Enhanced expression of *Rhizobium etli* cbb$_3$ oxidase improves drought tolerance of common bean symbiotic nitrogen fixation

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Abstract

To investigate the involvement of *Rhizobium etli* cbb$_3$ oxidase in the response of *Phaseolus vulgaris* to drought, common bean plants were inoculated with the *R. etli* strain, CFNX713, overexpressing this oxidase in bacteroids (cbb$_3$) and subjected to drought conditions. The negative effect of drought on plant and nodule dryweight, nitrogen content, and nodule functionality was more pronounced in plants inoculated with the wild-type (WT) strain than in those inoculated with the cbb$_3$ strain. Regardless of the plant treatment, bacteroids produced by the cbb$_3$ strain showed higher respiratory capacity than those produced by the WT strain. Inoculation of plants with the cbb$_3$ strain alleviated the negative effect of a moderate drought on the respiratory capacity of bacteroids and the energy charge of the nodules. Expression of the FixP and FixO components of the cbb$_3$ oxidase was higher in bacteroids of the cbb$_3$ strain than in those of the WT strain under all experimental conditions. The decline in sucrose synthase activity and the decrease in dicarboxylic acids provoked by moderate drought stress were more pronounced in nodules from plants inoculated with the WT strain than in those inoculated with the cbb$_3$ strain. Taken together, these results suggest that inoculation of plants with a *R. etli* strain having enhanced expression of cbb$_3$ oxidase in bacteroids reduces the sensitivity of *P. vulgaris*–*R. etli* symbiosis to drought and can modulate carbon metabolism in nodules.

Key words: Bacteroidal respiration, carbon metabolism, nodules, *Phaseolus vulgaris*, *Rhizobium etli*.

Introduction

Grain legumes such as common bean are important crops in agriculture, accounting for 27% of the world’s primary crop production (Graham and Vance, 2003). One of their main characteristics is the ability to establish symbiotic associations with N$_2$-fixing soil bacteria. Legume cultivation has been continuously losing appeal due to environmental fluctuations such as soil salinity (Delgado *et al.*, 1993; Zahran, 1999), cold (Nayyar *et al.*, 2005), low temperature (Prasad *et al.*, 1994), presence of heavy metals (Sandialio *et al.*, 2001), and drought stress (Arrese-Igor *et al.*, 2011). Drought is the major environmental factor limiting crop production and has a particularly negative impact on symbiotic nitrogen fixation (SNF) (Zahran, 1999; Serraj, 2003; Arrese-Igor *et al.*, 2011). The negative impact of drought on SNF is made up of three different responses: (i) the effects on the infection of legumes by rhizobia; (ii) the effects on nodule growth and development; and finally (iii) the direct effects on nodule functioning. The effects of drought stress on nodule functioning have usually been perceived as a consequence of straightforward physiological responses acting on nitrogenase activity and involving these factors: (i) a certain direct effect on nodule oxygen permeability; (ii) less water for the transport of N-products away from the nodule or feedback regulation by nitrogen.
accumulation; and (iii) the alteration of nodule carbon metabolism (González et al., 1998; Serraj, 2003; Arrese-Igor et al., 2011). The responses of nodule functioning to most environmental constraints can be related to the closure of the oxygen diffusion barrier present in the cortex of nodules, provoking a decrease in oxygen availability for bacteroidal respiration and consequently a lack of energy to support the highly demanding SNF (Minchin et al., 2008).

SNF also depends on the supply of sucrose delivered from the leaves to the nodules. The decline in SNF before any significant change in photosynthesis under drought conditions would appear to rule out any significant role played by plant carbohydrate metabolism in drought-stressed nodules (Vance, 2008). Sucrose can be hydrolysed by either sucrose synthase (SS) or alkaline invertingase. While alkaline invertingase in nodules plays a predominant role in providing hexoses from sucrose, SS produces UDP-glucose for the formation of other nucleotide sugars and polysaccharides, such as starch and cellulose (Morell and Copeland, 1984, 1985). Studies of pea mutants have shown that SS is essential for nodule functioning (Gordon et al., 1999). Using an antisense approach to knock down the nodule-enhanced SS, a similar conclusion has recently been reached in relation to the model legume Medicago truncatula (Baier et al., 2007). SS has been reported to play an important role under environmental constraints, since SS has been shown to be the first nodule enzyme activity to decline under drought conditions in both tropical legumes, such as soybeans (González et al., 1995), and temperate legumes, such as peas (González et al., 1998), leading to reduced availability of organic acids for bacteroids (Gálvez et al., 2005). Using a proteomic approach, this has recently been confirmed for M. truncatula (Larrazanz et al., 2007). It has also been shown that soybean cultivars with different responses to drought also differ in their SS activity (Ladrera et al., 2007).

