* 125, 955-968.

Veatch , ME, Smith SE and Vandemark G (2004) Shoot Biomass Production among Accessions of *Medicago truncatula* Exposed to NaCl. *Crop Sci*. **44:** 1008-1013

Verdoy D, Coba De La Pena T, Redondo FJ, Lucas MM, Pueyo JJ (2006) Transgenic *Medicago truncatula* plants that accumulate proline display nitrogen-fixing activity with enhanced tolerance to osmotic stress. *Plant Cell Environ*. **29:** 1913-1923

Vinocur B and Altman A (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Curr Opin Biotech*. **16:**123-132

Voesenek, L. A., Benschop, J. J., Bou, J., Cox, M. C., Groeneveld, H. W., Millenaar, F. F., Vreeburg, R. A. and Peeters, A. J. (2003a). Interactions between plant hormones regulate submergence-induced shoot elongation in the flooding-tolerant dicot Rumex palustris. *Ann Bot* 91 Spec No, 205-11.

Voesenek, L. A., Jackson, M. B., Toebes, A. H., Huibers, W., Vriezen, W. H. and Colmer, T. D. (2003b). De-submergence-induced ethylene production in Rumex palustris: regulation and ecophysiological significance. *Plant J* **33**, 341-52.

Ward, D. E., Kengen, S. W. M., van der Oost, J. and de Vos, W. M. (2000). Purification and Characterization of the Alanine Aminotransferase from the Hyperthermophilic Archaeon Pyrococcus furiosus and Its Role in Alanine Production. *J. Bacteriol.* 182, 2559-2566

Winicov, I (1993) cDNA encoding putative zinc finger motifs from salt-tolerant alfalfa (*Medicago sativa* L.) cells. *Plant Physiol*. **102:** 681-682

Winicov, I and Bastola, DR (1999). Transgenic overexpression of the transcription factor Alfin1 enhances expression of the endogenous MsPRP2 gene in alfalfa and improves salinity tolerance of the plants. *Plant physiol*. **120**: 473-480

Winicov I (2000) Alfin1 transcription factor overexpression enhances plant root growth under normal and saline conditions and improves salt tolerance in alfalfa. *Planta* **210:** 416-422

Zhang JY, Broeckling CD, Sumner LW, Wang ZY. (2007) Heterologous expression of two *Medicago truncatula* putative ERF transcription factor genes, WXP1 and WXP2, in

Arabidopsis led to increased leaf wax accumulation and improved drought tolerance, but differential response in freezing tolerance. *Plant Mol Biol* **64**, 265-278

Zahran HH (1999) *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol Mol Biol Rev* **63:** 968-989