Improvement of Drought Tolerance and Grain Yield in Common Bean by Overexpressing Trehalose-6-Phosphate Synthase in Rhizobia

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Improving stress tolerance and yield in crops are major goals for agriculture. Here, we show a new strategy to increase drought tolerance and yield in legumes by overexpressing trehalose-6-phosphate synthase in the symbiotic bacterium *Rhizobium etli*. *Phaseolus vulgaris* (common beans) plants inoculated with *R. etli* overexpressing trehalose-6-phosphate synthase gene had more nodules with increased nitrogenase activity and higher biomass compared with plants inoculated with wild-type *R. etli*. In contrast, plants inoculated with an *R. etli* mutant in trehalose-6-phosphate synthase gene had fewer nodules and less nitrogenase activity and biomass. Three-week-old plants subjected to drought stress fully recovered whereas plants inoculated with a wild-type or mutant strain wilted and died. The yield of bean plants inoculated with *R. etli* overexpressing trehalose-6-phosphate synthase gene and grown with constant irrigation increased more than 50%. Macroarray analysis of 7,200 expressed sequence tags from nodules of plants inoculated with the strain overexpressing trehalose-6-phosphate synthase gene revealed upregulation of genes involved in stress tolerance and carbon and nitrogen metabolism, suggesting a signaling mechanism for trehalose. Thus, trehalose metabolism in rhizobia is key for signaling plant growth, yield, and adaptation to abiotic stress, and its manipulation has a major agronomical impact on leguminous plants.

Additional keywords: *OtsA*, *otsA*[−], TPS.

Environmental conditions are severe limiting factors for growth and yield in crops. Among these factors are drought stress, salinity, and extreme temperatures, which dehydrate the plant tissues and cause irreversible cellular damage and death (Bartels and Sunkar 2005; Bray et al. 2000). However, there are some organisms that can withstand long periods in a dried state and have the capacity to rehydrate and restart their metabolic functions after being in contact with water (Clegg 2001; Crowe et al. 1992). These anhydrobiotic organisms are rare; among them are resurrection plants such as *Selaginella lepidophylla* and some fungi such as *Saccharomyces cerevisiae*. All these organisms have in common the capacity of synthesizing

and accumulating large concentrations of trehalose (Elbein et al. 2003). This is a nonreducing disaccharide (α -D-glucopyranosyl-1, 1- α -D-glucopyranoside) formed by two molecules of glucose linked through their anomeric carbons.

There are five different pathways for trehalose biosynthesis; the most common is a two-step process involving glucose transfer from UDP-glucose to glucose-6-phosphate to form trehalose-6-phosphate and UDP (Avonce et al. 2006). This reaction is catalyzed by the enzyme trehalose-6-phosphate synthase (TPS) encoded by the *OtsA* gene in bacteria (Ström and Kaasen 1993). The second step results from the dephosphorylation of trehalose-6-phosphate and is catalyzed by trehalose-6-phosphate phosphatase (TPP). The enzyme trehalase degrades trehalose into two molecules of α -D-glucose and is present in most organisms (Aeschbacher et al. 1999). Trehalose in bacteria dramatically accumulates in osmotic or thermal stress conditions. Experiments done in *Escherichia coli* showed that mutation of *OtsA* restricts its capacity to grow in stress conditions (Giaever et al. 1988; Kandror et al. 2002).

Symbiotic nitrogen-fixing bacteria such as *Rhizobium* and related genera have the capacity of synthesizing trehalose, and its accumulation has been detected in bacteroids as well as in nodules (Müller et al. 2001; Streeter 1985). *Rhizobium etli*, a symbiont of *Phaseolus vulgaris*, has the capacity to induce nodule formation on the roots of bean plants (Sessitsch et al. 2002). Recently, the full genome sequence of *R. etli* has been reported (González et al. 2006).

Bean crops (*P. vulgaris*) are a staple food of great economic importance around the world, in particular in developing countries; nevertheless, their growth is affected by climatic conditions restricting grain yield (Subbarao et al. 1995). Mineral nitrogen often limits plant growth, and agriculture and food supplies largely depend on the nitrogen cycle. Although nitrogenous fertilizers have allowed the expansion of crop yields, they have dramatically contributed to air and water pollution, acid rain, and the greenhouse effect (Socolow 1999). Symbiotic relationships have evolved the capacity to fix atmospheric nitrogen in the rhizobia-colonizing legume nodules and are responsible for converting it to 70 million tons of ammonia each year (Brockwell et al. 1995).

