RESEARCH PAPER

Medicago truncatula improves salt tolerance when nodulated by an indole-3-acetic acid-overproducing Sinorhizobium meliloti strain

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Abstract

The abiotic stress resistance of wild-type Sinorhizobium meliloti 1021 was compared with that of RD64, a derivative of the 1021 strain harbouring an additional pathway for the synthesis of indole-3-acetic acid (IAA), expressed in both free-living bacteria and bacteroids. It is shown here that the IAA-overproducing RD64 strain accumulated a higher level of trehalose as its endogenous osmolyte and showed an increased tolerance to several stress conditions (55 °C, 4 °C, UV-irradiation, 0.5 M NaCl, and pH 3). Medicago truncatula plants nodulated by RD64 (Mt-RD64) showed re-modulation of phytohormones, with a higher IAA content in nodules and roots and a decreased IAA level in shoots as compared with plants nodulated by the wild-type strain 1021 (Mt-1021). The response of nodulated M. truncatula plants to salt stress, when 0.3 M NaCl was applied, was analysed. For Mt-RD64 plants higher internal proline contents, almost unchanged hydrogen peroxide levels, and enhanced activity of antioxidant enzymes (superoxide dismutase, total peroxidase, glutathione reductase, and ascorbate peroxidase) were found compared with Mt-1021 plants. These results were positively correlated with reduced symptoms of senescence, lower expression of ethylene signalling genes, lower reduction of shoot dry weight, and better nitrogen-fixing capacity observed for these plants. Upon re-watering, after 0.3 M NaCl treatment, Mt-1021 plants almost die whereas Mt-RD64 plants showed visual signs of recovery. Finally, the shoot dry weight of Mt-RD64 plants treated with 0.15 M NaCl was not statistically different from that of Mt-1021 plants grown under non-stressed conditions.

Key words: Antioxidant enzymes, hydrogen peroxide, legumes, proline, salinity.

Introduction

Among the abiotic stresses, salinity, even caused by irrigation, is the major limiting factor for plant growth, and will soon become even more severe: it is expected that >50% of all arable land will have salinity problems by the year 2050 (Vinocur and Altman, 2005). Legumes are very important plants both ecologically and agriculturally because they are able to interact symbiotically with soil microorganisms, rhizobia, to form root nodules where biological nitrogen fixation takes place (Spaink, 2000; Perret et al., 2000) and are thus used as pioneer plants growing in marginal land and under unfavourable environments (Zaharan, 2001). Hence, legumes are interesting candidates for improving soil fertility and incorporating salty soil into agriculture.

Unlike their host legumes, rhizobia can survive in the presence of high levels of salt, showing a marked variation in salt tolerance: some strains are inhibited by 100 mM NaCl, whereas strains of Rhizobium meliloti and R. fredii grow at salt concentrations >300 mM. The response and adaptation of rhizobia to salt stress are complex phenomena involving many physiological and biochemical processes that reflect changes in gene expression and in the activity of enzymes and transport proteins (Ferreras et al., 2006). Many species of bacteria adapt to saline conditions by the
intracellular accumulation of low molecular weight organic solutes called osmolytes. The accumulation of osmolytes, such as trehalose, is thought to counteract the dehydration effect of low water activity in the medium but does not interfere with macromolecular structure or function (Zahran, 1999).

The salinity response of legumes, like that of most cultivated crops, varies greatly and depends on climatic conditions, soil properties, and growth stage (Zahran, 1999). Legumes with a high tolerance to salt stress usually exhibit osmotic adjustment (Zhu et al., 1992) that is partly accounted for by changing cell turgor and by accumulation of metabolites, which are described as compatible solutes because they do not interfere with plant metabolism (Yancey et al., 1982). Proline accumulation is one of the most frequently reported modifications induced by salt stress in plants, and it is often considered to be involved in stress resistance mechanisms (Lutts et al., 1999). Indeed, transgenic plants (Verdoy et al., 2006), or insertion mutants (Yu et al., 2008) that accumulate high levels of proline, display enhanced osmotosolerance.

It is well documented that abiotic stresses, including salt stress, exert at least part of their effects by causing oxidative damage. Oxidative damage is caused by reactive oxygen species (ROS) that can react with a large variety of biomolecules causing irreversible damage and leading to cell necrosis and death (Kim et al., 2005; Pitzschke et al., 2006; Rivero et al., 2007). In plants, ROS are also continuously produced as by-products of various metabolic pathways that are localized in different cellular compartments. Hence, because ROS are toxic but also participate in signalling events, plant cells are equipped with at least two different mechanisms to regulate their intracellular ROS concentrations by scavenging ROS: one enabling the fine modulation of low levels of ROS for signalling purposes [peroxidase (POX), superoxide dismutase (SOD), and catalase (CAT)], and one regenerating the oxidized antioxidants [ascorbate peroxidase (APX) and glutathione reductase (GR)] (Mittler, 2002). The Rhizobium–legume symbiosis is more sensitive to salinity than the free-living bacteria. Unsuccessful symbiosis under salt stress may be due to the following factors: (i) failure in the establishment of rhizobia in the rhizosphere; (ii) failure of the infection process due to the effect of salinity; and (iii) the depressive effect on nitrogen fixation due to the inhibition of nitrogenase activity and nodule respiration (Zahran, 1999).