In the nodule, maintenance of nitrogenase activity is subject to a delicate equilibrium. A high rate of oxygen respiration is necessary to supply the energy demands of the nitrogen reduction process. However, oxygen irreversibly inactivates the nitrogenase complex. These conflicting demands are met by controlling oxygen flux through the oxygen diffusion barrier, which greatly limits permeability to oxygen (Minchin et al., 2008). Oxygen is then delivered to the bacteroids by the plant oxygen carrier, leghaemoglobin, present exclusively in the nodule (Downie, 2005). To cope with the low ambient oxygen concentration in the nodule (10–50 nM O2), nitrogen-fixing bacteroids induce a high-affinity cytochrome cbb3-type oxidase. Genes encoding the cbb3 oxidase complex were isolated initially from rhizobial species and named fixNOQP, given their role in symbiotic N2 fixation (Preisig et al., 1993; Mandon et al., 1994). Several studies have reported that genetically modified rhizobial strains that overproduce the cbb3 oxidase are more efficient in biological nitrogen fixation under optimal conditions compared to their parental strains (Yurgel et al., 1998; Soberón et al., 1999).

Rhizobium etli CNF42, the microsymbiont of common bean, possesses two copies of the fixNOQP operon (fixNOQPd, fixNOQPI), although only fixNOQPd is required for optimal symbiotic nitrogen fixation (Girard et al., 2000). Microaerobic induction of the R. etli fixNOQPd operon is controlled by at least three transcriptional regulators, FixKf, FnrNd, and FnrNchr, belonging to the Crp/Fnr family (Girard et al., 2000; Clark et al., 2001; López et al., 2001). Recently two novel Fnr/Crp-type regulators (StoRd and StoRF, symbiotic terminal oxidase regulators) have recently been described in R. etli CNF42. A mutation in stoRd (strain CNFX713) has also been reported to increase the synthesis of the cbb3-oxidase and the nitrogen fixation capacity of R. etli CNF42 (Granados-Baeza et al., 2007). In this respect, a possible strategy to improve the tolerance of R. etli–Phaseolus vulgaris symbiosis to drought could be the inoculation of plants with stoRd mutant strain CNFX713. The aim of this study is to evaluate the ability of strain CNFX713, exhibiting overexpression of the cbb3-oxidase, to improve the drought tolerance of symbiotic nitrogen fixation in P. vulgaris plants.

Materials and methods

Bacterial material, growth conditions, and drought treatment

Seeds of common bean (P. vulgaris, var. Negro jamapa) were surface sterilized in 96% (v/v) ethanol for 30 s, in 5% sodium hypochlorite for 5 min and then thoroughly washed five times with sterilized water (Delgado et al., 1994). The sterilized seeds were incubated for 2 h in sterile distilled water in darkness. The imbibed seeds were placed on plates containing 1% water-agar and were allowed to germinate at 30 ºC for 60 h. Three days after sowing, selected uniform seedlings were planted in sterile 2-kg pots (four/pot) containing a vermiculite/sand mixture (1:1, v/v) as substrate. Each seedling was inoculated with 1 ml of a single bacterial strain (1010 cells ml-1). Plants were grown in controlled environmental chambers (16/8 light/dark cycle, 25/19 ºC, photosynthetic photon flux 400 μmol m-2 s-1 and relative humidity 60–70%) (Peraltá et al., 2004). Plants were alternately watered with N-free mineral solution (Rigaud and Puppo, 1975) and water. When the plants were 3 weeks old, they were randomly separated into control and drought stress sets. Drought stress was applied by withholding water for 5–7 days (moderate drought) until the plants reached a leaf water potential (Ψw) of –1.5 ± 0.25 MPa, and for 10–12 days (severe drought), reaching values of –2.5 ± 0.3 MPa. Control plants were supplied daily with water to field capacity and they had a leaf Ψw of –1.0 ± 0.4 MPa. Each water stress treatment had its own control, since different time periods were required in order to achieve a given plant water potential. Leaf Ψw was measured in the first fully expanded leaf using a C52 sample chamber connected to a HR-33T psychrometer (Wescor, Logan UT, USA).