To gain understanding of the role of trehalose metabolism in rhizobia, we overexpressed the *OtsA* gene in *R. etli*, isolated an insertion mutant in the same gene, and analyzed the effect on free-living bacteria and symbiotic bacteroids at the molecular, biochemical, and physiological levels. Here, we show that common bean plants inoculated with *R. etli* overexpressing

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OtsA are stress tolerant, and that there is a substantial increase in grain yield.

RESULTS

The sequence of the *OtsA* gene from *R. etli* (*ReOtsA*) revealed an open reading frame (ORF) encoding a 469-amino-acid deduced protein. It showed significant sequence identity with other genes encoding TPS, such as 74% to *Sinorhizobium meliloti* and 33% to *Saccharomyces cerevisiae* homologs at the amino acid level. The full-length gene sequence of 1,621 bp includes the 5' and 3' untranslated regions. To overexpress the *ReOtsA* gene, we used the broad-host-range pBBR1MCS5 vector that allows gene expression under the control of the *lac* promoter (Kovach et al. 1995). The *ReOtsA* 1.4-kb ORF was amplified by polymerase chain reaction (PCR), subcloned into this vector, and mobilized to *R. etli*. In addition, an insertion mutant in the *OtsA* gene was isolated at a frequency of 1×10^{-9} by homologous recombination after knocking out the *OtsA* gene with a tetracycline resistant marker.

The *R. etli* wild-type (WT), *OtsA* mutant (*otsA*⁻), and overexpressing *OtsA* (Ox) strains were grown in minimal medium for 2 days. After 24 h, the Ox strain continued growing, whereas the WT and *otsA*⁻ started to decline (Fig. 1A). When the three strains were grown in the presence of 0.5 M NaCl, the Ox strain grew at a significantly higher rate than the WT and *otsA*⁻ strains (Fig. 1B). In fact, growth of the *otsA*⁻ mutant in 0.5 M NaCl was negligible. The Ox strain also survived better than WT and *otsA*⁻ strains if exponentially grown cells were heat shocked or freeze thawed before plating (data not shown). These results indicate that the overexpression of the *OtsA* gene provides stress tolerance to *R. etli*. Trehalose quantification was carried in *otsA*⁻, Ox, and WT strains grown in media without osmotic stress (Fig. 1C) or containing 0.5 M NaCl (Fig. 1D). There was a higher trehalose concentration in Ox cells compared with WT due to the expression of *ReOtsA* driven by the constitutive *lac* promoter, whereas the *otsA*⁻ strain has a lower concentration of the disaccharide (Fig. 1C). The trehalose content in the Ox strain was significantly higher after 24 h of growth in the presence of NaCl, suggesting a stress induction of *OtsB* (encodes for TPP) and possibly of the other trehalose biosynthetic genes that we found in the *R. etli* genome after a bioinformatics analysis. These genes are a *ReOtsA* homolog which shares 45% identity and 60% similarity at the amino acid level, and genes of other trehalose biosynthetic pathways, namely two encoding trehalose synthase which converts maltose into trehalose and one gene encoding a maltotrioglucosyl trehalose synthase that synthesizes trehalose from maltotrioglucosaccharides (Avonce et al. 2006). The mutant strain also had a slight increase in trehalose content upon stress but at a lower level compared with the WT strain. Thus, the trehalose concentration correlated with improved stress tolerance in free-living *R. etli* cells.

In order to determine the effect of the overexpression or mutation of the *OtsA* gene in *R. etli* in symbiosis with nodulating *P. vulgaris*, 2-day-old bean plantlets were inoculated with the WT, Ox, or *otsA*⁻ strains and allowed to grow up to 21 days post inoculation (dpi). Plants nodulated with the Ox strain showed a 27% increase in nodule number compared with the WT, whereas a decrease of 26% was observed in bean plants inoculated with the *otsA*⁻ mutant (Fig. 2A). To gain further understanding at the biochemical level, the nitrogenase activity was determined as acetylene reduction activity by gas chromatography analysis from nodules of plants inoculated with the three strains. Plants nodulated with the Ox strain showed a 38% increase in nitrogenase activity, whereas a 45% decrease in activity was observed in plants inoculated with the *otsA*⁻