The protective response of plants to both biotic and abiotic stresses is primarily regulated by phytohormones such as indole-3-acetic acid (IAA), salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA), and ethylene. These endogenous low molecular weight molecules lead, via synergistic and antagonistic actions, to the expression of different but overlapping suites of genes, which is referred to as signalling cross-talk (Raghavan et al., 2006; Kraft et al., 2007).

Furthermore, in different bacteria interacting with plants, the levels of IAA, used to enhance the colonization surface and the exudation of nutrients for bacterial growth by stimulating proliferation of plant tissues (Malhotra and Srivastava, 2006; Spaepen et al., 2007), are finely modulated in response to environmental stresses (such as acid pH, osmotic stress, and carbon limitation) (Spaepen et al., 2007) associated with the soil and plant environment.

A positive effect of IAA overproduction in a recombinant strain of Azospirillum on the growth of sorghum host plant has been also demonstrated (Malhotra and Srivavata, 2006).

It was recently shown that IAA triggers the activation of the central energy metabolism of Escherichia coli cells (Bianco et al., 2006b) and is also able to enhance protection of these cells against various abiotic stresses such as UV, high salt, and low pH (Bianco et al., 2006a).

In order to study the effect of abiotic stress on the Rhizobium–legume symbiosis, the response on both partners, separately and during symbiosis, was investigated. The wild-type strain Sinorhizobium meliloti 1021 and its IAA-overproducing derivative, RD64 (Imperlini et al., 2009), were used to inoculate Medicago truncatula plants. Due to the different sensitivity against various abiotic stresses observed for the two strains, it was of considerable interest to: (i) study the effect of salt stress on the growth of nodulated Medicago plants and thus on their ability to fix nitrogen; (ii) evaluate the contribution of organic solutes to osmotic adjustment under controlled saline conditions in plants; (iii) determine the activity of antioxidant enzymes involved in scavenging active ROS generated during salt stress; and (iv) analyse the interactions and the alterations of signalling molecules (auxin and ethylene) in plant salt stress responses. The investigation provided evidence that the overexpression of IAA in S. meliloti 1021 played a positive role in the adaptation to osmotic stress both in free-living bacteria and in nodulated plants.

Materials and methods

Bacterial strains, growth conditions, and stress tests

The S. meliloti wild-type strain used in this study was the sequenced strain 1021 (strR) (Galibert et al., 2001). The S. meliloti IAA-overproducing strain (RD64) was generated by introducing the p-iaaMms2 construct into S. meliloti 1021, as previously described (Defez and Spena, 1998; Pii et al., 2007; Camerini et al., 2008; Imperlini et al., 2009). Streptomycin (200 µg ml⁻¹) and spectinomycin (200 µg ml⁻¹) were included as required. Solid media contained 15 g l⁻¹ agar (Difco) in TYR (0.3% yeast extract, 0.5% tryptone, and 6 mM CaCl₂) or minimal medium. The bacteria were grown aerobically at 30 °C, on a shaker at 200 rpm, in minimal mannitol ammonium chloride (MMN) medium (Hooykaas et al., 1977).

For IAA treatment, exponentially growing S. meliloti 1021 cultures (OD₆₀₀=0.6) were split into two aliquots; to one aliquot, an IAA solution was added to a final concentration of 0.5 mM, and the second aliquot was left untreated (control). After 1.5 h (OD₆₀₀=0.9 for both cultures),
different cell batches, taken from IAA-treated, RD64, and control cells, were aliquoted, frozen in liquid nitrogen for 5 min, and stored at −80 °C for use in experiments.

Stress tests were performed as previously described (Bianco et al., 2006a) except that different treatment times were used for osmotic shock (t=3 h), heat shock (t=10 min), and acid pH assay (t=35 min). At least five independent experiments were performed for all the results presented in this work.

Trehalose determination

Unincorporated growth substrates and saccharides were removed by washing the cells twice in a solution containing 68 mM NaCl, 3 mM KCl, 1.5 mM KH2PO4, and 9 mM NaH2PO4. The cells were then extracted with hot (65 °C) 80% (v/v) ethanol/water. After heating at 65 °C for 15 min, the mixtures were chilled and centrifuged (8000 g for 15 min). The supernatant, containing the cytosolic osmolyte, was evaporated to dryness at 30 °C and dissolved in H2O. The trehalose content of four independent replicates was determined as previously described (Bianco et al., 2006a).