Plant and nodule biomass and nitrogen content

Plant and nodule dryweight were determined after drying fresh plant material that was heated at 60 ºC for 48 h. Total plant N was determined by the Kjeldahl method as described by Brouat and Crouzet (1965).

Leghaemoglobin content

Leghaemoglobin content was measured as previously described by LaRue and Child (1979). Essentially, nodules (0.3 g) were ground with 4 ml Lb extraction buffer (40 mM Na2HPO4, 2H2O, pH 7.4; 10 mM NaH2PO4, H2O, pH 7.4; 0.02% K3Fe(CN)6; 0.1% NaHCO3) supplemented with 0.1 g polyvinylpyrrolidone (PVPP). The homogenate was centrifuged at 12,000 g at 4 ºC for 20 min to retain
the supernatant. Clear supernatant (50 µl) and saturated oxalic acid (3 ml) were mixed in screw-capped tubes, which were sealed and autoclaved for 30 min at 120 °C and then allowed to cool to room temperature. The fluorescence of the solutions was measured using a Shimadzu spectrophotofluorometer (Shimadzu Scientific Instruments, Kyoto, Japan) equipped with a mercury-xenon lamp and a RF-549 red-sensitive photomultiplier. The excitation wavelength was 405 nm and the emission monochromator setting was 650 nm. The difference in fluorescence between heated and unheated samples was proportional to haem protein content.

Bacteroid fractionation and haem c staining
Bacteroids were prepared as previously described (Mesa et al., 2004). Briefly, 1.5 g of fresh nodules was ground in 7.5 ml TRIS-HCl (pH 7.5) supplemented with 250 mM mannitol. The homogenate was filtered through four layers of cheesecloth and was centrifuged at 250 g at 4 °C for 5 min to remove nodule debris. The resulting supernatant was centrifuged twice at 12,000 g at 4 °C for 10 min and was washed twice in 50 mM potassium phosphate buffer (pH 7). Bacteroids were resuspended in 3 ml of 50 mM potassium phosphate buffer (pH 7) containing 100 µM 4-amidino phenylmethanesulphonyl fluoride (PMSF), RNase (20 µg ml⁻¹), and DNase I (20 µg ml⁻¹). Cells were disrupted using a French pressure cell (SLM Aminco, Jessup, MD, USA). The cell extract was centrifuged at 20,000 g for 20 min to remove unbroken cells and the supernatant was then centrifuged at 140,000 g for 1 h. The membrane pellet was resuspended in 100 µl of the same buffer. Membrane protein aliquots (30 µg) were diluted in the sample buffer (124 mM TRIS-HCl, pH 7.0; 20% glycerol; 4.6% sodium dodecyl sulphate (SDS); and 50 mM 2-mercaptoethanol), incubated at room temperature for 10 min, separated at 4 °C in SDS/12% polyacrylamide gel electrophoresis, transferred to a nitrocellulose filter, and stained for haem-dependent peroxidase activity by chemiluminescence (Vargas et al., 1993) using the detection kit Super Signal (Pierce, Thermo Fisher Scientific, IL, USA).

Bacteroidal O₂ consumption
Bacteroidal respiration was measured by incubating bacteroids in the chamber of an O₂ electrode (Hansatech, Norfolk, England) as previously described by Delgado et al. (1993). The incubation medium contained 2 ml of 25 mM phosphate buffer (pH 7.5) at 28 °C, and a bacteroidal preparation (1–2 mg protein) without adding any exogenous substrate. The time taken to consume the oxygen present in the system was used to calculate the rate of oxygen consumption.