Fig. 1. Survival of free-living *Rhizobium etli* with overexpressed or mutated *OtsA* gene upon stress. Wild-type (triangles), overexpressing *OtsA* gene (squares), and *otsA*⁻ mutant (rhombus) strains of *R. etli* were grown at 30°C in **A**, liquid minimal medium alone or **B**, containing 0.5 M NaCl. At the indicated times, equal amounts of cells were plated on petri dishes, transferred to 30°C, and grown for 48 h before surviving colonies were counted. Wild-type (white bars), overexpressing *OtsA* gene (black bars), and *otsA*⁻ mutant (gray bars) strains of *R. etli* were grown as described before, and aliquots were taken to measure intracellular trehalose content from the same cultures used to count viable cells in **C**, minimal medium alone or **D**, containing 0.5 M NaCl. Both figures represent the results of three independent experiments. Asterisks indicate that the means of the samples are different at the 0.05 level ($P < 0.05$).

mutant (Fig. 2B). Histological analysis of nodules from constantly irrigated (Fig. 2C) and drought-stressed plants (Fig. 2D) showed a larger area of infected cells in the nodules of plants inoculated with the *R. etli* Ox strain and smaller infected area in nodules of plants inoculated with *otsA*⁻ mutant after comparison with the WT strain. All nodules from stressed plants shrank and, thus, look smaller than nodules from unstressed plants (Fig. 2C and D).

A major objective in the present work was to determine whether overexpression of the *OtsA* gene of *R. etli* improves tolerance to drought stress of inoculated bean plants. Plants inoculated with WT, Ox, or *otsA*⁻ strains were grown with continuous watering for 21 days. Afterward, watering was stopped for 3 weeks and then watering was resumed. Plants inoculated with the Ox strain showed evident drought tolerance compared

with those inoculated with the WT or *otsA*⁻ strains that did not survive stress (Fig. 3A). Two days after watering was resumed, 87% of the plants inoculated with *R. etli* overexpressing the *OtsA* gene had the capacity for recovering from drought stress whereas only 7% of plants inoculated with the WT strain and less than 1% of plants inoculated with the *otsA*⁻ mutant survived (Fig. 3B). The relative water content (RWC) of the plants and soil gravimetric water content (SGWC) were determined during the experimental time. At 21 dpi, just before drought stress was applied, RWC and SGWC were at their highest values (taken as 100%) and fairly equal for all plants (Fig. 3C and D). However, at 42 dpi that corresponded to 3 weeks of water deprivation, plants lost approximately 90% of their water content. There was a slightly higher RWC in the bean plants inoculated with the Ox strain (14.7%) compared with plants inoculated

Fig. 2. Biochemical and histological analyses of bean nodules. **A**, Number of nodules and **B**, nitrogenase activity were determined from common bean plants inoculated with the wild type (WT), *R. etli* strain overexpressing the *OtsA* gene (Ox), or *OtsA* mutant (*otsA*⁻). These data represent the results of three independent experiments. Bright-field photographs of transversal sections from **C**, a representative nodule of irrigated plants or **D**, drought-stressed plants inoculated with either strain. Single asterisks indicate that the means of the samples are different from WT, and double asterisks indicate that means of the samples are different from WT and Ox, at the 0.05 level ($P < 0.05$).

with *R. etli* WT (9.64%) or *otsA*⁻ (9.20%) strain after drought stress. The SGWC also dropped to approximately 25% after 21 days without watering.

The quantification of trehalose content in plants revealed significantly higher levels of the disaccharide in nodules than in shoots. In fact, trehalose increased two times when plants were subjected to drought stress (Fig. 3E). However, an increase of approximately 10% in the disaccharide content was observed in nodules of plants inoculated with the Ox strain compared with nodules inoculated with the WT. In contrast, the trehalose levels decreased significantly in nodules of plants inoculated with the *otsA*⁻ mutant (Fig. 3E). No significant differences were detected in trehalose concentration in shoots of plants inoculated with the three different *R. etli* strains. The disaccharide was not detected in roots free of nodules of plants inoculated with either strain.

The biomass of plants inoculated with the Ox strain was higher than of plants inoculated with WT and lower for plants inoculated with *otsA*⁻, after measuring both fresh and dry weight (Fig. 4A and B). There was a sharper difference considering constant watering conditions, indicating that the overexpression of *ReOtsA* provoked up to 92% of water retention, whereas the *otsA*⁻ mutant led to a 15% drop of water retention compared with the WT (Fig. 4A). Upon drought stress, the biomass of all plants inoculated with either strain decreased significantly; however, the differences still correlated to the corresponding strain as shown before (Fig. 4A and B).