Plant material and growth conditions

Seeds of *M. truncatula* cv Jemalong lines 2HA (Istituto Sperimentale per le Colture Foraggere, Lodi, Italy) and A17 (INRA, Centre de Montpellier, France) were surface sterilized as previously reported (Bucciarelli et al., 2006) and subjected to cold imbibition (48 h at 4 °C, in the dark) in Petri dishes with two filter papers moistened with 10 ml of distilled water. The Petri dishes were then kept for 48 h in the dark at 20 °C for seed germination. Germinated seeds were transferred in hydroponic units (plastic baskets in the dark at 20 °C) and incubated in distilled water. The Petri dishes were then kept for 48 h in Petri dishes with two filter papers moistened with 10 ml sterilized as previously reported (INRA, Centre de Montpellier, France) and A17 (Sperimentale per le Colture Foraggere, Lodi, Italy) and A17 (Sinorhizobium meliloti) strains used for inoculation were identified by their antibiotic resistance patterns. The results of nine independent replicates are given in μmol IAA g fresh weight (FW)−1.

Shoot water content

The water content of individual shoots was determined according to the formula used by Sibole et al. (2003) considering the fresh and dry weights of the shoot instead of an individual leaf.

Soluble proteins from shoot tissue

Frozen material was homogenized with 20 mM TRIS-HCl buffer (pH 8.0) supplemented with 1 mM MgCl2, 1 mM EDTA, 1 μl ml−1 β-mercaptoethanol, and 1% (w/v) polyvinylpolypyrrolidone. The homogenate was then centrifuged for 20 min at 13 000 g and at 4 °C. The protein concentration in the supernatant was measured by the Bradford protein assay using BSA as a standard.
Proline determination

Proline was measured as described by Bates et al. (1973) with some modifications. After homogenization, the residue was removed by centrifugation (15 min at 8000 g and 4 °C). A 0.5 ml aliquot of extract was incubated with 2 ml of glacial acetic acid and 2 ml of acid ninhydrin. The red coloration which developed was extracted with 2 ml of toluene.

H2O2 measurement

The direct release of H2O2 from leaf discs was determined as described by Zimmermann et al. (2006) except that a 1 h incubation at room temperature was used. The results are given in μmol H2O2 g FW−1.

Antioxidant enzymes assays

All enzymes (APX, non-specific POX, CAT, GR, and SOD) were extracted at 0–4 °C as described by Rubio et al. (2002) and activities were measured spectrophotometrically at 25 °C within the linear region for both time and enzyme concentration. APX (EC 1.11.1.11) activity was determined following the oxidation of ascorbate to dehydroascorbate (Asada, 1984). Non-specific POX (EC1.11.1.7) activity was determined by measuring peroxidation of hydrogen peroxide with guaiacol as an electron donor (Lee et al., 2001). CAT (EC 1.11.1.6) activity was assayed by measuring the conversion rate of hydrogen peroxide to water and oxygen molecules (Lee et al., 2001). GR (EC 1.6.4.2) activity was determined as described by Tegera et al. (2004) with some modifications. The assay mixture (1 ml final volume) contained 100 mM potassium phosphate buffer (pH 7.8), 0.5 mM EDTA, 0.03 mM NADPH, 0.5 mM oxidized glutathione (GSSG), and an appropriate volume of enzyme extract. Total SOD (EC 1.15.1.1) activity was assayed by determining the inhibition rate of cytochrome c as described by Rubio et al. (2002).

