Trehalose accumulation in *Azospirillum brasilense* improves drought tolerance and biomass in maize plants

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**Abstract**

Bacteria of the genus *Azospirillum* increase the grain yield of several grass crops. In this work the effect of inoculating maize plants with genetically engineered *Azospirillum brasilense* for trehalose biosynthesis was determined. Transformed bacteria with a plasmid harboring a trehalose biosynthesis gene-fusion from *Saccharomyces cerevisiae* were able to grow up to 0.5 M NaCl and to accumulate trehalose, whereas wild-type *A. brasilense* did not tolerate osmotic stress or accumulate significant levels of the disaccharide. Moreover, 85% of maize plants inoculated with transformed *A. brasilense* survived drought stress, in contrast with only 55% of plants inoculated with the wild-type strain. A 73% increase in biomass of maize plants inoculated with transformed *A. brasilense* compared with inoculation with the wild-type strain was found. In addition, there was a significant increase of leaf and root length in maize plants inoculated with transformed *A. brasilense*. Therefore, inoculation of maize plants with *A. brasilense* containing higher levels of trehalose confers drought tolerance and a significant increase in leaf and root biomass. This work opens the possibility that *A. brasilense* modified with a chimeric trehalose biosynthetic gene from yeast could increase the biomass, grain yield and stress tolerance in other relevant crops.

**Introduction**

Plant growth-promoting rhizobacteria stimulate growth by acting directly on plant metabolism, providing substances in short supply, fixing atmospheric nitrogen, solubilizing soil phosphorus and iron, or synthesizing phytoregulators (Bashan & de-Bashan, 2005). *Azospirillum brasilense* promotes growth of many economically important grass crops including wheat and maize (Heulin *et al*., 1989; Caballero-Mellado *et al*., 1992; Van de Broek *et al*., 1993; Dobbelaeere *et al*., 2001), mainly due to the accumulation and transport of indole-3-acetic acid to the plant (Umali-García *et al*., 1980; Hartmann *et al*., 1985; Dobbelaeere *et al*., 2003). This auxin promotes plant root elongation, which makes the uptake of mineral nutrients and water more efficient (Lin *et al*., 1983; Kapulnik *et al*., 1985; Dobbelaeere *et al*., 2003).

Maize is among the most prominent crops for human consumption, and, together with rice and wheat, is the staple food in most countries. One of the major challenges for the 21st century will be sufficient food production, given estimates that the human population will reach 10 billion by 2050. This means that a strong technological effort must be conducted to increase crop yield, without increasing arable land area. On the other hand, intensive agriculture generates serious environmental problems by using chemical fertilizers, plaguicides and herbicides, salinity and water shortage (Morrissey *et al*., 2004). All these are aggravated by climate change that is severely affecting crop productivity, and therefore improving drought tolerance in maize is of great relevance.

Plants are intermittently exposed to a plethora of abiotic stress conditions such as low or high temperature, salinity and drought (Bray *et al*., 2000). It has been acknowledged for a long time that water availability is the stress condition that causes most losses to agriculture (Boyer, 1982). Abiotic stress triggers many common responses in plants. All of them involve cell dehydration, loss of turgor and accumulation of reactive oxygen species (ROS), which affect subcellular structures and enzyme activity. Under exposure to osmotic stress, plants exhibit a broad range of molecular and
biochemical adaptive mechanisms (Bartels & Sunkar, 2005). These comprise changes in morphology and development such as root growth inhibition, and ion transport and metabolic adjustments, which involve the synthesis of compatible solutes (osmoprotectants) that counteract dehydration and ROS by lowering osmotic potential and protecting membranes, enzymes and other subcellular structures against irreversible damage, denaturation and protein aggregation without altering physiological performance.

