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13 Salt tolerance in the *Rhizobium*-legume symbiosis: An overview

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Abstract

High salinity in soils induces a strong decrease in crop yield and productivity in arable land. Plants can develop tolerance mechanisms to overcome salt and environmental stresses, including changes in gene expression patterns and in metabolic homeostasis. Recent studies have focused on the elucidation of the physiological events leading to stress tolerance, with the aim of introducing crop plants with enhanced salt tolerance and productivity to soils with high salinity.

In conditions of deficient soil nitrogen availability, legumes have the particular capacity to establish a symbiotic association with several species of soil bacteria. A consequence of this symbiotic relationship is the development of root nodules, in which bacteria fix atmospheric nitrogen, which the host plant incorporates in the form of organic compounds. Nitrogen fixation in nodules is affected by salt stress prior to any other of the plant's processes. By augmenting legumes' capacity to resist stress, their ability to fix nitrogen and, thus, enrich the soil for this

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element can be maintained under conditions of stress.

In the present review, updated knowledge of several aspects of salt tolerance in the Rhizobium-legume symbiosis system is presented in the context of the physiology and molecular biology of plant salt stress. Topics of special interest, lines of research and some results emerging from our laboratory are underlined.

Introduction

Nitrogen availability is one of the most limiting factors in agricultural systems. Use of nitrogenated fertilizers involves considerable expense and the accumulation of toxic nitrogen compounds in the environment [1]. Biological nitrogen fixation, performed by nitrogen-fixing organisms, is a natural and efficient process and is responsible for the accumulation of a significant proportion of the soil's nitrogen [1, 2 and references therein]. Symbiotic associations of soil Gram-negative bacteria from the Rhizobiaceae subfamily with legumes play the most important role in biological nitrogen fixation.

Leguminosae contains more than 12,000 species, of which only few have been studied in the context of symbiosis and nodulation capacity. The members of this family display a wide geographical distribution and ecological success [2]. In association with rhizobia, nodulated plants have an enormous potential for the remediation of arid and saline soils and intercropped culture reafforestation, and thus, mitigating or avoiding the use of nitrogenated fertilizers.

Rhizobia have the capacity to infect the roots (and, in some cases, the stems) of diverse leguminous (and some non-leguminous) plants, in conditions of deficient soil nitrogen content. This association induces the formation of a specialized new organ, the root nodule. Nodule tissues provide suitable conditions for bacterial nitrogen fixation. In this way, the plant becomes independent from soil nitrogen availability and fixing bacteria are supplied with nutrients by their legume host.

Symbiosis and nodule functions are very sensitive to salt stress, more so than the host legume or the rhizobia [3-7]. It has been observed that salt stress induces a reduction in the number of rhizobia that colonise the root [8], and in the number and shape of root hairs, and interferes with infection thread formation [9]. Initial phases of nodulation are more sensitive to salt stress than later phases [10, 11]. Salt stress induces a decrease in nodule weight and number [12, 13]. Decreases in the activity of the enzymes involved in ammonium assimilation [7] and in photosynthate influx to the nodule [14, 15] have also been observed in nodules subjected to salt stress.

Genetic diversity of legumes, responsible for varying degrees of salt tolerance, can be studied and exploited by techniques including breeding and intercropping [16, 17]. Similarly, the selection of bacterial strains adapted to saline and arid conditions can be attempted [1, 2]. Genetic manipulation of legumes and/or bacteria can also be performed.

A detailed study of the different aspects of salt stress effects on bacteria, the plant, the symbiotic process, and the nodule, as well as an overview of the strategies available for improving salt tolerance in the *Rhizobium*-legume symbiosis, are presented in the following sections. References cited are representative only and not exhaustive.

Rhizobia and salt stress

In general, symbiotic *Rhizobium* bacteria are more resistant to salt stress than their host legumes. Different rhizobial species and strains show diverse patterns of sensitivity

to salt stress; fast-growing bacteria are seen to be more salt-tolerant than slow-growing bacteria [for reviews and examples, see 2, 7]. Several rhizobial genes involved in salt stress and related to nodulation have been identified [18].

Morphological alterations of bacteria subjected to salt stress have been reported. Such alterations include cell expansion and distortion of the cell envelope [19]. Surface components, some of which are necessary for the establishment of symbiosis, can also suffer changes in a saline environment, e.g. a decrease in exopolysaccharide synthesis [20], changes in side chain length of lipopolysaccharides [21-23] and suppression or alteration of periplasmic oligosaccharides [24], which are involved in bacterial osmotic adaptation as reported by Miller *et al.* [25].

Compatible solute (osmolyte) intracellular accumulation has been observed in some species of rhizobia upon their subjection to salt stress. Higher intracellular concentrations of glycine betaine, trehalose, glutamate and N-acetylglutaminyl-glutamine amide have been reported [26]. It is thought that these substances act as genuine osmoprotectants. Other osmolytes, such as ectoine and sucrose, can improve the growth of some salt-stressed rhizobia species, but no intracellular accumulation of these substances has been observed under conditions of salt stress. Intracellular accumulation of potassium and some polyamines has also been reported [2, 7 and references therein].