Quantitative real-time PCR analysis

Total RNA was isolated from shoots using an RNeasy plant mini kit (Qiagen) following the manufacturer’s instructions except that, after the addition of the RLT buffer (RNeasy mini handbook, Qiagen) to the tissue powder, the tissue was homogenized. A 4 min at a frequency of 30 s−1. Residual DNA present in the RNA preparations was removed by incubation at 37 °C with 8 U of DNase I (Epicentre Technologies) and 20 U of RNase Block ribonuclease inhibitor (Stratagene) for 30 min. The isolated RNA was further extracted with phenol/chloroform/soyamyl alcohol and chloroform/soyamyl alcohol, precipitated with 2.5 vols of ethanol and then dissolved in diethylpyrocarbonate-treated water. After purification and quality checking by agarose gel electrophoresis, the RNA concentration was determined by absorbance at 260 nm and the RNA was stored at −20 °C until further use. First-strand cDNA was synthesized from 2 μg of total RNA with the RETROscript kit (Applied Biosystems) and a random deca-mer, according to the manufacturer’s instructions. Quantita-
tive PCR was performed with the Power SYBR PCR Master Mix (Applied Biosystems), with 500 nM of each primer and 0.25 μl of reverse transcription reaction product in a final volume of 20 μl. One ‘no RT’ control (without reverse transcriptase) for each RNA sample and one ‘no RNA’ control (replacing RNA with dH2O) for each primer and probe set were also performed. Reactions were run and analysed on the iCycler iQ (Bio-Rad). The thermocycling condition were: 10 min at 95 °C, 40 cycles of denaturation at 95 °C for 15 s, followed by annealing at 60 °C for 1 min and extension for 1 min at 72 °C. A melting curve was run after the PCR cycles. Specific primer pairs for the genes MtP5CS1, MtP5CS2, and Mtc27 were those reported by Verdooy et al. (2006). Specific primer pairs for nifH, GH3, ETR1, CTR1, Actin1, and Actin2, and Actin genes, designed using the Primer3 software, were as follows: nifH (SMa0825), 5′-TCCACGACCTC-CAATAC-3′ and 5′-CGCAGTCCTGCACTGGAA-3′; GH3 (TC106906), 5′-ACCTAATGGTGCCACTGACC-3′ and 5′-AACGACGCGCTCCATTTCC-3′; ETR1 (TC95393), 5′-AAAAAGAAGCAGCATTTCA-3′ and 5′-TGGGATCCTGGAGTAGA-3′; CTR1 (TC102141), 5′-GTGGAGAAGAACTTGTTGAT-3′ and 5′-GGAAAGCCCAAAATCACA-3′; Actin1 (TC102970), 5′-GGTTCTTTGGGGATTTGTT-3′ and 5′-TGTTTGTGGCCAATTTG-3′; and Actin (TC106785), 5′-ACGAGGCGTTTACATGAGTA-3′ and 5′-ACCTCCGATCCAGCAGA-3′. Primers for Mtc27 (constitutively expressed gene) and Actin were included in all the quantitative RT-PCR analyses for the purpose of data normalization. RT-PCR amplification for each cDNA sample was performed in triplicate wells. During the reactions, the fluorescence signal due to SYBR Green intercalation was monitored to quantify the double-stranded DNA product formed in each PCR cycle. Results were recorded as relative gene expression changes after normalizing for each cDNA sample for the comparative CT method (2−DDCT) as previously described (Bianco et al., 2006b).

The 2−DDCT value was >1 for genes more highly expressed in salt-stressed plants and <1 for genes more highly expressed in non-stressed plants.

Statistical analysis

The results presented in the text, tables, and figures are the means ± SD of at least five measurements each performed on independent samples collected at independent times. Data were subjected to statistical evaluation using one-way analysis of variance (ANOVA). Tukey’s multiple comparison test was performed to compare the means of single variables.

Results

Effect of various abiotic stresses on S. meliloti viability

It has previously been shown that IAA addition to E. coli cells (Bianco et al., 2006a) triggered the induction of genes related to bacterial stress resistance. To investigate whether
IAA could trigger a similar effect in soil bacteria and whether such an effect might somehow influence the symbiotic plant response to stress, this analysis was extended to *Sinorhizobium meliloti*. The results listed in Table 1 indicate that, when various abiotic stresses were applied, the number of viable bacterial cells increased both in IAA-treated and IAA-overproducing strains as compared with the non-stressed strains. However, a higher number of viable cells was recorded for the RD64 strain. This effect was much more evident in the case of osmotic shock where surviving colonies counted for RD64 cells were 40% higher than those counted for 1021 cells. As shown in Fig. 1, the number of colony-forming units (cfu) was measured in a time course experiment at 4 °C. It was found that, even after 60 d of cold treatment, ~50% of IAA-treated 1021 cells and RD64 cells are viable, whereas half of this percentage was found for untreated 1021 cells.

**Determination of intracellular trehalose**

To investigate one of the protection systems activated in *S. meliloti* cells under stress conditions, the intracellular content of trehalose, a sugar that acts as a stress protector under different environmental insults, was analysed. It was found that IAA induced trehalose accumulation both in the IAA-treated 1021 strain (388±55 nmol mg protein⁻¹, n=5, *P* <0.0005) and in the RD64 strain (971±166 nmol mg protein⁻¹; n=5, *P* <0.0005) compared with the control strain (277±27 nmol mg protein⁻¹, n=5, *P* <0.0005).

**IAA levels in Medicago plants**

ELISA experiments were performed to estimate the IAA levels in different organs of *Medicago* plants. The IAA concentration in the shoot of *Mt*-RD64 plants was lower than that measured for *Mt*-1021 plants (Table 2). In contrast, the IAA levels measured both in the roots containing nodules and in nodules alone of *Mt*-RD64 plants were higher than those of *Mt*-1021 plants (Table 2).