Sucrose synthase activity
Sucrose synthase (SS) activity was determined according to Wright et al. (1998). Nodules (200 mg) were homogenized in liquid nitrogen by using a mortar and pestle with 50 mM HEPES-KOH (pH 8), 0.1 mM PMSE, 1 mM DTT, 2 mM EDTA, 20 mM MnCl₂, 1 mM benzenzidime, 5 mM MgCl₂, and 1 mM CaCl₂. The homogenate was centrifuged at 15,700 g for 15 min at 4 °C. Samples up to 20 µl were incubated at 37 °C for 30 min in a reaction mixture containing 50 mM HEPES-KOH (pH 7.5), 1 mM MgCl₂, 400 mM sucrose, and 5 mM UDP in a total volume of 100 µl. After incubation, the reaction was stopped by heating at 95 °C for 4 min. SS activity was analysed after addition of 100 µl 10 mM NAD, 5 µl of UDP-glucose dehydrogenase (0.01 units), and 793 µl of 200 mM glucose-KOH (pH 8.9). NADH produced from NAD+ reduction was monitored at 340 nm for 30 min using a Shimadzu UV-1603 spectrophotometer. The protein concentration was estimated by using the Bio-Rad assay, with a standard curve of varying bovine serum albumin concentrations.

Sucrose content
Sucrose content was measured in nodule cytosol according to González et al. (1995). Carbohydrates were extracted from frozen nodules (200 mg) with trichloroacetic acid, followed by further washing with diethyl ether. After centrifugation at 15,700 g for 20 min at 4 °C, samples (up to 5 µl) were incubated in a microplate reader with a reaction mixture containing 200 µl buffer (100 mM HEPES, pH 7.3; 1 mM MgCl₂; 0.75 mM NADP; 0.85 mM ATP; 0.1 units glucose-6-phosphate dehydrogenase and 0.1 units hexokinase). Sucrose was measured spectrophotometrically at 340 nm, after adding invertase (20 units per well) to the reaction mixture, by determining the production of NADPH.

Organic acids and adenylate energy charge analyses
Organic acid and adenylates (ATP, ADP, and AMP) were extracted from nodules (0.2 g) in 5% w/v trichloroacetic acid, and samples were processed as described by Galvez et al. (2005). Malate, citrate, succinate, and 3-ketogluutarate were quantified using ion chromatography in a DX-500 system ( Dionex, Sunnyvale, CA, USA) through gradient separation with an IonPac AS11 column (Dionex) according to the method recommended by the supplier (2.5 mol m⁻³ NaOH/18% methanol to 45 mol m⁻³ NaOH/18% methanol for 15 min).

The adenylate energy charge (AECh) was calculated as (ATP + 0.5 ADP)/(ATP + ADP + AMP), was evaluated by measuring the proportion of each adenylate using high-performance capillary electrophoresis in a PACE system 5500 (Beckman Instruments, Fullerton, CA, USA). The electrodyes used were 20 mM TRIS and 20 mM Na₂HPO₄·H₂O pH 7, containing 100 mM DTAB and 1 mM EDTA. The potential applied was 30 kV. A capillary of 50 µm in diameter and 62/69 cm in length and a UV detector at 260 nm was used. Quantification was performed by interpolation with AMP, ADP, and ATP standards in a concentration range of 0.005–0.1 mM.

Transcript levels
Total RNA was extracted from nodules harvested from the different treatments using the RNeasy Plant Mini Kit (Qiagen), treated with DNasel Amplification Grade (Invitrogen) and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen). For each RNA sample, control experiments, in which the reverse transcriptase step was omitted, were carried out to confirm the absence of any residual DNA. The complementary DNA was amplified with the specific primers FwSSPv (5’-GCCAGGTGCCCTCTGTAACCG-3’) and RwSSPv (5’-CTTGAAGATCCATTGGCAAC-3’) for the P₇SSn gene and FwActinPv (5’-GTATGTGTCACCTAGTTTCTT-3’) and RwActinPv (5’-CGACCCGCAAGATCAAGACGAGAGA-3’) for the P₇Actin gene, according to Silvante et al. (2003) and Valdés-López et al. (2008). The initial denaturation stage was at 95 °C for 5 min, followed by 30 cycles of amplification (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min) and a final elongation stage at 72 °C for 5 min. PCR products were analysed by electrophoresis in agarose gels.

Statistical analysis
The total numbers of replicates are given in each figure. For each parameter, results were analysed by one-way ANOVA, and means were separated using the Tukey HSD test at P ≤ 0.05 with SPSS software.

Results

Plant growth and symbiotic nitrogen fixation
Plants subjected to drought stress by stopping irrigation for 5–7 days (moderate drought) reached a water potential (Ψw)
of about –1.5 ± 0.25 MPa. When watering was stopped for 10–12 days (severe drought), \( \Psi_w \) reached values of about –2.5 ± 0.3 MPa. Control plants maintained a leaf \( \Psi_w \) of –1 ± 0.4 MPa. The effect of either moderate or severe drought stress on the leaf \( \Psi_w \) of plants inoculated with the CFNX713 (cbb\(_5\)) strain was similar to that of plants inoculated with the wild-type (WT) strain (data not shown).