An interesting question was whether the overexpression or mutation of the *OtsA* gene in *R. etli* interacting with bean plants would have an energetic cost for the plant, reflected in the grain yield. Therefore, three groups of 10 plants each were inoculated with the WT, Ox, or *otsA*⁻ strains of *R. etli* and kept under continuous watering until obtaining mature seed. The yield (see Material and Methods), which considered number of pods (legume fruits) and seed and their dry weight, showed an increase of 57% in plants inoculated with the Ox strain compared with plants nodulated with the WT strain, whereas a drop of 43% in yield was observed in plants inoculated with the *otsA*⁻ mutant, when plants were constantly irrigated (Fig. 4C). Upon drought stress, the yield sharply decreased but was still 38% higher in plants inoculated with the Ox strain and negligible for plants nodulated with the *otsA*⁻ mutant (Fig. 4C).

The overexpression of a *TPS* gene in *Arabidopsis* leads to an altered gene-expression profile, and it has been suggested a signaling role for trehalose and trehalose-6-phosphate (Avonce et al. 2004; Schluepmann et al. 2004). To explore if the changes in yield and stress tolerance observed in common bean inoculated with either Ox or *otsA*⁻ strains were due to a change in the plant gene-expression pattern, a macroarray

approach was used. Nylon filter arrays with a redundant set of 7,200 expressed sequence tags (EST), containing 1,806 unique genes, from a *P. vulgaris* nodule cDNA library (Ramírez et al. 2005) were hybridized with cDNA from total RNA nodules of bean plants inoculated with either the *R. etli* WT, Ox, or *otsA*⁻ strain. After hybridization, the signal intensity of each spot was quantified and the expression ratio of each gene in nod-

Fig. 3. Survival of plants subjected to drought stress. Well-watered, 3-week-old common bean plants were subjected to drought stress by withholding watering for another 3 weeks. In each pot, five plants inoculated with *Rhizobium etli* wild-type (WT), overexpressing the *OtsA* gene (Ox), or *otsA*⁻ strain were present. **A**, Photographs were taken 2 days after rewatering. Survival of imposed drought stress on plants nodulated with either strain was determined considering 15 plants of each condition, and three independent experiments were conducted. **B**, Survival of plants upon continual watering was taken as 100%. **C**, Relative water content (RWC) and **D**, soil gravimetric water content (SGWC) were determined before (21 days postinoculation [dpi]) and after (42 dpi) water stress. Trehalose was determined in shoots and nodules from plants nodulated with the WT, Ox, or *otsA*⁻ strain after 3 weeks of drought treatment or **E**, continual watering. Asterisks indicate that the means of the samples are different at the 0.05 level ($P < 0.05$).

ules of plants inoculated with either the Ox or *otsA*⁻ strain compared with WT-inoculated plants was calculated. Such comparison was done in *P. vulgaris* plants subjected to drought stress and in continually irrigated plants. Overall, 3,485 EST were upregulated in plants subjected to drought stress whereas only 177 EST were induced in plants under continual irrigation, after comparing the ratio of genes induced in plants inoculated with Ox versus WT. Among the most highly upregulated genes in nodules of Ox-inoculated plants were those involved in nodule function, nitrogen metabolism, tolerance to abiotic stress, and carbon metabolism; the expression ratios of some of these genes are shown in Table 1.

The analyzed genes were highly induced in nodules of drought-stressed plants inoculated with the Ox strain compared with plants inoculated with the WT strain, showing expression ratios over 30-fold. In the irrigated plants, only some genes showed upregulation in Ox-elicited nodules, with lower ex-

pression ratios compared with WT nodules. Genes that are highly expressed in nodules are widely represented in the nodule cDNA library and macroarray used (Ramírez et al. 2005); in agreement, several EST from these contigs show high Ox/WT expression ratios (Table 1). The most conspicuous transcripts highly induced in nodules elicited by Ox strain were those that encoded leghemoglobin 2, which transports oxygen to the bacteroid; nodulin 30 transcript, so far of unknown function; glutamine synthetase, involved in NH₄ assimilation; uricase II, which synthesizes ureids; sucrose synthase, PEP carboxylase, and malate dehydrogenase, which are involved in producing carbon skeletons to feed the symbiotic bacteria; and several detoxification enzymes such as homogluthathione synthetase, catalase, cytochrome P450, glutaredoxin, and ascorbate peroxidase are key to helping plants recover from drought stress (Table 1). None of the analyzed genes were induced in nodules from plants inoculated with the *otsA*⁻ mutant strain, whether irrigated or subjected to drought stress (data not shown).

DISCUSSION

Breeding has had limited success in improving drought tolerance in crops due to the multigenic nature of the trait. A more promising strategy is the overexpression of genes conferring tolerance to abiotic stress. Trehalose is involved in stress tolerance in ahydrobiotic organisms and in transgenic animals and plants. In the present work, the overexpression and mutation of the *ReOtsA* gene in *R. etli* was evaluated in both free-living and symbiotic conditions.