Among the different osmoprotectants, trehalose is one of the most effective at counteracting several types of abiotic stress (Elbein et al., 2003). Trehalose (α-D-glucopyranosyl-1, 1-α-D-glucopyranoside) is a nonreducing disaccharide present in a wide variety of organisms known as anhydrobions, which include certain species of plants, fungi, bacteria and invertebrates, and that are capable of reviving in the presence of water in a few hours after being completely dehydrated for months or years (Paul et al., 2008). There are five known trehalose biosynthetic pathways (Avonce et al., 2006), but the best characterized and the one present in many bacteria and plants consists of the condensation of UDP-glucose and glucose-6-phosphate by means of trehalose-6-phosphate synthase (TPS) to form trehalose-6-phosphate, which is subsequently dephosphorylated to trehalose by trehalose-6-phosphate phosphatase (TPP).

In previous reports we have shown that the overexpression of OtsA (bacterial TPS) gene in Rhizobium etli increases its osmotic stress tolerance, and using it to inoculate common bean plants improves drought tolerance and grain yield (Suárez et al., 2008). Also, we have demonstrated that transformation of Arabidopsis thaliana with a yeast bifunctional TPS–TPP enzyme confers multiple stress protection to plants, namely freeze, heat, drought and salt tolerance (Miranda et al., 2007).

Here, we analyzed the effect of inoculating maize plants with genetically engineered A. brasilense strains that over-accumulate trehalose.

**Materials and methods**

**Biological material**

Azoospirillum brasilense strain Cd (Eskew et al., 1977) and maize (Zea mays landrace Chalqueño, Mexico) plants were used. For gene construction and plasmid mobilization to A. brasilense, Escherichia coli S17-1 and DH5α strains were used, respectively.

**Growth conditions**

To inoculate plants, Azoospirillum strains were grown on nickel fluoroborate (NFB) liquid medium (Döbereiner et al., 1976) supplemented with NH₄Cl (0.2 g L⁻¹) for 2 days at 30 °C to 250 r.p.m. Bacteria were harvested by centrifugation (7000 g for 10 min) and washed twice in 10 mM MgSO₄, 7H₂O. The Congo red (Sigma Chemical Co., St. Louis, MO) agar medium (Rodríguez-Cáceres, 1982) for growing A. brasilense was routinely used to check culture purity and count bacteria. Maize plants were grown in 3-L plastic pots containing sterilized river sand for 2 weeks and watered five times with 200 mL of sterile distilled water. All incubations were performed in a greenhouse (24 ± 4 °C, natural illumination).

**Molecular techniques**

The 1452-bp ReOtsA coding region (Suárez et al., 2008) was amplified with forward primer (5’CCGCTCGAGAAGGAA AACCCCATGAGGCTTTAT) containing XhoI restriction site (italics), Shine–Dalgarno sequence (underlined) and initiation codon (bold) and reverse primer (5’CCCAAGCT TAGCGGCTAAAGAGGCTTGCTTG) containing HindIII restriction site (italics) and stop codon (bold). To construct a chimeric gene (BIF gene) encoding the TPS–TPP bifunctional enzyme from Saccharomyces cerevisiae, the full-length TPS1 gene was fused to the three-end part of TPS2 (Miranda et al., 2007). It is known that when the N-terminal 504 amino acids are removed from TPS2-encoded protein, the truncation allele is still fully active as the remaining 392-amino acid carboxy-terminus encompasses the homology blocks present in all TPS enzymes (Vogel et al., 1998). Therefore, we fused a TPS1 1526-bp fragment (encoding 493 amino acids plus two linker amino acids) to a 1353-bp fragment corresponding to the carboxy end of TPS2 (encoding 397 amino acid plus two linker amino acids) to yield a 2873-bp DNA fragment fused through the BamH linker site, which showed a continuous ORF encoding the predicted 892-amino acid sequence after DNA sequencing (Miranda et al., 2007). The 2873-bp TPS1-TPS2 encoding the chimeric translational fusion, here designated BIF gene, was amplified with forward primer (5’CCGCTCGAGAAGGAAAAACCCCATGACTACGGGATAAC) containing XhoI restriction site (italics), Shine–Dalgarno sequence (underlined) and initiation codon (bold) and reverse primer (5’CCGGGTTACCATGGTGGGTTCCAGAC) containing KpnI restriction site (italics) and stop codon (bold). The PCR program used to amplify these sequences with High Expand DNA polymerase (Roche, Indianapolis, IN) was one cycle at 94 °C for 3 min; and 30 cycles of 94 °C for 1 min; 55 °C for 1 min; and 72 °C for 1.5 min. Both genes were checked by DNA sequencing before subcloning in the broad-host-range pBBR1MCS5 vector, which allows gene expression under the control of the lac promotor (Kovach et al., 1995). The pBBR1M::ReOtsA construct containing ReOtsA (bacterial TPS from R. etli) was described previously (Suárez et al., 2008). In the present work, the BIF gene subcloned in pBBR1MCS5 vector was denominated pBBR1M::BIF. Colonies from both plasmid constructs were...
selected on gentamycin (30 μg mL⁻¹). Plasmids were mobilized to \textit{A. brasilense} by conjugation with \textit{E. coli} S17-1 harboring the corresponding construct. Wild-type \textit{A. brasilense} was selected on ampicillin (100 μg mL⁻¹) and selection of transformed \textit{A. brasilense} with either of the described plasmids was selected on gentamycin as well. Standard molecular techniques for DNA digestion, plasmid isolation and bacterial transformation were used (Sambrook & Russell, 2001).