A moderate drought provoked a decrease of about 33% in the plant dryweight (PDW) of plants inoculated with the WT strain (Table 1). In plants inoculated with the cbb\(_5\) strain and subjected to moderate drought, PDW fell by only 24% compared to control plants. Specific N content was reduced by moderate drought in plants inoculated with the WT strain (Table 1) (by 38% compared to control plants). However, this parameter was not significantly affected in plants inoculated with the cbb\(_5\) strain (Table 1). After a severe drought, a 60% and 42% reduction in PDW and N content, respectively, was observed in plants inoculated with the WT strain, while a reduction of only 21% and 15%, respectively, was observed in those inoculated with the cbb\(_5\) strain compared to control plants. Similarly, plants inoculated with the WT strain and subjected to moderate drought showed a reduction of about 58% in total nitrogen (TN) content compared to control plants. This parameter fell drastically under severe drought (by 76% as compared to control plants) (Table 1). However, in plants inoculated with the cbb\(_5\) strain, the decrease in TN was only about 35% when plants were subjected to moderate or severe drought as compared to control plants (Table 1).

The effect of inoculation with the WT or cbb\(_5\) strain on the nodule number (NN) and nodule dry weight (NDW) of plants subjected to water deficit was also assessed. Regardless of the plant treatment, no significant differences in NN were observed between plants inoculated with the WT and cbb\(_5\) strains (data not shown). Conversely, NDW per plant was negatively affected by moderate water deficit, with values being more drastically reduced for plants inoculated with the WT strain (39%) than those of plants inoculated with the cbb\(_5\) strain (23%) as compared to control plants. A sharper decrease in NDW was observed in plants subjected to severe stress and inoculated with the WT (69%) or cbb\(_5\) (62%) strain as compared to control plants (Table 2). These results indicate that inoculation of plants with the cbb\(_5\) strain produced a positive effect on nodule growth as compared to plants inoculated with the WT strain either in plants subjected to normal irrigation or drought conditions.

The leghaemoglobin (Lb) content of the nodules as an estimation of nodule functionality was also measured (Table 2). Independently of the plant treatment, inoculation of plants with the cbb\(_5\) strain enhanced Lb content compared to those plants inoculated with the WT strain. Similarly, Granados-Baeza and colleagues (2007) have reported that inoculation of common bean plants with R. etli CFNX713 enhanced nitrogen-fixing capacity of nodules. Moderate drought provoked a significant decline in Lb content of approximately 42% in plants inoculated with the WT strain compared to control plants (Table 2). By contrast, this parameter was not affected in plants inoculated with the cbb\(_5\) strain and subjected to a moderate drought. When plants were subjected to severe drought, a negative effect on Lb content was observed which was more pronounced in plants inoculated with the WT strain than in those inoculated with the cbb\(_5\) strain (52% versus 26%, respectively) (Table 2).

### Bacteroidal respiratory capacity

Bacteroids of the cbb\(_5\) strain expressed greater respiratory capacity than WT bacteroids after isolation from the nodules of plants grown under either control or stress.

### Table 2. Nodule dry weight and leghaemoglobin of plants inoculated with R. etli CFN42 (WT), and CFNX713 (cbb\(_5\))

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Moderate drought</th>
<th>Severe drought</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PDW N TN</td>
<td>NDW Lb</td>
</tr>
<tr>
<td>WT</td>
<td>Control</td>
<td>0.125 ± 0.02(a) 24 ± 0.3(a) 13 ± 0.2(a)</td>
<td>0.134 ± 0.03(b) 57 ± 3(b) 72 ± 6(b)</td>
</tr>
<tr>
<td></td>
<td>Stress</td>
<td>0.076 ± 0.03(c) 15 ± 0.3(c) 5.4 ± 0.1(c)</td>
<td>0.041 ± 0.04(a) 33 ± 2(a) 34 ± 2(a)</td>
</tr>
</tbody>
</table>
| cbb\(_5\) | Control | 0.168 ± 0.03\(a\) 85 ± 4\(a\) 15.5 ± 0.1\(a\) | 0.181 ± 0.03\(a\) 91 ± 3\(a\)
|        | Stress     | 0.130 ± 0.02\(a\) 85 ± 3\(a\) 0.069 ± 0.02\(a\) 67 ± 6\(a\) |