**Effect of IAA overexpression and salt treatment on Medicago growth**

The analysis of the effects of bacterial IAA overproduction on nodulation and plant growth was performed, under non-stressed conditions, for both *Mt*-A17 and *Mt*-2HA plants. For *Mt*-A17, 4 weeks after infection, no statistically significant differences were found in the growth of aerial parts; however, the number of nodules per plant and growth of the lateral roots were higher in plants infected by the IAA-overproducing strain when compared with the control (data not shown), as previously found by Pii et al. (2007). For *Mt*-2HA, in contrast, the biomass production of the aerial parts (measured 4 weeks after infection as shoot fresh and dry weight) increased in *Mt*-RD64 plants as compared with *Mt*-1021 plants (Table 2); this enhanced dry matter production was positively correlated to a higher development of the whole root apparatus (for *Mt*-1021, root fresh weight=174 mg, *n*=30, *P*<0.001; for *Mt*-RD64, root fresh weight=243 mg, *n*=30, *P*<0.001).

The growth of *Mt*-2HA plants, inoculated with either the IAA-overproducing or control *S. meliloti* strain, was also

*Fig. 1.* Cell viability at 4 °C. Surviving colonies were determined at the indicated times as described in Materials and methods. Data are means ±SD of independent biological experiments (*n*=5, *P* <0.01). At least three technical replicates were performed for each biological replicate.

| Table 1. Cell resistance of *Sinorhizobium meliloti* cells to various abiotic stresses |
|-----------------|-----------------|-----------------|
| **Treatment**   | **Survival (%)** |
| Acid shock (pH 3.0) | (1.05±0.02)×10⁻³ | (2.3±0.6)×10⁻³ | (2.0±0.1)×10⁻³ |
| Osmotic shock (>0.5 M NaCl) | 49±2 | 78±1 | 90±4 |
| UV-irradiation (100 J m⁻²) | 67±6 | 86±1 | 92±8 |
| Heat shock (55 °C) | 0.165±0.005 | 0.196±0.021 | 0.297±0.025 |

| Table 2. Estimation of IAA levels in 2HA *M. truncatula* plants |
|-----------------|----------------|----------------|-----------------|
| **Sample**      | **IAA content (µmol g FW⁻¹)** | **Ratio** | **P-value** |
|                 | *Mt*-1021      | *Mt*-RD64     |
| Shoot           | 4.0±0.4        | 2.7±0.3        | 0.67            | <0.003        |
| Root-1 (total)  | 1.9±0.1        | 3.3±0.3        | 1.7             | <0.0002       |
| Root-2 (without nodules) | 5.2±0.7       | 3.9±0.3        | 0.75            | <0.005        |
| Nodules         | 2.5±0.1        | 4.4±0.5        | 1.8             | <0.0005       |

* Calculation of the ratio was based on the control plants.
evaluated under salt stress conditions. The genotype 2HA was chosen because of its higher regeneration ability as compared with the genotype Jemalong A17.

After 3 d of salt stress (0.3 M NaCl), visual signs of ion toxicity were present in the leaves of Medicago plants (Fig. 2). Symptoms of senescence and zones of necrosis on the leaves of Mt-RD64 plants were less evident as compared with the control. In addition, different values of water and soluble protein content were found in the shoot of control and experimental plants (Table 3). In particular, the total protein content of Mt-1021 plants decreased slightly whereas that of plants infected by RD64 significantly increased (up to 46%). When a long-term (10 d) NaCl treatment was carried out, it was found that the growth of both Mt-1021 and Mt-RD64 plants was not significantly affected by salinity at 100 mM NaCl (data not shown). In contrast, when 150 mM NaCl was applied to the plants it caused a growth reduction for both Mt-1021 and Mt-RD64 plants (Table 3). Nevertheless, the reduction of shoot dry weight was less severe for Mt-RD64 plants; indeed, the absolute value of shoot dry weight for these plants subjected to salt stress was not statistically different from that of Mt-1021 plants grown under non-stressed conditions (Table 3). Upon re-watering, Mt-1021 plants did not recover from the stress and almost died, whereas Mt-RD64 plants showed recovery signs such as an increase in leaf turgor (data not shown).

**Internal proline content**

The analysis of the leaf proline accumulation after the salt treatment showed that for both control and experimental plants a positive response was achieved (Table 3). However, when the Mt-RD64 plants were subjected to salt stress, the proline content showed a 2-fold increase over that of control.

**Hydrogen peroxide content**

The changes in H$_2$O$_2$ content in the leaves of Medicago plants subjected to short-term salt stress and recovery are shown in Fig. 3. Different response patterns can be observed. The H$_2$O$_2$ content in the leaves of Mt-1021 plants increased 1.4-fold after salt stress ($t=0$ recovery) and remained relatively constant during the recovery time (2.5 d) as compared with the unstressed plants. On the other hand, the H$_2$O$_2$ content in the leaves of Mt-RD64 plants, at the same times, was not statistically significantly different from that found in the shoot of unstressed plants.