**Stress tolerance tests in \textit{A. brasilense}\**

\textit{Azospirillum brasilense} precultures were grown at 30 °C for 1 day in Luria–Bertani liquid media supplemented with ampicillin (100 μg mL⁻¹) for the wild-type strain, and 30 μg mL⁻¹ gentamycin and ampicillin for strains harboring either pBBRM1::BIF or pBBRM1::ReOtsA plasmid. Cells were spun down and grown to mid-logarithmic phase (0.5 OD₆₀₀ nm) in NFb liquid media with antibiotics. To determine survival following osmotic stress, serial dilutions were plated and grown on Congo red agar medium with different NaCl concentrations (0.1–0.5 M) at 30 °C for 3 days. The surviving colonies were counted afterwards.

**Plant inoculation and stress tolerance tests\**

Maize seeds were surface sterilized in 25% commercial bleach solution (Clorox®; 6% sodium hypochlorite) for 25 min, and then washed three times with distilled sterile water. Maize seeds were germinated on 0.7% water agar plates for 24 h at 29 °C; thereafter, the pregerminated seeds were sown and the seedlings were inoculated on the radicle with 1 mL bacterial suspension (1 × 10⁸ CFU mL⁻¹) in 10 mM MgSO₄·7H₂O. Inoculated seeds were set on pre-viously watered pots with 200 mL of nutritive solution (1 mM K₂HPO₄, 1.5 mM KH₂PO₄, 3 mM NaCl, 1.5 mM CaCl₂, 4.5 mM MgSO₄, 5 mM KNO₃, 40 μM Ca₃(PO₄)₂, 40 μM H₂BO₃, 13 μM MnSO₄·7H₂O, 1.2 μM ZnSO₄, 0.5 μM CuSO₄ and 0.4 μM Na₂MoO₄, pH 6.3) and were allowed to grow for 2 weeks. Thereafter, irrigation was stopped for 10 days and was re-established afterwards. Plant recovery was observed 15 days after rewatering and the relative water content (RWC) was also determined.

**Isolation of \textit{A. brasilense} from maize roots\**

A detached section of maize root from each plant inoculated with the different bacterial strains was rinsed in sterile water and weighed (1–2 g), before adding 10 mM MgSO₄·8H₂O of tissue and shaking vigorously. Decimal dilutions were prepared from resuspended bacteria and plated in Congo red media with the corresponding antibiotic. After 3 days of incubation at 30 °C, the CFU were determined.

