Evidence for Transcriptional and Post-Translational Regulation of Sucrose Synthase in Pea Nodules by the Cellular Redox State

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Nitrogen fixation (NF) in legume nodules is very sensitive to environmental constraints. Nodule sucrose synthase (SS; EC 2.4.1.13) has been suggested to play a crucial role in those circumstances because its downregulation leads to an impaired glycolytic carbon flux and, therefore, a depletion of carbon substrates for bacteroids. In the present study, the likelihood of SS being regulated by oxidative signaling has been addressed by the in vivo supply of paraquat (PQ) to nodulated pea plants and the in vitro effects of oxidizing and reducing agents on nodule SS. PQ produced cellular redox imbalance leading to an inhibition of NF. This was preceded by the downregulation of SS gene expression, protein content, and activity. In vitro, oxidizing agents were able to inhibit SS activity and this inhibition was completely reversed by the addition of dithiothreitol. The overall results are consistent with a regulation model of nodule SS exerted by the cellular redox state at both the transcriptional and post-translational levels. The importance of such mechanisms for the regulation of NF in response to environmental stresses are discussed.

Additional keywords: carbon metabolism, *Pisum sativum* L.

Nitrogen fixation (NF) in nodule legumes is an energy-demanding process that ultimately depends on photosynthate supply that is transported from shoots via the phloem, mainly in the form of sucrose. Hexoses and hexose-phosphates derived from sucrose cleavage are metabolized through the glycolytic pathway to phosphoenolpyruvate, which is converted to malate via the combined action of phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) and malate dehydrogenase (MDH; EC 1.1.1.82). Multiple evidence supports the idea that C4-dicarboxylates are the main products of sucrose breakdown supplied to bacteroids to fuel NF (Lodwig and Poole 2003). Nodule NF has been shown to be more sensitive to environmental constraints than photosynthetic CO₂ assimilation (Durand et al. 1987). This nodule activity decline has been

related to an impairment of sucrose cleavage activity which, together with the maintenance of sucrose phloem flux, leads to an accumulation of sucrose in nodules (González et al. 1995) and a depletion of malate (Gálvez et al. 2005). Thus, sucrose cleavage has been revealed to be a critical regulation point for nodule metabolism and, therefore, NF.

Enzymes that degrade sucrose are classified either as hydrolases (invertases) or glucosyltransferases (sucrose synthase [SS]). Invertases (β -D-fructofuranosidase) hydrolyze sucrose to glucose and fructose, whereas SS catalyses the readily reversible reaction $\text{sucrose} + \text{UDP} + \text{H}^+ \rightleftharpoons \text{fructose} + \text{UDP-glucose}$, although it is thought to function primarily in the direction of sucrose cleavage in plant sink tissues, which have a high demand for carbon in biosynthetic and respiratory pathways (Winter et al. 1997). There is general agreement that acid invertase is unlikely to be responsible for the nodule metabolism of sucrose (Gordon et al. 1999). Indeed, acid invertase transcripts have not been identified from nodule expressed sequence tag libraries and only very low acid invertase activities have been detected in this organ (Flemetakis et al. 2006). Sucrose may be cleaved in nodules by either alkaline invertase (AI; EC 3.2.1.26) or SS. Early reports suggested AI to be the predominant sucrose-cleaving enzyme in developing soybean nodules, although both activities contributed to the cleavage of sucrose at maturity (Morell and Copeland 1984). Also, enzyme kinetic data suggested that partitioning of sucrose between both reactions might be favored by AI, because of a higher affinity, whereas SS may provide UDP-glucose for the formation of other nucleotide sugars and polysaccharides (Morell and Copeland 1985). Moreover, after digestion of nodule slices to separate cortical and central tissues, SS activity was not detected in the central tissues (Copeland et al. 1989). However, there is now a wealth of evidence that suggests that SS is the prevailing activity in sucrose cleavage in nodules of most, if not all, legume species studied so far. This evidence for a major role of SS in nodule metabolism is provided by its identification as a major nodule-enhanced protein (nodulin-100) in soybean (Thummler and Verma 1987) and *Medicago truncatula* root nodules (Hohnjec et al. 1999) and the fact that the levels of SS protein and RNA are higher in the N₂-fixing nodules than in the other tissues of the legume plants (Hohnjec et al. 2003). Moreover, the supply of 1'-fluorosucrose, which is an extremely poor substrate for AI but can be cleaved by SS

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(Hitz et al. 1985), suggest that, in young nodules, SS is almost entirely responsible for sucrose metabolism and even represents more than 70% in older nodules (Harrison 1995). Finally, mutations at the *rug4* locus of pea lie in a gene encoding SS, resulting in the loss of most of the nodule SS activity, showed that SS is essential for nodule functioning (Craig et al. 1999; Gordon et al. 1999). Using an antisense approach to knock down the nodule-enhanced SS, a similar conclusion recently was reached for the model legume *M. truncatula* (Baier et al. 2007).