We showed that free-living *R. etli* has a significantly higher survival capacity to osmotic stress when the bacteria overexpressed *ReOtsA* (Fig. 1). Similarly, the overexpression of *OtsAB* genes in *E. coli* increased tolerance to osmotic stress (Purvis et al. 2005). Addition of exogenous trehalose to *Bradyrhizobium japonicum* confers tolerance to dehydration stress (Streeter 2003). In contrast, mutation of *ReOtsA* impaired stress tolerance (Fig. 1). However, we still could detect trehalose in the *otsA*⁻ mutant, although at relatively lower levels compared with the WT strain. This is probably due to the presence of three trehalose biosynthetic pathways in *R. etli* that we found by bioinformatics analysis: an *OtsA* homolog that encodes TPS, a trehalose synthase gene, and a trehalose maltotoligosyl trehalose synthase gene. Interestingly, these same three routes have been found in *B. japonicum* as well, and their significant transcription induction correlates with an elevated level of trehalose, suggesting an important role of this disaccharide in desiccation tolerance (Cytryn et al. 2007). Another striking result is the hyperaccumulation of trehalose when the Ox strain was subjected to osmotic stress (Fig. 1). This could be explained by the stress induction of *OtsB*, the *OtsA* homolog, or the other trehalose biosynthetic genes present in the *R. etli* genome.

We also examined the effects of overexpressing or mutating *ReOtsA* in *R. etli* in symbiosis with *P. vulgaris* and, for this aim, several parameters were evaluated. First, a higher number of nodules and nitrogenase activity were found in plants inoculated with the Ox strain, whereas a lower amount of both were present in plants inoculated with the *otsA*⁻ mutant strain (Fig. 2). Also, we have shown that the expression of the leghemoglobin 2 gene in nodules from plants inoculated with the Ox strain was increased significantly, suggesting a possible increase in bacteroid respiration and, thus, a higher nitrogenase activity and nitrogen fixation. Trehalose might also protect nitrogenase from the inactivation caused by oxygen. It has been shown that trehalose has an antioxidant effect (Gonzalez-Parraga et al. 2003).

The increase in nodule number and nitrogenase activity in *P. vulgaris* plants overexpressing *ReOtsA* suggested that bacterial

Fig. 4. Plant biomass and grain yield. **A**, Foliage fresh weight or **B**, dry weight of bean plants at 21 days after inoculation with *Rhizobium etli* wild-type (WT), overexpressing the *OtsA* gene (Ox) or *otsA*⁻ strains was determined. **C**, Grain yield in *Phaseolus vulgaris* plants nodulated with WT, Ox, or *otsA*⁻ strains was determined in three independent experiments. Percentages were calculated taking the value of the WT strain as 100%. White bars denote continually irrigated plants and black bars plants subjected to drought stress. Asterisks indicate that the means of the samples are different at the 0.05 level ($P < 0.05$).

trehalose triggered a morphological and biochemical response leading to a higher biological nitrogen fixation. Therefore, we analyzed a possible effect at the subcellular level in nodules. There was a greater quantity of infected nodule cells in plants inoculated with the Ox strain (Fig. 2). These results could be explained because the accumulation of trehalose in this strain provides it with a greater capacity to infect or to escape the defense mechanisms in nodulating plants, thus inducing the differentiation of a greater number of cells containing bacteroids. It has been reported that the mutation of the *OtsA* gene of *Mycobacterium tuberculosis* causes a decrease in the infectivity of mice (Murphy et al. 2005). Similarly, a Δ *tpsI* mutant in the rice blast fungus *Magnaporthe grisea* was greatly attenuated in pathogenicity (Foster et al. 2003). Moreover, *TpsI* in *M. grisea* plays a central role in the establishment of plant disease (Wilson et al. 2007). Recently, it has been shown that trehalose catabolism in *Sinorhizobium meliloti* plays a critical role in alfalfa root colonization (Jensen et al. 2005). Therefore, trehalose seems to be involved in the animal- and plant-microorganism interactions.

One of the essential questions of this work was to determine whether the overexpression of *ReOtsA* gene in *R. etli* in symbiosis with common bean plants caused an increase in drought tolerance. In fact, *P. vulgaris* plants inoculated with the Ox strain had a significant increase in drought tolerance compared with plants inoculated with the WT strain (Fig. 3). As far as we know, this is the first report of the improvement to drought stress of a legume species by a different method than plant breeding or plant transformation.