**Biomass and water content analyses\**

To determine plant biomass, leaves were separated from roots and oven dried at 65 °C for 3 days and weighed (dry weight). RWC and soil gravimetric water content (SGWC) were determined as reported previously (Gaxiola et al., 2001). Briefly, leaves were excised, and their fresh weight was determined. After floating them in deionized water at 4 °C overnight, their rehydrated weight was determined. Plant RWC was calculated by measuring (fresh weight – dry weight)/(rehydrated weight – dry weight). SGWC was determined in fully watered soil and after 10 days without watering.

**Trehalose determination\**

For trehalose determination, \textit{Azospirillum} strains were grown on NFb liquid medium for 1 day at 30 °C. Cultures were centrifuged, washed with water and resuspended in 80% ethanol. After incubation at 85 °C during 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 HPLC using a ZORBAX carbohydrate analysis column (Agilent Technologies, Santa Clara, CA) eluted with acetonitrile:water (65:35 v/v). Purified trehalose (Sigma Chemical Co.) was used as a standard to determine the concentration.

**Statistical analysis\**

All experiments were repeated at least two times independently unless otherwise stated in the figure legend. The data were processed by ANOVA using Student’s t-test followed by Duncan–Waller mean analysis.

**Results\**

**Trehalose accumulation in \textit{A. brasilense} confers stress tolerance\**

To overaccumulate trehalose in \textit{A. brasilense} we used the broad-host-range pBBR1MCSS vector that allows gene expression under the control of the lac promoter (Kovach et al., 1995). Two plasmids were constructed using this vector as a backbone: the first contained the ReOtsA gene reported previously (Suárez et al., 2008). The other construct consisted of a chimeric translational fusion of TPS and TPP from \textit{S. cerevisiae} that we have tested before in yeast and plants (Miranda et al., 2007).

To determine the possible stress tolerance in \textit{A. brasilense} transformed with trehalose biosynthesis genes, these strains together with \textit{A. brasilense} wild type were grown with different NaCl concentrations. The bacteria with BIF construction or ReOtsA gene grew equally well up to 0.3 M...
NaCl, whereas the wild-type strain could not grow at this salt concentration. At higher NaCl concentrations reaching 0.5 M, the strain harboring BIF gene grew better than \textit{A. brasilense} with ReOtsA gene (Fig. 1a). These results demonstrate that the trehalose biosynthesis genes expressed in \textit{A. brasilense} confer tolerance to osmotic stress, and also shows that the BIF gene is more efficient than ReOtsA.

To further substantiate that tolerance to osmotic stress of the \textit{A. brasilense} recombinant strains is due to the presence of trehalose, the disaccharide concentration of bacteria grown in media with 0.5 M NaCl or without salt was measured. The trehalose levels detected in \textit{A. brasilense} harboring ReOtsA or BIF constructions rose 1.5 and 2 times, respectively, when they were grown under osmotic stress (Fig. 1b), whereas without stress, the trehalose concentration was the same in both cases and increased 1.4 times in comparison with the wild type. This strain displayed basal levels of trehalose without osmotic stress and none was detected after subjecting it to stress because it was unable to grow at 0.5 M NaCl (Fig. 1b). Thus, the increase in trehalose concentration correlated with improved stress tolerance in \textit{A. brasilense} cells.

\textbf{Improvement of drought tolerance in maize inoculated with \textit{A. brasilense} containing trehalose}

With the aim to determine a possible effect of \textit{A. brasilense} recombinant strains on enhancing maize drought tolerance, plants were inoculated with \textit{A. brasilense} harboring ReOtsA or BIF gene. Plants inoculated with \textit{A. brasilense} wild-type strain or noninoculated plants were used as controls. Watering was stopped for 10 days in well-irrigated 2-week-old maize plants. All plants were negatively affected, with the noninoculated plants displaying the most severe wilting symptoms (Fig. 2a). At the end of the drought episode, irrigation was reestablished and plants were allowed to recover for 2 weeks. Maize plants inoculated with either of the different \textit{A. brasilense} strains recovered much better than noninoculated plants (Fig. 2b). Plant survival was higher with those inoculated with \textit{A. brasilense} harboring the BIF
gene, which displayed 85% recovery, twice that of noninoculated plants (Fig. 3a). No significant ($P < 0.05$) survival difference in drought stress tolerance was found between noninoculated plants and those inoculated with either the wild-type strain or bacteria harboring ReOtsA.