Salt-tolerant rhizobia strains have been identified and characterised [27]. Some of these have been isolated from nodules of wild legumes growing in arid zones, often in the presence of high salt concentrations in the soil. These bacterial strains show broad host specificity [1, 2 and references therein] and can be used to inoculate crop legumes to enhance plant growth and nitrogen fixation under conditions of severe stress. Successful examples of this approach have been reported [28].

Genetically modified bacteria have also been used to improve nitrogen fixation in salt-stressed legumes. El-Saidi and Ali [29] obtained an enhanced-salt tolerant *R. leguminosarum* strain by transformation with DNA from a salt tolerant *Bacillus* species. Host legumes nodulated by this transformed strain also showed increased salt tolerance in arid soils. In a field experiment, Van Dillewijn *et al.* [30] found that a genetically modified *Sinorhizobium meliloti* strain overexpressing the *putA* gene, which codes for proline dehydrogenase (an enzyme that catalyses the conversion of proline present in root exudates to glutamate) has a competitive advantage over natural rhizobia populations in regard to infecting and nodulating alfalfa roots submitted to drought stress.

Nodule structure, nodule respiration, nitrogenase activity and the oxygen diffusion barrier under salt stress

Salt stress induces morphological and ultrastructural alterations in the nodule. These alterations have been studied in salt-stressed nodules of *Medicago sativa* [31], *Glycine max* [32], *Phaseolus vulgaris* [33], and *Vigna radiata* [34] among others. Ultrastructural alterations reported include cytoplasm disintegration and loss of cell wall rigidity [35], a decrease in the packaging of inner cortex cells [36], the appearance of lobulated nuclei and variations in chromatin condensation [32], a decrease in the volume of intercellular spaces [33], and an increase in epidermis and cortex membrane surface due to an increase in vesicules numbers [35].

Nitrogenase catalyses the fixation of atmospheric dinitrogen into ammonia, and its enzymatic activity is inhibited by free oxygen. A microaerobic environment is present in the nodule by the combined action of the oxygen diffusion barrier, localised in the nodular inner cortex, and leghemoglobin, which transports and supplies oxygen to bacteroids for respiration activity. A decrease in nitrogen fixation, nitrogenase activity, respiration and an increase in fermentative activity have been observed in nodules subjected to salt and drought stress. It is thought that this effect is principally due to the observed decrease in O_2 permeability in the oxygen diffusion barrier in drought and salt stressed nodules [7, 37], and to a decrease in leghemoglobin levels [3].

The mechanisms by which the diffusion barrier regulates and changes its conductivity of O_2 under different environmental conditions have not been clearly elucidated. It seems that there is a correlation between those changes and cellular elongation and glycoprotein extrusion in the extracellular spaces of the inner cortex [38, 39]. Glycoproteins include the MAC236 and MAC265 antigens [40], a peanut lectin [41], and the nodulin ENOD2 [42]. Alfalfa nodules grown under different oxygen pressures did not show significant changes in ENOD2 RNA and protein levels [43]. Ultrastructural cellular modifications induced by salt stress and with a putative role in permeability alterations have been observed in the inner cortex [36]. Intercellular water present in inner cortex intercellular spaces was also thought to have a role in permeability regulation [44], but recent results seem to confound this hypothesis [45]. It has been observed that a nodule-specific carbonic anhydrase expressed in the inner cortex of the nodule [46] could be involved in the control of oxygen diffusion [47]. It would be of interest to study the expression of this nodulin in salt stressed nodules and to determine oxygen diffusion properties in transformed plants with altered levels of carbonic anhydrase transcripts and proteins. Dalton *et al.* [48] have observed high levels of ascorbate peroxidase in the endodermis of the nodule, and suggest that this enzyme is involved in the scavenging of reactive oxygen species (ROS) generated by O_2 consumption in the endodermis or parenchyma.

Addition of NaCl to a suspension of bacteroids also induces a decrease in respiration [3, 49, 50]. A direct inhibitory effect of different salts on nitrogenase activity has been observed in crude extracts [51]. Del Castillo and Layzell [52, 53] have proposed that oxygen limitation is a consequence, rather than a cause, of respiratory capacity inhibition, and hence of nitrogenase activity and metabolism, in soybean nodules under drought stress. Sucrose availability may also be an important factor involved in the inhibition of nitrogen fixation, as a correlation between nitrogenase activity and a decrease in sucrose synthase activity and transcript levels has been observed in nodules under conditions of drought and salt stress [54]. ABA (abscisic acid) treatment induces a decrease in nitrogen fixation and leghemoglobin content, but not in sucrose synthase activity; therefore, ABA may be involved in an independent regulatory pathway in nodules subjected to drought stress [55].

Ascorbate and ascorbate peroxidase activities also seem to be important in regulating nitrogenase activity and dinitrogen fixation in legumes [56]. Serraj *et al.* [57] have proposed that nodule nitrogen fixation is inhibited by feedback regulation of ureide accumulation in the shoot during drought stress; this effect would not be direct, but mediated through an intermediary compound such as asparagine.