It is noteworthy that plants inoculated with the Ox strain showed an increase of approximately 10% in trehalose levels in nodules and leaves. Similar small increases of trehalose have been observed in other instances where a *TPS* gene was overexpressed in transgenic tobacco, potato, rice, *Arabidopsis*, or animal cells, and still there was an enhanced drought tolerance (Avonce et al. 2004; Garg et al. 2002; Guo et al. 2000; Holmström et al. 1996; Yeo et al. 2000). The trehalose concentration found was too low to explain an effect of trehalose as osmoprotector (Gaff 1996). Thus, one possibility is that treha-

lose could be functioning as a signal molecule activating stress tolerance pathways in the plant (Paul 2007). It is well established that sugars signal plant growth, development, and stress responses, with hexokinase (HXK) having a central role as a glucose sensor (Rolland et al. 2006). In *Arabidopsis*, a *tpsI* knock-out is developmentally arrested in the torpedo stage, which is associated with initiation of storage reserve accumulation (Eastmond et al. 2002). Alterations of trehalose-6-phosphate levels have demonstrated that this metabolic intermediate is indispensable for carbohydrate utilization for plant growth (Schluepmann et al. 2003). *Arabidopsis* plants overexpressing *AtTPS1* are drought-tolerant and insensitive to glucose and ABA, and showed an altered expression of *HXK1* and *ABI4* genes (Avonce et al. 2004). Recently, the maize *ramosa* mutant with abnormal inflorescences and lower grain yield was shown to be knocked-out in a *TPP* gene demonstrating the role of a trehalose metabolic enzyme in development (Satoh-Nagasawa et al. 2006). All these results strongly support the role of trehalose as a signal molecule.

The possible trehalose transport from rhizobia to the nodule plant cells still needs to be demonstrated. However if the latter occurs, we propose that trehalose may be acting as a signal molecule to activate transcription in nodule cells of genes involved in stress tolerance and carbon and nitrogen metabolism (Table 1). Interestingly, in this study, an unexpected link between trehalose metabolism in *Rhizobium* spp. and plant yield was found: plants inoculated with the overexpressing strain showed up to 57% improvement in the grain yield (Fig. 4). These results are important because they demonstrate that the overexpression of *OtsA* gene in *R. etli* increases crop yield. The increase in biomass and grain yield of plants inoculated with *R. etli* overexpressing the *OtsA* gene is also a consequence of a higher nitrogen fixation because several genes related to this process are upregulated (Table 1). For instance, the leghemoglobin 2 was the highest upregulated gene when bean plants were inoculated with the overexpressing strain. In fact, the heterologous expression of a *Vitreoscilla* sp. hemoglobin gene in *R. etli* led to an increase in respiratory activity in free-living rhizobia and

Table 1. Upregulated plant genes in nodules responsive to *ReOtsA* overexpression in *Rhizobium etli*

Annotation ^a	Pathway	Irrigated plants		Drought-stressed plants	
		Ox/WT ^b	No. of EST	Ox/WT ^b	No. of EST
Nodulins and nitrogen metabolism					
Leghemoglobin 2	Oxygen transport	7	38	37	209
Nodulin 30	Unknown function	7	15	31	149
Glutamine synthetase	NH ₄ assimilation	2	1	11	23
Uricase II	Ureide synthesis	–	0	7	15
Nitrate reductase	NH ₄ assimilation	2	1	3	3
Carbon metabolism					
Sucrose synthase	Sucrose cleavage	–	0	6	17
Malate dehydrogenase	Malate synthesis	2	1	5	10
PEP carboxylase	Oxaloacetate synthesis	2	1	3	5
Trehalase	Trehalose cleavage	–	0	3	3
Abiotic stress					
Homogluthathione synthetase	Detoxification	6	1	32	1
Catalase	Detoxification	–	0	16	3
Cytochrome P450	Detoxification	–	0	10	12
Glutaredoxin	Detoxification	–	0	9	6
Ascorbate peroxidase	Detoxification	–	0	6	13
Heat-shock transcription factor	Heat tolerance	–	0	6	3
Zn/Cu superoxide dismutase	Detoxification	2	1	4	3
Heat-shock 70 kDa protein	Heat tolerance	–	0	4	1
K ⁺ /Na ⁺ symporter HKT1	Salt tolerance	–	0	3	1
Glutathion peroxidase	Detoxification	–	0	2	1

^a Enlisted genes had an enhanced expression, as evidenced by macroarrays of all the corresponding expressed sequence tags (EST) spotted in the membrane.

^b Expression ratio. The EST's highest expression ratio is shown for the corresponding nodule gene of plants inoculated with *R. etli* overexpressing *ReOtsA* (Ox) and wild-type (WT) strains.

higher nitrogenase activity and total nitrogen content in bean plants (Ramírez et al. 1999).