### Table 1. Plant dry weight, specific nitrogen content, and total nitrogen content of plants inoculated with R. etli CFN42 (WT), or R. etli CFNX713 (cbb\(_5\)) strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Moderate drought</th>
<th>Severe drought</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PDW N TN</td>
<td>NDW Lb</td>
</tr>
<tr>
<td>WT</td>
<td>Control</td>
<td>0.54 ± 0.07(a) 24 ± 0.3(a) 13 ± 0.2(a)</td>
<td>0.69 ± 0.03(a) 24 ± 0.6(a) 16.5 ± 0.2(a)</td>
</tr>
<tr>
<td></td>
<td>Stress</td>
<td>0.36 ± 0.02(a) 15 ± 0.3(a) 5.4 ± 0.1(a)</td>
<td>0.28 ± 0.06(a) 14 ± 0.1(a) 4 ± 0.1(a)</td>
</tr>
<tr>
<td>cbb(_5)</td>
<td>Control</td>
<td>0.62 ± 0.05(a) 25 ± 0.2(a) 15.5 ± 0.1(a)</td>
<td>0.76 ± 0.07(a) 32 ± 0.7(a) 24 ± 0.5(a)</td>
</tr>
<tr>
<td></td>
<td>Stress</td>
<td>0.47 ± 0.07(a) 21 ± 0.3(a) 10 ± 0.2(a)</td>
<td>0.69 ± 0.04(a) 27 ± 0.1(a) 16 ± 0.4(a)</td>
</tr>
</tbody>
</table>

Plants were subjected to moderate or severe drought by stopping irrigation for 5–7 or 10–12 days, respectively. Within columns, values followed by the same lower-case letter are not significantly different as determined by the Tukey HSD test at \( P \leq 0.05 \) (\( n = 9 \)). PDW, plant dryweight (g plant\(^{-1}\)); N, specific nitrogen content (mg (g plant\(^{-1}\))); TN, total nitrogen content (mg plant\(^{-1}\)).
conditions (Fig. 1a). A moderate drought decreased the respiratory capacity of WT bacteroids by about 39% compared to the respiration levels observed in bacteroids isolated from control plants. By contrast, the respiratory capacity of cbb\textsuperscript{3} bacteroids from plants subjected to moderate drought increased by about 13% compared to that observed in bacteroids from control plants (Fig. 1a). A severe drought reduced the respiratory capacity of WT and cbb\textsuperscript{3} bacteroids by about 40% and 20%, respectively (Fig. 1a).

After haem c staining analysis of proteins from bacteroid membranes, two bands, of 32 and 27 kDa, were detected (Fig. 1b). These proteins were previously identified as the FixP and FixO components of the cbb\textsubscript{3} terminal oxidase of \textit{R. etli} (Granados-Baeza et al., 2007). As previously reported by Granados-Baeza and colleagues (2007), bacteroids of the cbb\textsuperscript{3} strain showed an increase of about 60% in the expression of FixP and FixO compared to those of the WT strain after their isolation from control plants (Fig. 1b, c). Similarly, under moderate drought conditions, expression of FixP and FixO proteins was about 40% higher in cbb\textsuperscript{3} bacteroids than in WT bacteroids (Fig. 1b, c). Interestingly, both WT and cbb\textsuperscript{3} bacteroids responded to a moderate drought by increasing the expression of FixP and FixO by about 40% and 20%, respectively, compared to that of bacteroids isolated from control plants (Fig. 1b, c).

Under moderate drought conditions, the adenylate energy charge (AEC) in WT nodules was found to be significantly lower (0.38 ± 0.03) than with normal irrigation (0.54 ± 0.06) (Fig. 1d). By contrast, the AEC was not affected by a moderate drought in nodules produced by the cbb\textsuperscript{3} strain and remained at 0.60 ± 0.01. A significant decrease in AEC was observed in nodules from plants subjected to severe drought stress and inoculated with any of the strains (Fig. 1d).