**Antioxidant activity**

The activity of oxygen-scavenging enzymes in the shoot of stressed Medicago plants was measured (Figs 4, 5). After short-term salt stress, the APX activity decreased in Mt-1021 plants whereas it was induced in Mt-RD64 plants.
Table 3. Effect of salt-stress on 2HA M. truncatula growth
NaCl treatment was as described in Fig. 2. The values reported in the table are the averages ±SD

<table>
<thead>
<tr>
<th></th>
<th>Water content (g g DW⁻¹)</th>
<th>Protein content (mg g FW⁻¹)</th>
<th>Proline content (μmol g FW⁻¹)</th>
<th>Shoot DW (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mt-1021</td>
<td>3.2±0.3</td>
<td>6.2±0.2</td>
<td>0.90±0.13</td>
<td>44±5</td>
</tr>
<tr>
<td>Mt-1021—salt stress</td>
<td>0.8±0.1</td>
<td>5.1±0.5</td>
<td>1.4±0.2</td>
<td>27±3</td>
</tr>
<tr>
<td>Mt-RD64</td>
<td>3.6±0.3</td>
<td>5.0±0.4</td>
<td>0.91±0.08</td>
<td>62±6</td>
</tr>
<tr>
<td>Mt-RD64—salt stress</td>
<td>1.2±0.1</td>
<td>7.0±0.5</td>
<td>2.1±0.2</td>
<td>48±5</td>
</tr>
</tbody>
</table>

* Short-term salt stress (0.3 M NaCl); n=20, P <0.0001.
† Short-term salt stress (0.3 M NaCl); n=8, P <0.0001.
‡ Long-term salt stress (0.15 M NaCl); n=15, P <0.0001.
§ Long-term salt stress (0.15 M NaCl); n=15, P <0.0001.

when compared with the unstressed plants. After the recovery period, the activity of this enzyme was more severely repressed in Mt-1021 plants and only slightly decreased in Mt-RD64 plants (Fig. 4A).

The GR (Fig. 4B) and POX (Fig. 5A) activities of Mt-1021 plants were not affected or slightly increased, after short-term salt stress, as compared with the controls. A similar trend was observed after the recovery period. In contrast, the activity of these enzymes in the shoot of Mt-RD64 plants was positively affected: after a short-term salt stress, the GR and POX activities increased by 32% and 130%, respectively, as compared with the control. These increments remained relatively constant thereafter, during recovery.

For the SOD enzyme, Mt-1021 plants showed almost the same activity as control plants after both short-term salt stress and recovery (Fig. 5B). In contrast, salt stress induced an increase (41% higher) of total SOD activity in Mt-RD64 plants (Fig. 5B). Upon re-watering, the SOD activity of these salt-stressed plants declined below control values (34% lower) (Fig. 5B).

CAT activity behaved completely differently from SOD during both stress and recovery treatments: its activity was reduced in both Mt-1021 and Mt-RD64 plants during salt stress as compared with the control; however, the decrease observed for the latter plants was lower (Fig. 5C). After the recovery period, the activity of this enzyme was almost completely inhibited (96% less than the control) in Mt-1021 plants whereas in Mt-RD64 plants its activity remained relatively constant (Fig. 5C).

Expression analyses by real-time PCR

nifH gene: To verify the effect of severe salt stress on the nitrogen-fixing capacity (nitrogenase activity), a quantitative RT-PCR analysis of the nifH gene, encoding the Fe-protein of the nitrogenase enzyme, was performed. The mRNA level of this gene was reduced in both Mt-1021 and Mt-RD64 plants, although the decrease was less severe in Mt-RD64 plants (0.32±0.06, n=4) as compared with the control plants (0.19±0.01, n=4).

GH3 gene: Transcript levels of the auxin response gene GH3 were evaluated by using RT-PCR studies. The results of this analysis showed good agreement with those obtained...
in ELISA experiments: the expression level of the GH3 gene was strongly induced in the roots (20.0±0.3, n=6) and down-regulated in the shoot (0.39±0.03, n=6) of Mt-RD64 plants as compared with Mt-1021 plants.

Ethylene signalling genes: Experiments were conducted to examine whether IAA induced alterations in the main genes involved in ethylene signalling, whose modulation may affect salt stress responses in plants. After salt stress, the expression level of the ETR1 receptor was induced in both Mt-1021 (2.34±0.07, n=4) and Mt-RD64 (1.84±0.46, n=4) plants. In contrast, the CTR1 gene was strongly inhibited in Mt-1021 plants (0.50±0.08, n=4) and slightly induced in Mt-RD64 plants (1.43±0.01, n=4) when compared with the controls. For the positive regulator EIN2 higher up-regulation was observed in Mt-1021 plants (2.50±0.15, n=4) than in Mt-RD64 plants (0.90±0.01, n=4).