The macroarray data suggest that the possible signaling role of trehalose coordinates the gene expression of nitrogen metabolism genes such as glutamine synthetase and uricase II, with the carbon metabolism genes of sucrose synthase, PEP carboxylase, and malate dehydrogenase required to increase the availability of carbon skeletons to the bacteroid; concomitantly, stress-adaptive genes are expressed to remove free radicals or other toxic molecules and to help plants recover from drought stress. Upon drought stress, a significantly higher expression of all gene categories was observed in plants inoculated with the *R. etli* overexpressing *OtsA* (Table 1), supporting the role of trehalose in triggering stress response.

Conclusion.

The overexpression of *ReOtsA* confers tolerance to osmotic stress in free-living *R. etli*, providing the inoculant with a greater survival capacity and symbiotic efficiency. In plant nodules, the symbiotic bacteroid with higher trehalose content results in an increased nodulation and nitrogenase activity, which leads to a higher biomass and grain yield. Trehalose is a signal, possibly traveling from the bacteroid to the plant, that also triggers an enhanced drought tolerance. Thus, the overexpression of TPS in rhizobia establishes a new method to obtain a biofertilizer that could be used in agriculture to improve grain production and drought tolerance in legume crops.

MATERIALS AND METHODS

Biological material and plant growth.

R. etli CE3 strain and *P. vulgaris* var. Negro Jamapa plants were used in the present study. Inoculation of bean plants with *R. etli* WT, mutant, and overexpressing strains was done in the following way. Seed were surface sterilized with sodium hypochlorite 10% solution for 15 min. After washing the seed with water, they were transferred to a tray containing paper towels moistened in water, and thereafter covered with aluminum foil to allow seed germination at 27°C during 72 h. After germination, each seedling planted in pots containing sterile vermiculite was inoculated with 1 ml of *R. etli* culture. Plants were watered with Hoagland's solution (Sigma, St. Louis) every other day.

Cloning *R. etli* *OtsA* gene and mutant isolation.

To clone the *OtsA* gene from *R. etli*, two oligonucleotides (forward, 5'-ATCATCGGTGTCGATCGCCT and reverse, 5'-GGATCGTCCGGATCCTGC) were designed to flank a conserved region from the *OtsA* gene from *Mesorhizobium loti*, *S. meliloti*, and *Rhizobium* sp. NGR234, and were used to amplify a DNA fragment by PCR (1 cycle at 94°C for 3 min; 30 cycles at 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min; and 1 cycle at 72°C for 10 min) using as a template the *R. etli* genome. The resulting 370-bp fragment was cloned in PCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, U.S.A.) and sequenced. For *R. etli* mutagenesis by homologous recombination, *E. coli* S17-1 strain hosting the pSUP202 suicide vector (Simon et al. 1983), which had the *ReOtsA* 370-bp fragment, was used. Two *R. etli* mutants were obtained after selecting in tetracycline, which were characterized by Southern blot and sequencing. To clone the full-length *OtsA* gene from *R. etli*, a genomic library from this species was constructed in pBluescript (Stratagene, La Jolla, CA, U.S.A.). After library screening, four clones hybridizing to the 370-bp probe were isolated. Sequencing both ends of each clone showed that the gene was cloned in parts. To obtain the full-length coding-sequence, forward (5'-CCGCTCGAGAAGGAAAACCCCAT

GAGCCGTCTTATC, which contains a *XhoI* site and the *R. etli* ribosome binding site; the initiation codon is underlined) and reverse (5'-CCCAAGCTTAGCGCGCTAAAGAGCCTATCCGTG, which contains a *HindIII* site; a STOP codon is underlined) oligonucleotides were used to amplify it by PCR (1 cycle at 94°C for 5 min; 30 cycles at 94°C for 1.5 min, 55°C for 1 min, and 72°C for 1.75 min; and 1 cycle at 72°C for 10 min) using *R. etli* genome as a template. A predicted 1.4-kb fragment was obtained. The *ReOtsA* gene was then subcloned in the broad-host-range pBBR1MCS5 expression vector (Kovach et al. 1995) after transformation of *E. coli* DH5 α strain. The resulting plasmid was named pBBR1M::*ReOtsA* and was used to transform *E. coli* S17-1 strain and mobilized to *R. etli* by conjugation.

Stress tolerance tests in *R. etli*.