Sucrose synthase activity, gene expression, and carbon metabolites

As shown in Fig. 2a, inoculation of plants with the cbb\textsuperscript{3} strain resulted in increased SS activity in the nodules compared to those from plants inoculated with the WT strain either under control or moderate drought conditions. A moderate drought caused a significant decline in the SS activity of nodules, which was more pronounced in those produced by the WT strain (49%) than in those produced by the cbb\textsuperscript{3} strain (only 15%) compared to control plants (Fig. 2a). A severe drought provoked a dramatic decline in SS activity in nodules produced by the WT and cbb\textsuperscript{3} strains (71% and 67%, respectively, compared to control plants) (Fig. 2a). The decline in SS activity observed in nodules subjected to a moderate drought correlated with the accumulation of sucrose, which was higher in WT nodules than that observed in cbb\textsuperscript{3} nodules (60% versus 21%, respectively, compared to control plants) (Fig. 2b).

Nodules produced by the cbb\textsuperscript{3} strain showed approximately a 1.8-fold increase in expression of the \textit{Pv}SSn gene compared to those produced by the WT strain after isolation from control plants (Fig. 2c, d). Similarly, in plants subjected to a moderate drought, expression of the \textit{Pv}SSn gene in cbb\textsuperscript{3} nodules was about 1.6 times higher than that observed in WT nodules (Fig. 2c, d). In both WT and cbb\textsuperscript{3} nodules, a moderate drought provoked only a slight decrease in \textit{Pv}SSn gene expression (Fig. 2c, d).

The application of moderate drought stress produced a decline in malate content in nodules which was more marked in WT nodules (41%) than that observed in nodules produced by the cbb\textsuperscript{3} strain (23%) compared to control nodules (Fig. 3a). A moderate drought also provoked a decline in the nodule content of other dicarboxylic acids, such as \textit{z}-ketoglutarate, succinate, and citrate, and was more significant in nodules produced by the WT strain than in those produced by the cbb\textsuperscript{3} strain compared with the values observed for control nodules (Fig. 3b, c, and d, respectively). Severe drought had a dramatic negative effect
on malate content in nodules formed by the WT and \(cbb^3\) strains (reductions of 57% and 54%, respectively) compared to control plants (Fig. 3a). Under severe drought conditions, sharp declines in \(\alpha\)-ketoglutarate, succinate, and citrate content in WT and \(cbb^3\) nodules were also observed (Fig. 3 b, c, and d, respectively).

**Discussion**

Oxygen is critical for SNF since it is a high energy-consuming process that occurs under extremely low \(O_2\) concentrations, due to the sensitivity of the nitrogenase complex to \(O_2\). Therefore, symbiosis requires a respiratory chain with a high affinity for oxygen, which is closely coupled to ATP production. This requirement is fulfilled in bacteroids by a special three-subunit terminal oxidase, the cytochrome \(cbb_3\) oxidase encoded by the \(fixNOQP\) operon (Preisig et al., 1993). *R. etli* CFN42 contains two copies of the \(fixNOQP\) genes, although only the copy located on the symbiotic plasmid (\(fixNOQP_d\)) is required in order to establish an effective symbiosis (Girard et al., 2000). In previous studies, isolation of *R. etli* mutants with derepressed expression of cytochrome terminal oxidases produced more nitrogen fixation during symbiosis than did the wild type (Miranda et al., 1996; Silvente et al., 2002). More recently, it has been reported that inactivation of the *R. etli* Crp/Fnr-type transcriptional regulator StoRd (strain CFNX713) increases \(cbb_3\) expression and showed an enhanced nitrogen fixation capacity (Granados-Baeza et al., 2007). One of the nodule responses to environmental constraints and, in particular, to drought is the closure of...
the oxygen diffusion barrier and a decrease in oxygen flux in nodules, resulting in the limitation of oxygen availability for bacteriodal respiration. In this context, inhibition of the respiratory capacity of bacteriods has been observed in soybean plants subjected to drought as compared with unstressed plants (Díaz del Castillo and Layzell, 1995). Similarly, the inhibitory effect of NaCl on SNF by pea nodules has been associated with a decrease in bacteriodal respiration (Delgado et al., 1993, 1994). According to these observations, the results of this study demonstrate that drought inhibits the respiratory capacity of bacteriods from common bean nodules produced by R. etli CFN42.