Proline biosynthesis-related genes: Quantitative real-time PCR was used to determine transcript levels of the M. truncatula genes MtP5CS1 (Δ1-pyrroline-5-carboxylate synthase 1) and MtP5CS2 (Δ1-pyrroline-5-carboxylate synthase 2) involved in proline biosynthesis from glutamic acid. In response to 0.3 M NaCl treatment, the expression level of MtP5CS1 was slightly reduced in Mt-1021 plants (0.63±0.06, n=6) whereas it was induced in Mt-RD64 plants (2.2±0.1, n=6). In contrast, salt stress induced an increase of the MtP5CS2 transcript level in the leaves of both Mt-1021 and Mt-RD64 plants, although a higher level of transcript induction was detected in RD64-nodulated plants (3.9±0.2, n=6) when compared with the control plants (2.1±0.2, n=6).

Discussion

In this work, an engineered S. meliloti 1021 strain (RD64), able to release up to 78-fold more IAA in liquid growth medium when compared with the wild-type strain (Imperlini et al., 2009), was tested for its ability to overcome adverse environmental conditions. The effects of this strain on the growth of the model legume M. truncatula under salt stress, a major constraint limiting legume production in semi-arid regions and in irrigated fields, was also studied. The genotype 2HA was chosen because of its higher regeneration ability compared with the genotype Jemalong A17. The results show that the RD64 strain is less sensitive to various selected stresses, such as salinity and acidity. This enhanced resistance might be connected to the accumulation of the higher levels of trehalose observed in the analyses (Zahran, 1999).

Several studies have shown that, in the Rhizobium–legume symbiosis under conditions of high soil salinity, the legume salt tolerance is the main factor in determining the success of the N2 fixation process. Therefore, a competitive and persistent rhizobial strain is not expected to express its full capacity for nitrogen fixation if factors impose limitations on the vigour of the host legume (Zahran et al., 1999).

In the present study, a higher salt tolerance was found for both the RD64 strain and Mt-RD64 plants. Indeed, at the vegetative developmental stage, the leaves of Mt-RD64 plants showed a reduction in chlorosis, necrosis, and drying compared with the control plants. The finding of higher levels of water, protein content, and shoot dry weight confirmed the reduction of the visual senescence symptoms observed for these plants as compared with the Mt-1021 plants. These results were connected to the lower down-regulation of the nitrogenase gene, nifH, observed for these plants, and are consistent with the nitrogen-fixing ability previously found for Mt-RD64 plants grown under normal conditions: nitrogenase activity detected in
6-week-old Mt-RD64 plants increased by 40% when compared with Mt-1021 plants (Imperlini et al., 2009).

Furthermore, the leaves of Mt-RD64 plants were able to accumulate proline to an increased extent (i.e. up to 60% more than control leaves). The measurement of expression levels of two genes involved in the first two steps of proline biosynthesis from glutamic acid, MtP5CSI and MtP5CS2, confirmed these results: both genes were overexpressed in the leaves of Mt-RD64 plants as compared with the Mt-1021 plants. These results are in agreement with those of Verdoey et al. (2006) and reinforce the suggestion that proline is an important component of the salt stress response in plants.

It is known that, in plants, salt stress induces the accumulation of ROS species such as H₂O₂. To avoid or to alleviate the risk of ROS accumulation, plants respond by activating different scavenging pathways (Levine et al., 1994; Apel and Hirt, 2004; Fujita et al., 2006). Among the scavenging systems, SOD is the primary scavenger in the detoxification of ROS induced by oxidative stress in plants. Other scavenging enzymes, including the ascorbate–glutathione cycle enzymes (APX and GR) and CAT, play an important role in the detoxification of the cellular toxic products of SOD (i.e., H₂O₂) in plant cells (Mittler, 2002).

After salt stress, the activity of SOD, APX, GR, and POX enzymes was induced in the leaves of Mt-RD64 plants, whereas these enzymes were unaffected, slightly induced, or repressed in Mt-1021 plants when compared with the unstressed plants. The catalase enzyme was repressed in the leaves of both Mt-1021 and Mt-RD64 plants, although the effect was more evident for Mt-1021 plants. CAT down-regulation after salt stress is also reported for other plants species (Moran et al., 1994; Dat et al., 1998; Lee et al., 2001).