Compatible solute accumulation in legume root nodules

In general, plants under conditions of salt stress accumulate compatible solutes (osmolytes). The accumulated compatible solute composition differs among different plant species, but in general they include betaine and betaine-derived molecules (glycine betaine), poly-alcohols, sugars (mannitol, ononitol, sorbitol) and amino acids (proline). It has been proposed that this accumulation induces an increase in salt tolerance brought about by the osmotic role of these osmolytes [58-61].

Symbiotic nodules under salt stress accumulate proline in both the cytosol and in bacteroids; lactic acid, pinitol, fructose and sucrose concentrations also increase in salt-stressed nodules [7, 37, 62, 63]. Proline accumulation in the symbiosome has also been reported [64]. An osmoregulatory function has been proposed for these compounds. In our laboratory, transcript accumulation of proline-rich proteins has also been observed in stressed nodules [65].

Variously transformed plant species (over)expressing genes involved in osmolyte synthesis and accumulation usually presented an increase in organ compatible solute concentration and a stronger salt and osmotic tolerance compared to non-tolerant, non-transformed plants [61, 66]. Some examples are set out below and a summary of transgenic approaches to salt stress tolerance is displayed in Table 1.

Tobacco transformed with the Δ^1-pyrroline-5-carboxylate-synthetase (*P5CS*) gene from *Vigna aconitifolia*, an enzyme involved in the first two steps of proline biosynthesis [67], accumulated higher proline concentrations, resulting in stronger tolerance to salt and osmotic stresses compared with control plants [68]. *P5CS* displays feedback inhibition by proline. Tobacco plants transformed with a mutated *P5CS* gene lacking proline inhibition, *P5CSF129A*, showed even higher proline levels and salt tolerance [69]. *Arabidopsis* plants transformed with an antisense construct of the proline dehydrogenase (*ProDH*) gene, that catalyses proline degradation, also presented an increase in proline accumulation and concentration, and in salt tolerance [70]. On the other hand, carrot cells transformed with *P5CS* overaccumulate proline, did not show enhanced salt tolerance [71].

Glycine betaine is a compatible solute widely distributed in plants. In *Arthrobacter globiformis*, its biosynthesis is catalysed by the enzyme choline oxidase (*codA*) [72, 73]. In glycine betaine-accumulating plants (spinach, sugar beet), this reaction is performed by two enzymes and the osmolyte is synthesised in the chloroplast. Non-accumulating plants, such as *Arabidopsis thaliana*, *Brassica juncea* and *Oryza sativa*, have been transformed with *codA*, resulting in glycine betaine accumulation and an increase in salt tolerance. However, glycine betaine concentrations in transformed plants were lower than those observed in stressed naturally accumulating plants [74].

Arabidopsis and tobacco plants overexpressing the mannitol-1-phosphate dehydrogenase (*mtlD*) gene from *E. coli*, an enzyme involved in the synthesis of mannitol, accumulated this compatible solute and presented higher tolerance to salt stress [75, 76]. In the case of *Arabidopsis*, transformed seeds could germinate in a medium containing a four-fold salt concentration than control seeds [77]. Tobacco plants transformed with a gene encoding a myo-inositol O-methyltransferase (*Imt1*) from *Mesembryanthemum crystallinum*, an enzyme involved in D-ononitol biosynthesis, accumulated increased levels of D-ononitol and were more tolerant to drought and salt stress [78]. Tobacco plants transformed with the gene *Stpd1* from apple, which

Table 1. Transgenic approaches to salt stress tolerance.