Barratt and associates (2001) described three SS isoforms (Sus1, Sus2, and Sus3) and their corresponding coding sequences (*PsSus1*, *PsSus2*, and *PsSus3*) having distinct patterns of expression in different organs of the plant and during organ development in pea plants, but mentioned hints for five different genes encoding SS-like proteins, corresponding to the situation in *M. truncatula* (Hohnjec et al. 2003). Currently, there is evidence for six genes encoding SS proteins in *Arabidopsis thaliana* (Bieniawska et al. 2007), where different isoforms have been suggested to carry out distinct metabolic functions, because they are strongly differentially expressed: spatially, developmentally, and in response to low temperature, anoxia, or carbon availability (Marana et al. 1990; Martin et al. 1993; Silvente et al. 2003; Zeng et al. 1998). Moreover, SS can be post-translationally modified by phosphorylation in vivo and in vitro and different functions have been proposed for this modification, such as changes in catalytic properties, intracellular distribution, and targeting for degradation (Hardin et al. 2004; Komina et al. 2002). A very recent report suggests that sucrose concentration and phosphorylation may regulate SS oligomerization and F-actin association in maize kernels and seedlings (Duncan and Huber 2007).

Nodule SS function also has been shown to be relevant under environmental constraints, because SS has been shown to be the first nodule enzyme activity that declines under drought in both tropical legumes, such as soybean (González et al. 1995), and temperate legumes, such as pea (González et al. 1998) and also is responsive to other abiotic stresses (Gordon et al. 1997), leading to a reduced availability of organic acids to bacteroids (Gálvez et al. 2005). This fact has been confirmed recently in a proteomic approach performed in nodules of the model legume *M. truncatula* (Larrainzar et al. 2007) and it has been shown that soybean cultivars with contrasting responses to drought also differ in their SS activity behavior (Ladrera et al. 2007).

Little is known so far about the signaling pathways that link stress perception and the decline in SS activity, but it has been shown that SS responses to environmental constraints are independent of ABA-mediated signals in both *Arabidopsis* (Déjardin et al. 1999) and pea nodules (González et al. 2001). Recent studies, using split-root systems, have shown that NF and SS activity only declined in the water-deprived, half-root system, suggesting a local rather than a systemic control for NF under drought with some indication of an involvement of cellular redox imbalance (Marino et al. 2007b). Indeed, it has been shown that drought effects on carbon and nitrogen metabolism of pea nodules can be mimicked by paraquat (PQ) (Marino et al. 2006). Although it still remains unclear which signal transduction pathway could be responsible for regulating SS under abiotic stresses, these studies raised the issue that, in addition to the oxidative damage already described by other authors under environmental constraints, a likely regulation mechanism mediated by reactive oxygen species (ROS) signaling may occur at an earlier stage.

Nodules have a great capacity to produce ROS due to their high respiration rates, the high concentration of leghemoglobin in the cytosol of infected cells, the abundance of catalytic Fe,

and the presence of several redox proteins with the capacity to transfer electrons to O₂ (Becana et al. 2000). The equilibrium between production and scavenging of ROS may be perturbed by environmental constraints, such as water stress, pathogens, high irradiance, or high temperatures carrying out oxidative stress and cell damage (Apel and Hirt 2004). Prior to these deleterious effects, an imbalance between production and scavenging of ROS may lead to oxidative signaling (Foyer and Noctor 2005). Cell redox state is known to be involved in signaling processes mediating responses to several stimuli (Buchanan and Balmer 2005; Neill et al. 2002) such as stomatal closure (Pei et al. 2000) or programmed cell death (Beers and McDowell 2001). Moreover, ROS produced in different subcellular compartments influence the expression of a large number of genes (Neill et al. 2002). A microarray study in *Arabidopsis* showed that the expression of 1 to 2% of genes was altered by H₂O₂ (Desikan et al. 2001). An involvement of H₂O₂ in the regulation of NF also had been theoretically suggested (Minchin 1997), likely associated with increases in internal oxygen concentration of nodules and, indeed, H₂O₂ has been detected in nodules associated with nodule formation (Santos et al. 2001) and senescence (Alesandrini et al. 2003; Escuredo et al. 1996; Evans et al. 1999).