After 2.5 d of re-watering, Mt-1021 plants showed unchanged SOD, GR, and POX activity as compared with non-stressed plants. In contrast, for Mt-RD64 plants, SOD activity decreased whereas GR and POX maintained the increment shown after salt stress. CAT and APX activity was severely deactivated in the leaves of Mt-1021 plants, whereas it was less inhibited in the leaves of Mt-RD64 plants. Such a lower decrease may be supported by the ability of proline, which accumulated much more in these cells, to work as an enzyme protectant under stress, as previously reported (Khedr et al., 2003). Here it was found that the changes in the antioxidant enzymes activity were correlated with the alterations observed in H₂O₂ generation. Indeed, during both salt stress and recovery, the level of H₂O₂ in the leaves of Mt-1021 plants was much higher than that of Mt-RD64 plants. This result was in agreement with the reduced necrotic lesions observed for Mt-RD64 plants.

It is known that most of the abiotic stresses (salinity, cold, drought, and oxidative stress) can induce defence responses in plants through changes in hormone levels. Indeed, it was recently found that the gaseous hormone ethylene, a key regulator of plant growth and development, is involved in plant responses to abiotic stresses such as freezing, salt, and osmotic shocks (Chen et al., 2005). In Medicago, ethylene signal transduction involves a two-component histidine protein kinase receptor, ETR1. In air, this ethylene receptor maintains CTR1, the negative regulator of the mitogen-activated protein kinase (MAPK) pathway, in an active state. In the presence of ethylene, the repression is relieved. Binding of ethylene inactivates the receptor, thereby inactivating CTR1. As a result, EIN2, a central component in ethylene signalling, is activated and a transcriptional cascade, involving various transcription factors, is initiated to regulate ethylene responses (Ouaked et al., 2003; Guo and Ecker, 2004).

The analysis of the expression levels of the main ethylene signalling genes showed that severe salt stress triggered different alarm levels in Mt-1021 and Mt-RD64 plants: for Mt-1021 there was a higher induction of ethylene signalling, whereas a significant regulation of the key positive regulators of this signalling in Mt-RD64 plants was not found.

Furthermore, the accumulation of auxin in the root of Mt-RD64 plants may inhibit the acropetal transport of IAA to the root and, thus, its further synthesis in the shoot (Pennmetsa et al., 2003), as suggested from the results obtained in ELISA experiments and in RT-PCR analysis of the GH3 gene.

It is known that a cross-talk between different signalling molecules such as hormones is involved in both abiotic and biotic stress responses. Indeed, low concentrations of auxins have been found to inhibit ethylene formation and then leaf senescence (Grossmann, 2000; Schmelz et al., 2003). It is thus speculated that the alterations of auxin content in the shoot of Mt-RD64 plants are correlated to the salt stress response of these plants.

The reduced foliar senescence observed for Mt-RD64 plants might also be consistent with the alteration in the levels of other important hormones that promote leaf senescence and control plant growth such as ABA and JA (Grossmann, 2000; Guo and Ecker, 2004; Chen et al., 2005). Further studies need to be conducted to confirm this hypothesis.

Rhizobia with higher tolerance to stress conditions have been identified: these strains usually perform better, improving N₂ fixation symbioses in marginal lands with extreme conditions (Zahran, 1999; Essendoubi et al., 2007). On the other hand, Rhizobium mutants selected for their decreased salt tolerance under free-living conditions were symbiotically deficient (Nogales et al., 2002). Furthermore, genetic evidence showed that nitrogen-fixing bacteria might be under osmotic stress within the host cells (Nogales et al., 2002).

Thus, although the presence of stress tolerance in vitro does not guarantee that the RD64 strain will have stress resistance in the field under salty soil conditions, there are already indications that the S. meliloti strain RD64, with its ability to overproduce IAA, shows better survival and persistence under unfavourable conditions (Defez, 2006).

In summary, the data presented here show that the S. meliloti strain RD64 is more resistant to various abiotic stresses and that salt stress induces much less oxidative damage in the leaves of 2HA Medicago plants nodulated by
this strain. Analogous experiments have to be repeated with the Jemalong A17 line. This result may be due to the positive correlation between the internal proline content, the antioxidant enzymes activity, and the better nitrogen-fixing capacity associated with the enhanced salt stress response of these plants as a consequence of a general phytohormone re-modulation.

A different system to enhance stress tolerance in higher plants is proposed here. Transgenic plants overexpressing IAA or other hormones (Rivero et al., 2007) might have an imbalance affecting their development and differentiation. A transient system was used for the local and continuous delivery of IAA in the root nodule tissue. A similar result might be obtained by ectopically directing the IAA synthesis from a root-activated promoter.

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References


Raghavan C, Kok Ong E, Dalling MJ, Stevenson TV. 2006. Regulation of genes associated with auxin, ethylene and ABA pathways by 2,4-dichlorophenoxyacetic acid in Arabidopsis. Functional and Integrative Genomics 6, 60–70.