GENE	HOST PLANT	EFFECT	ENHANCED TOLERANCE	REFERENCE
Δ^1-pyrroline-5-carboxylate synthetase *(P5CS)*	*Nicotiana tabacum*	proline accumulation	Salt and Osmotic	[68]
A mutated form of Δ^1-pyrroline-5-carboxylate synthetase *(P5CSF129a)*	*Nicotiana tabacum*	proline accumulation	Salt	[69]
Antisense form of proline dehydrogenase *(Anti- ProDH)*	*Arabidopsis thaliana*	proline accumulation	Salt	[70]
Myo-inositol *O*-methyltransferase *(Imt1)*	*Nicotiana tabacum*	D-ononitol accumulation	Drought and Salt	[78]
	Arabidopsis thaliana	D-ononitol accumulation	Drought and Salt	[141, 142]
Choline oxidase *(codA)*	*Brassica juncea*	glycine betaine accumulation	Salt	[143]
	Oryza sativa	glycine betaine accumulation	Salt	[72]
	Arabidopsis thaliana	glycine betaine accumulation	Salt	[75]
Mannitol-1-phosphate dehydrogenase *(mtlD)*	*Nicotiana tabacum*	mannitol accumulation	Salt	[76]
	Arabidopsis thaliana	mannitol accumulation	Salt	[77]
Sorbitol-6-phosphate dehydrogenase (S6PDH)	*Nicotiana tabacum*	sorbitol accumulation	Salt	[79]
Barley LEA protein (HVA1)	*Oryza sativa*	protein accumulation	Drought and Salt	[111]
	Triticum aestivum	protein accumulation	Drought	[112]
Mn superoxide dismutase (MnSOD)	*Medicago sativa*	enzyme overexpression	Drought	[139]
Fe superoxide dismutase (FeSOD)	*Nicotiana tabacum*	enzyme overexpression	Oxidative	[144]
O-Acetylserine(thiol) lyase (OASTL)	*Nicotiana tabacum*	glutathione accumulation	Oxidative	[138]
Glutathione S-transferase (GST)	*Nicotiana tabacum*	enzyme overexpression	Salt	[136]
Glutathione peroxidase (GPX)	*Nicotiana tabacum*	enzyme overexpression	Salt	[136]
Ascorbate peroxidase (APX)	*Nicotiana tabacum*	enzyme overexpression	Oxidative	[145]
	Arabidopsis thaliana	enzyme overexpression	Heat	[146]
Glutamine synthetase (GS)	*Oryza sativa*	glutamine accumulation	Salt	[147]
Aldose / aldehyde reductase	*Nicotiana tabacum*	enzyme overexpression	Oxidative and drought	[137]
Glyceraldehyde-3-phosphate dehydrogenase (GPD)	*Solanum tuberosum*	enzyme overexpression	Salt	[148]
Vacuolar Na^+/H^+ antiporter (NHX1)	*Arabidopsis thaliana*	antiporter overexpression	Salt	[149]
Salt overly sensitive protein (SOS1)	*Arabidopsis thaliana*	plasma membrane Na^+/H^+ antiporter overexpression	Salt	[150]

overexpressed sorbitol-6-phosphate dehydrogenase (S6PDH) and accumulated sorbitol, displayed increased salt tolerance [79].

In accordance with the strategies outlined above, *M. truncatula* has been transformed in our laboratory with the above mentioned *P5CS*, *codA*, *mtlD* and *Imt1* genes, under the control of the CaMV 35S promoter (Verdoy *et al.*, unpublished). We will determine whether compatible solute accumulation occurs in symbiotic nodules and other organs, and whether salt tolerance is increased in transformed plants as compared with controls in terms of nitrogen-fixing activity.

In order to explain the effect of compatible solutes, it is suggested that these molecules induce an osmotic adjustment, inducing a decrease in osmotic potential to

allow an increase in water absorption and a re-establishment of intracellular salt concentration. However, studies performed on transformed plants accumulating proline and other compatible solutes have demonstrated that osmolyte concentrations were not high enough to induce an osmotic effect [80]. As regards the above, it has been reported that osmolyte concentrations in different subcellular compartments must be taken into account to fully determine the osmotic importance of these compounds, and such concentrations have not yet been determined [81]. At present, other action mechanisms have been proposed for these solutes (principally for amino acids): changes in membrane potential and membrane permeability for the transport of different ions (H^+, OH^-, K^+) in the presence of high concentrations of amino acids, protection of enzymes against denaturation by direct molecular interaction, and a scavenger role for reactive oxygen species produced during stress [59, 60, 81, 82, and references therein].

Some proteins related to salt stress

Aquaporins are water channel proteins localized in several plant membrane compartments, including the plasma and vacuolar membranes. The aquaporin molecule is an integral membrane protein with six transmembrane domains and two extracellular loops. They regulate the water flow across biological membranes depending on the water potential gradient. In some cases, aquaporins can also transport neutral solutes of small molecular size, such as urea and glycerol [83-85]. Numerous aquaporin genes have been identified in plants. They show high homology with the major membrane intrinsic protein (MIP) family, and it is thought that they have a key role in cytosolic osmoregulation, root water uptake, intracellular and extracellular water transport and water homeostasis of plants. *Arabidopsis* plants transformed with an antisense construct of a plant plasma membrane aquaporin showed reduced cellular membrane water permeability and increased root surface [86].

Some aquaporin genes are constitutively expressed, but others show gene expression induction or repression under different environmental conditions, including drought or salt stress, or upon hormone addition, including ABA [87-90]. It has been observed that salt stress induces a strong decrease in water permeability or conductivity in plant roots [91, 92]. Changes in aquaporin protein activity or gene expression could be involved in this physiological event.

Regarding the *Rhizobium*-legume symbiosis, the nodulin 26 (NOD26) gene is an aquaporin expressed in the peribacteroid membrane of soybean root nodules [93, 94]. NOD26 was purified from the symbiosome membrane and characterised. In addition to water, this protein can also transport glycerol and formamide, and probably has an osmoregulatory function in symbiosis [95, 96]. NOD26 was the first member of the NOD26-like MIPs (NIPs) to be characterised. Homologous genes have been identified in other non-legume plant species, but their cellular localization has not been yet determined. Two cDNAs encoding aquaporins have been identified in nodules of *Lotus japonicus* using an RT-PCR approach [97]; *LIMP 1* (Lotus intrinsic membrane protein 1) is expressed in nodules and root tissues, and is similar in sequence to tonoplast aquaporins; *LIMP 2* is similar to NOD26, and it is a nodulin expressed in mature nitrogen fixing nodules. Drevon *et al.* [98] have observed through using immuno-localization the expression of an aquaporin (*Pvaq*) in the inner cortex of *P. vulgaris* nodules. Expression and functional characteristics of nodule aquaporins during drought

and salt-stress remain to be investigated.