RWC content before drought stress shows that all plants had a very similar water status at the beginning of the experiment, whereas 10 days after the stress episode, plants inoculated with A. brasilense containing the BIF gene construct retained more than twice as much water as plants inoculated with ReOtsA strain, or wild-type plants. After stress, practically no water was found in noninoculated maize plants (Fig. 3b). The SGWC was similar for all treatments before stress, and it dropped to 60% after the drought episode in all soil samples (Fig. 3c), indicating that the difference in plant RWC was not due to the soil water status.

**Biomass increase in maize inoculated with A. brasilense containing trehalose**

The root and aerial parts of plants inoculated with the different A. brasilense strains or without inoculation were weighed (Fig. 4a). Plants inoculated with A. brasilense harboring the BIF gene had a significantly increased biomass, 1.7 times greater than that of the noninoculated plants and 1.2 times (nonsignificant) greater than that of plants inoculated with A. brasilense wild type or harboring ReOtsA construct (Fig. 4a). There was no substantial increase in biomass in plants inoculated with the wild-type strain compared with noninoculated controls. These results show that there is a greater increase in maize biomass in both root and aerial parts.
and foliage of *A. brasilense* expressing the BIF gene (Fig. 4a). Additionally, plants inoculated with *A. brasilense* containing the BIF gene exhibited a significantly higher plant height and root length compared with noninoculated plants or plants inoculated with the wild-type strain, or *A. brasilense* carrying the *ReOtsA* gene (Fig. 4b). These results correlate with the previously described increase in biomass using the same bacterial strain. An interesting observation is the remarkable bulky aspect of maize roots inoculated with *A. brasilense* containing the BIF gene compared with plants inoculated with the other strains (Fig. 5a).

To determine if trehalose was translocating from *A. brasilense* to the maize tissues, the trehalose content in maize roots and leaves was determined. No significant levels of the disaccharide could be detected in any of the analyzed tissues from plants inoculated with the different *A. brasilense* strains (data not shown).

To corroborate the presence of the different *A. brasilense* strains in the maize plants after drought stress treatment, a root sample from each plant inoculated with the different strains was taken and grown in Congo red plates with their appropriate antibiotic. *Azospirillum brasilense* (WT, *ReOtsA* or BIF) was recovered in comparable concentrations in all cases, except for noninoculated plants, which had no bacteria attached to their roots (Fig. 5c). This clearly shows that the strains used to inoculate maize plants remained present throughout the experiment.

**Discussion**

Decades of breeding have 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 biosynthesis has been successfully engineered in animal cells and plants to improve stress tolerance (Elbein *et al*., 2003; Avonce *et al*., 2004; Paul *et al*., 2008). In the present work, we developed a novel approach to engineer stress tolerance, namely the overexpression of trehalose biosynthesis genes in *A. brasilense*, which were then used to inoculate maize plants. We used a yeast TPS–TPP bifunctional enzyme for several reasons: this chimeric construct works in distantly related species such as plants (Miranda *et al*., 2007), and it has a strong TPP activity, thus avoiding the accumulation of free trehalose-6-phosphate in the cytosol, which is known to provoke pleiotropic effects (De Virgilio *et al*., 1994; Schluepmann *et al*., 2004). Secondly, among the five trehalose biosynthetic routes, the TPS–TPP pathway is widely distributed in bacteria, fungi, invertebrates and plants, and the corresponding genes display significant identity. Thirdly, at present, there are no homolog genes available in databases from *A. brasilense* or closely related species.