In M. truncatula–Sinorhizobium meliloti (Aydi et al., 2004) and Cicer arietinum–Mesorhizobium ciceri (L’taief et al., 2007), it has been reported that plant tolerance to salinity is associated with higher nodule conductance and oxygen uptake capacity. Thus, inoculation with rhizobia strains having a greater respiratory capacity could be a promising strategy to improve symbiotic nitrogen fixation tolerance to drought. In this study, it has been demonstrated for the first time that inoculation of plants with R. etli CFNX713, which overexpresses the cbb3 oxidase, alleviated the negative effect of drought on the respiratory capacity of bacteriods and increased the tolerance of symbiotic nitrogen fixation to drought.

Contrary to the drought effect on the respiratory capacity of bacteriods, an increase in the expression of the FixP and FixO components of the cbb3 oxidase was observed as a response to a moderate drought. Since R. etli fixNOQPd genes are induced by low oxygen conditions (Girard et al., 2000), a decrease in oxygen diffusion to nodules caused by drought may increase the expression of these genes. In spite of the induction of cbb3 expression in nodules subjected to moderate drought, the respiratory capacity of bacteriods isolated from these nodules and incubated under nonlimited oxygen conditions was inhibited. This suggests that, in addition to oxygen, other metabolic constraints such as carbon availability for respiration must be involved in the negative effect of drought on bacteriodal respiratory capacity.

In this study, the inhibition of SS and accumulation of sucrose were observed in common bean nodules in response to moderate or severe drought, confirming the findings of previous studies. Inoculation with the cbb3+ strain alleviated the negative effect of moderate drought on carbon metabolism. Environmental constraints may regulate SS functioning by downregulating SS gene expression, leading to a decline in SS activity (González et al., 1995, 1998; Gordon et al., 1997; Gálvez et al., 2005). In P. vulgaris, two SS genes have been identified (Silvente et al., 2003). One of these genes is expressed almost exclusively in nodules (PvSSn) and the other is expressed in all the tissues tested (PvSS). It has been shown that expression of PvSSn is dependent on C availability and is mediated by the status of nitrogen metabolism components in bean nodules (Silvente et al., 2003). The present study demonstrates that inoculation of common bean with strain CFNX713, which overexpresses cbb3 oxidase, induces the expression of the PvSSn gene in nodules. In line with these results, it has previously been reported that inoculation of P. vulgaris plants with a R. etli mutant (CFN037) having increased respiratory capacity produces an induction of SS activity (Silvente et al., 2002). In the present study, the decrease in SS activity observed in WT nodules in response to drought did not correlate with the PvSSn transcript levels. These results suggest that the negative effect of drought on the expression of nodule SS in P. vulgaris may occur at post-transcriptional levels. It has previously been suggested that regulation of nodule SS by the cellular redox state occurs at both the transcriptional and post-translational levels, which would confirm the present findings (Marino et al., 2008).

Sucrose is cleaved in mature nodules to produce UDP-glucose and -fructose, which, after phosphorylation by hexokinases, enter the glycolytic or oxidative pentose phosphate pathways and are metabolized to phosphoenolpyruvate (PEP), which is converted to malate via the combined action of PEP carboxylase (PEPC) and malate dehydrogenase. It has been suggested that the sharp decline in SS activity following drought in soybean nodules produces a subsequent potential decrease in the glycolytic flux (González et al., 1995; 1998). In pea plants, it has also been shown that the levels of hexoses, as well as malate, decrease in water-stressed nodules compared to unstressed nodules (Gálvez et al., 2005). Similarly, the present study has shown a dramatic decline in nodule malate content in drought-stressed nodules. Malate deprivation under drought conditions could be due to the inhibition of sucrose synthase activity or to impaired HCO3 binding to PEP to form oxalacetate and, subsequently, malate. However, analysis of PEPC activity indicates that this enzyme activity was not affected by drought (data not shown). Although it has been proposed that both PEPC (Schulze, 2004; Nomura et al., 2006) and carbonic anhydrase activities (Coba de la Peña et al., 1997; Gálvez et al., 2000) are essential for nodule performance, it remains to be demonstrated whether they play any significant role in SNF regulation under drought conditions.

Taken together, these results clearly demonstrate that inoculation of common bean with a strain overexpressing the cbb3 oxidase, and therefore having increased respiratory capacity, confers greater drought tolerance of symbiotic nitrogen fixation. It is also tempting to suggest that higher cbb3 expression, which leads to more efficient oxygen consumption, in turn provokes higher C-skeleton demand. This demand is supported by an induction of SS expression in nodules which consequently increases the levels of malate availability to bacteriods.

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