Late embryogenesis abundant protein (LEA) transcripts accumulate during desiccation in the last states of seed maturation [99, 100]. Expression of these genes in vegetative tissues and organs during osmotic, cold and water stress, and exogenous ABA application has been observed [101, 102]. LEA proteins belong to the more general group of hydrophilins, which are involved in adaptive responses to hyperosmotic environmental conditions and are strongly conserved among different organisms [103]. Expression of LEA proteins is generally related to adaptation mechanisms to different stresses, such as desiccation tolerance in young plants [104-106], salt stress [107] and cold tolerance [108, 109]. Yeast transformed with a LEA gene from tomato develops enhanced tolerance to salt and cold stress [110]. Transgenic rice constitutively expressing a barley LEA gene is more tolerant to drought and salt stress [111] and transgenic wheat expressing the same gene from barley showed stronger water-stress tolerance [112].

Different functions have been attributed to LEA proteins, such as the binding of water in order to minimizing water loss during stress, and membrane and protein stabilization [109, 113-115].

In our laboratory, a study of LEA expression in nodules under drought and salt stress has been performed. Following northern and western analysis, LEA transcript and protein accumulation in bean nodules submitted to salt stress has been detected [65]. Specific functions of LEA proteins in nodule protection during stress are being studied.

Nitrogen-containing inorganic salts are taken from the soil solution by the plant and incorporated into amino acids in the root (where amino acids derived from symbiotic nitrogen fixation are also present in the case of legumes) and mature leaves. A proportion of these amino acids forms proteins and other nitrogen-containing molecules in both sites of primary assimilation, and remaining amino acids are transported to other organs with nutritional needs via the xylem and the phloem. By functional complementation in yeast, a set of amino acid transporters has been identified in plants. These transporters have been classified into two superfamilies: the APC superfamily (amino acid, polyamine and choline transporters) and the amino acid transporter superfamily (ATF), which includes amino acid permeases, lysine histidine transporters, putative auxin transporters, aromatic and neutral amino acid transporters, and the subclass of proline transporters. Amino acid transporters are transmembrane proteins and, usually, proton-amino acid symporters [116-118].

Amino acid permeases are general amino acid transporters, show low affinity for different amino acids, and the expression of some of them is repressed during water stress. On the other hand, proline transporters (ProT) usually show high affinity and, in several cases, seem to be specifically involved in the redistribution of proline, betaine, GABA and/or other compatible solutes during water or salt stress. ProT genes were identified and characterised for the first time in *Arabidopsis*: *AtProT1* and *AtProT2* [119]. Both genes are expressed in all organs of the plant, but *AtProT1* seems to have an important role in supplying proline to the ovules, and *AtProT2* is induced upon water and salt stress. Schwacke *et al.* [120] have identified three proline transporter genes in tomato, and one of them, *LeProT1*, seems to play an important role during germination and growth of the pollen tube, probably avoiding its dehydration. Homologous genes have been cloned in rice - *OsProT*, which is not salt-induced [121] - and in barley roots -

HvProT, the expression of which is strongly induced in roots during salt stress [122]. Other salt-induced proline transporters have been identified in *Avicennia marina* (*AmT1*, *AmT2* and the partial clone *AmT3*), a betaine-accumulating plant. The corresponding protein products were characterised as betaine/proline transporters, and their transport activity was enhanced upon NaCl and KCl addition [123].

Interestingly, important changes in the concentrations of proline and other free amino acids were observed in the phloem sap of *M. sativa* during water stress. These changes may be related to osmotic adjustments occurring in some plant tissues [124]. Such results strongly suggest an involvement of proline and other high affinity amino acid transporters in amino acid and compatible solute specific transport during water and salt stress in alfalfa. Identification of amino acid and proline transporters in alfalfa will allow, for the first time, the characterisation of these transporters' expression and function in legumes and symbiotic nodules exposed to differing physiological conditions, including water and salt stress. So far, high accumulation of proline has been observed in nodules under conditions of salt stress, as it was mentioned above [62]. We have undertaken a search for putative proline transporter homologous genes in *M. sativa*. We have isolated two partial clones (670 and 418 bp) from alfalfa roots, and both of them show strong homology with previously identified plant proline transporters.

Reactive oxygen species

Generation of toxic reactive oxygen species (ROS) is an unavoidable consequence of aerobic metabolism. ROS include, among others, H_2O_2, superoxide ion, the strongly toxic hydroxyl radical, and nitric oxide. Oxygen species are principally generated in mitochondria and chloroplasts, as a consequence of electronic transport involved in respiration and photosynthesis, respectively. During stress, increases in ROS generation occur due to altered metabolism within these organelles. It has been proposed that ROS induce oxidative damage to several cellular components, such as proteins, nucleic acids and membrane lipids [59, 60]. Whether ROS induce direct damage in cellular structures or inhibit reparation mechanisms still remains unclear [61].