First of all, we showed that *A. brasilense* has an increased tolerance to osmotic stress when it overexpressed either *ReOtsA* or BIF gene; however, the latter was most efficient (Fig. 1). This might be due to the fact that a bifunctional enzyme, although from a distant phylogenetically related organism such as yeast, works much better in *A. brasilense* than TPS alone to make trehalose, probably because there is a higher trehalose-6-phosphate accumulation in *A. brasilense* expressing *OtsA* compared with cells expressing the BIF gene. Also, higher trehalose levels in *A. brasilense* correlated with increased stress tolerance. Similarly, the overexpression of *ReOtsA* in free-living *R. etli* increased its tolerance to osmotic stress (Suárez *et al*., 2008). In contrast, mutation of *ReOtsA* in *R. etli* impaired stress tolerance. Addition of exogenous trehalose to *Bradyrhizobium japonicum* confers tolerance to dehydration stress (Streeter, 2003). Interestingly, in other Alphaproteobacteria also capable of fixing atmospheric nitrogen, *Sinozobium melloti* and *B. japonicum*, three different trehalose biosynthetic routes have been found (Dominguez-Ferreras *et al*., 2006; Cytryn *et al*., 2007). Transcription induction of their corresponding genes correlates with an increased level of trehalose, supporting an
The important role of this disaccharide in desiccation tolerance. A striking result is the overaccumulation of trehalose when the *A. brasilense* strains harboring *ReOtsA* or *BIF* genes were subjected to osmotic stress (Fig. 1). We have observed the same effect in *R. etli* overexpressing trehalose with the same construct harboring *ReOtsA* gene and lacZ promoter (Suárez et al., 2008). This might be due either to the stress induction of the other trehalose biosynthetic pathways or to repression of the trehalose catabolic enzyme, or alternatively it is possible that the lacZ promoter used to overexpress the *BIF* gene in *R. etli* and *A. brasilense* might be stress induced.

We also examined the effects of inoculating maize plants with *A. brasilense* overexpressing either the *ReOtsA* or the *BIF* gene. Our results clearly show that the overexpression of the *BIF* gene in *A. brasilense* interacting with maize plants caused an increased drought tolerance (Figs 2 and 3). As far as we know, this is the first report of engineering drought tolerance in a grass crop by a different method than plant breeding or plant transformation. An intriguing question is whether trehalose is being transported from the bacteria to the plant, as we could not detect the disaccharide in plant tissues. We hypothesized that very small amounts of trehalose translocate to the maize roots and signal stress tolerance pathways in the plant. It is well established that trehalose plays a role as a signaling molecule (Paul et al., 2008). Sugars signal growth, development and stress responses in plants and are considered to be hormone-like molecules (Rolland et al., 2006). Alterations of trehalose-6-phosphate levels have demonstrated that this metabolic intermediate is indispensable for carbohydrate utilization for plant growth (Schluemmann et al., 2003). Arabidopsis plants overexpressing *AtTPS1* are drought tolerant, insensitive to glucose and abscisic acid, and have shown an altered expression of *HXK1* and *ABH4* genes (Avonce et al., 2004).

Another set of interesting results in the present study is a link between trehalose accumulation in *A. brasilense* and a significant increase in plant biomass. Remarkably, larger and bulky maize roots were found in plants inoculated with *A. brasilense* containing the *BIF* gene, suggesting that trehalose plays a role in root growth (Figs 4 and 5). Recently, we reported that inoculation of common bean plants with *R. etli* overexpressing trehalose caused a significant increase in biomass and grain yield (Suárez et al., 2008). A transcriptomics analysis of plant nodules in symbiosis with *R. etli* overexpressing trehalose, revealed induction of several genes involved in stress tolerance, carbon and nitrogen metabolism. However, so far there is no clear evidence as to how bacterial trehalose could regulate these plant genes. Although inoculation with *A. brasilense* Cd increased grain yield through improved utilization of soil moisture (Sarig et al., 1988), our present results show that the overexpression of the *BIF* gene in *A. brasilense* Cd led to an enhancement in aerial and root biomass over the wild-type Cd strain, suggesting that it might also lead to a greater increase in maize crop yield. Therefore, *A. brasilense* engineered with the *BIF* gene is a potential tool to improve drought tolerance, biomass and possibly yield in economically important grass crops.

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**References**


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