Rhizobia were grown at 30°C in PY media supplemented with nalidixic acid (20 μ g/ml) for the WT, tetracycline at 5 μ g/ml for the *otsA*⁻ mutant, and gentamycin at 30 μ g/ml for the Ox strain. Precultures were spun down to wash the antibiotics and grown up to midlogarithmic phase (optical density of 0.4 at 600 nm) in minimal medium supplemented with 0.4% glycerol. To determine survival upon osmotic stress, cells were cultured in 0.5 M NaCl for 2 days, and dilutions were made and plated on PY agar medium with its corresponding antibiotic at 30°C for 72 h. After this time, the surviving colonies were counted.

Drought tolerance tests and biomass determination in bean plants.

Bean plants inoculated with the *R. etli* WT, *otsA*⁻ mutant or Ox strains were subjected to drought stress for 3 weeks by stopping watering after 21 days of growth, and observed for wilting symptoms. Then, watering was reestablished for 1 day and photographed. To determine plant biomass, leaves were weighed immediately after detaching (fresh weight) and then placed in an oven at 70°C for 72 h before weighing again (dry weight). Relative water content was determined as reported before (Gaxiola et al. 2001).

Trehalose quantification.

To quantify the trehalose content in *R. etli* and bean plants, a standard protocol was used (Avonce et al. 2004). For bacteria, cultures were centrifuged, washed with water, and resuspended in 80% ethanol. After incubation at 85°C for 15 min, the samples were centrifuged and the supernatant recovered. The ethanol excess was evaporated and samples were resuspended in ultrapure water before being analyzed by high-performance liquid chromatography (HPLC) using a ZORBAX carbohydrate analysis column (Agilent Technologies, Santa Clara, CA, U.S.A.) eluted with acetonitrile/water (65:35, vol/vol). To quantify trehalose from *P. vulgaris*, tissues were weighed and frozen in liquid nitrogen, resuspended in water, and boiled before being deionized in a 1-ml column prepared with cationic and anionic resins (Avonce et al. 2004) and then analyzed by HPLC as described before.

Determination of nitrogenase activity.

Root nodules were counted, separated, and used to determine enzyme activity (Wacek and Brill 1976). Nitrogenase-specific activity was determined in detached 21-day postinoculated nodulated roots by the acetylene-reduction activity in closed jars and were expressed as nanomoles of ethylene per minute per gram of nodule (fresh weight). Thirty min after the air was extracted and replaced with acetylene, a sample was injected in a gas chromatographer. After the assay, the nodule weight sample were determined.

Microscopy.

Nodules from 21-day-old plants were harvested, rapidly fixed, and processed as reported (Trepp et al. 1999). The embedded tissues were sectioned in 7- μ m cuttings and placed in microscope slides covered with poly-L-lys. After paraffin elimination with xylene, the cuttings were stained in methylene blue solution, dehydrated, and mounted with Permount (Fisher Scientific, Pittsburgh). Sections were examined under a direct light microscope and photographed with a digital camera.

Yield quantification in bean plants.

Bean plants were inoculated with the *R. etli* WT, *otsA*⁻ mutant, or Ox strains, and had continual watering for 4 months until pods appeared. In order to determine the yield of inoculated plants with the different *R. etli* strains, the following parameters were taken: the number of pods and the number of seed on each pod were counted, and each seed was weighed. Yield = (number of pods per plant) $\times$ (number of seed per pod) $\times$ (seed weight) (Linkemer et al. 1998). Three independent experiments were carried out with three repetitions each time.

Macroarray analysis of nodule genes.

The EST sequences and nylon filter arrays were previously reported (Ramírez et al. 2005) and a full description of these data is available online. Total RNA was isolated from mature nodules 21 dpi from plants grown under drought stress or in normal conditions. Nodules were elicited with the *R. etli* WT, *otsA*⁻ mutant, or Ox strain. cDNA was in vitro synthesized from each RNA preparation and was radioactively labeled before using it as a probe to hybridize nylon membranes printed with *P. vulgaris* nodule EST as described before (Ramírez et al. 2005). The hybridization of macroarrays was repeated twice from each condition. The radioactivity of each spot was quantified using a Phosphor Screen imaging system (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and the signal intensity was determined using the Array-Pro Analyzer (Media Cybernetics, Silver Spring, MD, U.S.A.) with normalization against background. Gene expression was considered reliably expressed if they showed an intensity/background ratio greater than 2 throughout all related parallel hybridizations.

Statistical analysis.

The data were processed by analysis of variance by *t* Student test followed by Duncan-Waller mean analysis.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Center for Genomic Sciences (CCG) *Phaseolus vulgaris* EST Project:
www.ccg.unam.mx/phaseolusest/Data_download.htm