Living organisms have developed several antioxidant systems for scavenging ROS, among which are numbered catalase, superoxide dismutase (SOD), peroxidase and enzymes of the ascorbate-glutathione cycle (ascorbate peroxidase, glutathione-S-transferase, glutathione reductase, glutathione peroxidase, etc.) which determine concentrations of low molecular weight antioxidants, like ascorbic acid, reduced glutathione (GSH) and homoglutathione (hGSH) which is abundant in legumes. The action of genes coding for these antioxidant enzymes is being characterised in nodules, and antioxidant defences in nodules are under study. Puppo *et al.* [125] have isolated and characterised a peroxidase enzyme in soybean nodules. Dalton *et al.* [48] have observed high levels of ascorbate peroxidase in alfalfa nodule endodermis. Nodule glutathione and homoglutathione synthetases have been cloned and studied by Moran *et al.* [126] and Iturbe-Ormaetxe *et al.* [127]. Rubio *et al.* [128] have characterised superoxide dismutase expression and regulation in alfalfa nodules. Iturbe-Ormaetxe *et al.* [129] have studied ascorbate peroxidase, SOD and other ROS-scavenging enzymes, which are present in nodule mitochondria, and also homoglutathione flux among cytosol, mitochondria and bacteroids.

The level of ROS generation is critical for symbiotic nodule metabolism. Nodular nitrogenase is very sensitive to ROS, and leghemoblogin can suffer autooxidation [130, 131]. *Rhizobium* SODs are also critical for the protection of bacteria during the establishment of functional nodules, as SOD-deficient mutants fail to differentiate into bacteroids and to nodulate the host legume efficiently [132]. *Rhizobium* mutants defective in glutathione synthetase present a decreased tolerance to osmotic and other abiotic stresses, and colonise and infect less competently host legume roots [133]. These results suggest that regulation of ROS levels are very important in determining bacterial environmental stress tolerance and host legume infection and nodulation.

Salt stress can induce nodule senescence through ROS generation [134]. In fact, the activities of several ROS-scavenging enzymes, such as catalase, SOD and ascorbate peroxidase often decrease in nodules during salt or drought stress; however, peroxidase activity increases during salt stress in nodules of several species [37, 135, and references therein].

Some studies have suggested a correlation between antioxidant levels and the tolerance of several abiotic stresses. Transformed plants overexpressing antioxidant enzymes often show increased tolerance to salt and other abiotic stresses, such as drought, cold and heavy metal stress. Increased tolerance is also manifested during the induction of compatible solute accumulation, as indicated above, and a detoxification effect has been proposed for this type of compounds. Tobacco plants transformed with glutathione S-transferase and glutathione peroxidase show higher growth rates under salt and abiotic stress conditions than control plants [136]. Oberschall *et al.* [137] have transformed tobacco plants with an alfalfa NADPH-aldose/aldehyde reductase gene. These transgenic plants showed an increased tolerance to oxidative damage and drought stress and decreased lipid peroxidation. In tobacco plants transformed with O-Acetylserine(thiol) lyase from wheat, cysteine biosynthesis is enhanced, increasing GSH synthesis and SOD expression level. These transgenic plants showed a decrease in oxidative damage upon methyl viologen treatment [138]. In a recent report, Rubio *et al.* [139] obtained transgenic alfalfa plants overexpressing different SOD isozymes in leaves and nodules and observed a moderate increase in photosynthetic activity, as compared with control plants, when these plants were subjected to mild water stress.

Flavodoxin is a flavoprotein that can behave as antioxidant and ROS scavenger due to the stability of its semiquinone state [140]. It can function as a ROS scavenger or as regenerator of cellular ROS scavengers. It has been observed that tobacco plants, which expressed and accumulated a flavodoxin from *Anabaena* in the chloroplast, showed enhanced tolerance to oxidative stress induced by different abiotic conditions (Tognetti *et al.*, unpublished). Recent results in our laboratory showed that transformed *E. coli* expressing flavodoxin display enhanced tolerance to oxidative stress (Redondo *et al.*, unpublished). At present, we are transforming both alfalfa plants and *S. meliloti* as a double strategy for obtaining flavodoxin-expressing plants and nodules with enhanced oxidative and abiotic stress tolerance.

Plant signalling, regulation and transcription factors

Signal transduction events involved in plant sensing and responses to salt stress are being progressively elucidated, but many aspects of signalling and regulation components, specificity and cross-talk, remain unknown. Some recent reviews on this

subject [59, 151-155] distinguish three types of salt stress signalling: ionic and osmotic stress signalling (involved in the restoration of homeostasis under conditions of stress), detoxification signalling (involved in damage control and repair following stress damage), and signalling involved in control of cell and plant growth. In general, environmental stress signals (including ABA) are registered by the plant through primary sensors, but only one putative primary sensor has been identified in plants. These receptors induce MAPK- and phospholipid-cascades, generating ROS-mediated signalling and the alteration of intracellular Ca^{2+} concentrations (which can induce several rounds of signalling from an unique input event and has an effect on calcium-dependent protein kinase activities). These signal cascades are transmitted to the nucleus, where they induce phosphorylation of constitutive transcription factors, which subsequently go on to induce the rapid and transient expression of early response genes (inducible transcription factors). These genes, in turn, induce the expression of delayed-response genes, one of whose characteristics is sustained expression and which encode stress tolerance effectors. Effector genes involved in stress tolerance include ion transporters for Na^+ detoxification and homeostasis, enzymes involved in osmolyte biosynthesis and accumulation, water and osmolyte transport systems (probably including several aquaporins and amino acid transporters), LEA protein expression, cyclin-dependent kinase activity (CDK, involved in regulation of cyclin expression and activity, and hence cellular division and proliferation), molecular chaperones and proteinases. Cross-talk is common in the signalling of different abiotic stresses. No salt-induced signalling pathway has been elucidated in detail, except for the salt overly sensitive (SOS) pathway characterized by studying *Arabidopsis* mutants.

Few salt-induced signalling components have been identified in legumes and the symbiotic nodule system. A salt-induced MAP kinase (SIMK) activated upon moderate salt stress has been characterized in *Medicago* suspensions [156]. SIMK activation pathway is being elucidated. An upstream signalling element, SIMK kinase (SIMKK), has also been identified. This element interacts specifically with SIMK and enhances its salt- and pathogen-induced activation [157]. Recently, it has been reported that SIMK seems to be associated with the actin cytoskeleton and is involved in root hair growth [158]. Different protein kinases seem to mediate extreme hyperosmotic stress [156].

The expression of several genes that may be related to abiotic signalling has been studied in non-stressed nodules and other organs. Some of them are induced during nodule development. One example is a lipoxygenase expressed in some organs and in the nodule parenchyma of *Phaseolus vulgaris* [159]; this gene is induced in the hypocotyl upon ABA treatment or water stress. A phophatase 2C gene (*LjNNP2C1*) is induced in *L. japonicus*, specifically during nodule development [160]. Whether salt or drought stress induce changes in expression levels and localization of these genes in the nodule remains to be investigated. Calmodulin genes identified in nodules of *P. vulgaris* [161] and *M. truncatula* [162] could also have a putative role in abiotic stress-related signalling in the nodule and other organs.

Several classes of salt- and abiotic-stress-induced transcription factors have been identified in plants: the basic-region leucine zipper proteins (including ABA-responsive elements or ABREs), Myb and Myc-like proteins, homeodomain-leucine zipper (HD-ZIP) proteins and genes with AP2/DREBP (dehydration-responsive element-binding proteins) domain [106, 154]. A transcription factor identified in *M. sativa*, *Alfin1*, is

involved in gene expression related to the induction of salt tolerance. Transgenic alfalfa overexpressing this gene shows enhanced resistance to salt stress [163]. Genes directly regulated by *Alfin1* and the subsequent cascade of gene activation remain to be elucidated.

An involvement of heterotrimeric G proteins in several developmental and signalling processes has been observed in plants [for reviews, see 164, 165]. A low number of genes, compared to animals, showing homology with α, β and γ subunits of G proteins have been identified in plants. Studies on rice Gα-deficient mutants (affected in gibberellin signal transduction) [166] and Gα and Gβ-deficient plants [167-169] have suggested a role of heterotrimeric G proteins in seed germination, plant morphogenesis and development. Transgenic *Arabidopsis* plants overexpressing a Gα subunit showed inducible ectopic enhanced cell proliferation activity [170]. A putative G-coupled receptor has been identified in *Arabidopsis*, and its characterisation suggests a role in seed dormancy and flowering timing [171]. Different assays have suggested a role for heterotrimeric G proteins in Ca^{2+} channel activity [172], hormone signalling [166, 169, 173] and light signalling [174-177] in plants. Tomato plants expressing the A1 unit of the cholera toxin gene (an heterotrimeric G protein agonist) are more resistant to pathogen infection [178], which suggests an involvement of these G proteins in plant defence response signalling during microbial infection. By using specific inhibitors and agonists, an involvement of heterotrimeric G proteins in Nod factor signalling during *Rhizobium*-legume symbiosis has been proposed [179].

Little is known about the putative involvement of heterotrimeric G proteins in abiotic stress signalling [153, 180]. Some studies have demonstrated the modulation of enzymatic activity of ABA induced-phospholipase D (involved in dehydration signalling) by heterotrimeric G protein agonists and inhibitors [181, 182]. Transformed *Arabidopsis* carrying a null mutation in a Gα gene showed an altered regulation of ion channels in guard cells and no sensitivity to ABA [173]. At present, our laboratory is investigating the involvement of heterotrimeric G proteins in salt stress signalling in alfalfa through the analysis of expression levels of several specific salt stress-induced genes in *M. sativa* in the presence/absence of heterotrimeric G protein agonists and inhibitors.

Genomics and proteomics

Recent genomic and proteomic approaches are being progressively applied in the study of plant salt stress, and will soon be used in the elucidation of specific molecular events involved in salt tolerance in the context of nodulated legumes. These powerful techniques will allow the large-scale identification and characterisation of genes and proteins involved in salt tolerance. Identification and functional characterisation of new genes, signalling and regulation pathways are being performed with the aid of genomic sequencing programs in combination with large-scale expression analyses such as subtractive hybridization, "cold plaque screening", construction of expressed sequence tag (EST) libraries, DNA microarrays and cDNA-amplified fragment length polymorphism (cDNA-AFLP). These techniques are being applied in genomic studies of several model plant species such as *A. thaliana*, rice, maize, cotton, *M. truncatula*, the halophyte *Mesembryanthemum crystallinum*, and the resurrection plant *Craterostigma plantagineum*, among others, but relatively few studies have been carried out on

abiotically stressed plants [183, 184]. Comparison of induced gene expression between glycophytes and halophytes is revealing that signalling and regulation events involved in salt stress tolerance can be different in halophytes.

Several expression studies have been performed in the *Rhizobium*-legume symbiotic system. Gamas *et al.* [185] have identified, using subtractive hybridisation, specific genes induced during nodule organogenesis; Frugier *et al.* [186] have identified, using cold plaque screening, putative regulatory genes in alfalfa nodule development. Szczyglowsky *et al.* [187] have performed an expression analysis of ESTs of late nodulin genes in *L. japonicus*. Györgyey *et al.* [188] and Fedorova *et al.* [162] have analysed ESTs from an *M. truncatula* nodule cDNA library identifying symbiosis-related genes. Poulsen and Podenphant [189] have undertaken a similar study in roots and nodules of *L. japonicus*. Colebatch *et al.* [190] and Fedorova *et al.* [162] identified genes with enhanced expression in nodules by using a cDNA microarray approach. Simões-Araújo *et al.* [191] identified a set of genes differentially expressed during heat-shock treatment of cowpea nodules by using cDNA-AFLP. Several EST libraries from *G. max* leaves and shoots under salt, drought and cold stress can be found in the Public EST Project for Soybean site (http://soybean.ccgb.umn.edu). *M. truncatula* sequences with homology to previously identified salt-induced genes can be consulted in the TIGR *M. truncatula* Gene Index site (http://www.tigr.org/tdb/tgi/mtgi). Genes specifically up- or down-regulated during salt and abiotic stress in the nodule can be identified and functionally characterised by using these technical approaches. This research will be complemented by the more classical studies of targeted or random mutagenesis, functional mutant screening and complementation, and the overexpression or knockout of a particular gene in transformed plants.

Proteomics, including protein microarrays and yeast two-hybrid systems, allow the comparison of proteins maps, with the detection of different isozymes, in stressed and non-stressed plants [192]. Improved methods for the extraction of plasma membrane proteins in plants are also available [193]. The proteome differential analysis of *O. sativa* seedlings submitted to ozone stress has been performed [194]. Altered levels of pathogenesis-related proteins and some ROS-scavenging enzymes, among others, were detected. Similar analyses were performed on rice leaves during water-deficit stress [195]. Methods in proteomics that allow the comparison of protein expression profiles among different plant species are being developed [196]. Mathesius *et al.* [197] have obtained a proteome map of uninoculated (and non-stressed) *M. truncatula* roots, which will be continuously updated and can be used as reference for studies of different treatments of alfalfa roots. Proteins involved in metabolism (malate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, Rubisco subunits, etc.), defence and stress response (glutathione-S-transferase, heat shock proteins, SOD, PR10, glycine-rich RNA-binding proteins, etc.), protein synthesis and processing (chaperonins, cysteine proteinase inhibitor, etc.), hormone regulation (ABA- and auxin-responsive proteins, ripening-related proteins and others), membrane transport (F1 ATPase, vacuolar H^+-ATPase subunits), cytoskeleton (alpha and beta tubulin), transcriptional regulation (Hap3a transcription factor), cell wall synthesis (alfa-fucosidase, ENOD18) and others of unknown function were detected. Regarding symbiosis, a large set of proteins differentially expressed in *Melilotus albus* roots, nodules and in the corresponding free-living rhizobia bacteria and bacteroids were characterised by a proteomic approach and

using peptide mass fingerprinting [198]. Protein profiles obtained from the peribacteroid membrane and peribacteroid space have been characterised in pea symbiosomes [199]. It will be of interest to compare these profiles with those of salt-stressed bacteria, bacteroids, host legume roots, nodules and symbiosomes in order to identify proteins involved in conferring salt tolerance on this symbiotic system. Other techniques must be developed for the suitable detection of regulatory proteins, proteins probably of great importance to the induction of tolerance to salt stress, which are usually only present in low concentrations, and which display little change in transcript levels upon exposure to stressful conditions.

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