In the present study, in vivo and in vitro ROS effect on nodule SS was studied to ascertain the possible implication of cellular redox state on SS regulation and, as such, on the NF process inhibition provoked by environmental constraints. For this purpose, PQ, a compound known to provoke electron chain transport impairment leading to ROS over-overproduction, was fed to nodulated pea plants. In a second set of experiments, oxidizing and reducing agents were used in an in vitro assay with nodule cell-lysates to assess their effect on SS activity. The overall results are consistent with a regulation of nodule SS by the cellular redox state at both the transcriptional and post-translational levels.

RESULTS

In vivo PQ supply alters nodule cellular redox state and provoked the inhibition of NF and SS downregulation.

PQ supply to the nodulated root system modified the nodule cell redox state as shown by a decline of ascorbate (ASC) content (0.12 and 0.25 $\mu\text{mol g}^{-1}$ nodule fresh weight (NFW) in PQ-treated versus control nodules, respectively) and a corresponding increase in dehydroascorbate (DHA) content (0.68 versus 0.37 $\mu\text{mol g}^{-1}$ NFW in PQ-treated versus control nodules, respectively), leading to a decline in the ASC/(ASC+DHA) ratio of approximately 65%, although the reduced glutathione (GSH)/(GSH + oxidized glutathione [GSSG]) did not vary (data not shown). Furthermore, the enzymatic antioxidant defense system also was activated, as shown by the increase in activity displayed by the different isoforms of nodule superoxide dismutase (SOD) in nondenaturing gels (Fig. 1). Four SOD isozymes were detected and, according to their mobility and differential inhibition to H₂O₂ or KCN (data not shown), they were assigned as MnSOD, FeSOD, CuZnSOD I, and CuZnSOD II. Under control conditions, the first two were more prominent, with CuZnSOD I showing an intermediate activity, whereas CuZnSOD II signal was low. Following PQ treatment, the four isozymes showed an increase in their activity.

PQ provoked NF impairment 96 h after the onset of treatment (Fig. 2). This decline occurred despite the fact that PQ supply did not affect the bacteroid's nitrogenase content at this time (Fig. 2, inset).

The more significant nodular carbon and nitrogen-specific enzyme activities were monitored every 24 h following PQ supply. This included SS, AI, UDP-glucose pyrophosphorylase

(UDPGPP; EC 2.7.7.9), PEPC, MDH, isocitrate dehydrogenase (ICDH; EC 1.1.1.42), glutamate synthase (GOGAT; EC 1.4.1.14), and aspartate aminotransferase (AAT; EC 2.6.1.1). With the exception of SS, none of these activities was significantly affected for the first 72 h of treatment and only ICDH, with an increase of 33%, showed a significant difference at the end of treatment (a full description of enzyme activities throughout the study period is published in Supplemental Table S1).

The three genes encoding SS described in *Pisum sativum* had very different levels of expression in pea nodules under control conditions in the absence of PQ, *PsSus1* representing 98% of the overall expression, *PsSus2* 2%, and *PsSus3* being almost negligible, because this represented a level approximately 35,000-fold lower than *PsSus1*, thus probably having limited relevance for pea nodule metabolism (Table 1). SS gene expression, protein content, and enzyme activity was measured every 24 h following PQ supply. A detailed description of how each individual gene expression responded to PQ supply can be found in Table 1, whereas Figure 3A displays the overall *PsSus* expression (*PsSus1* + *PsSus2* + *PsSus3*). This was significantly affected from the beginning of treatment (within 24 h after PQ application) and decreased progressively until the end of the study period. This fact was due largely to the decrease in *PsSus1* expression that rapidly responded to the presence of PQ (within 24 h). The response of *PsSus3* was similar to *PsSus1* in terms of expression decline, although its response was slightly delayed and was detectable within 48 h. PQ did not have any effect on *PsSut1*, a sucrose transporter, and *PsSus2* gene expression, although the relative contribution of the latter to the overall *PsSus* gene expression increased with time because of the decline of the remaining genes. SS protein content experienced a decline (Fig. 3C) that was significant 3 days after the onset of the treatment. At the end of the study, SS content was 20% of control plants (Fig. 3C). The decline in gene expression and content led SS activity to experience a progressive activity decline that was significant after 72 h of PQ application (Fig. 3B).

Fig. 1. Time course of pea nodule superoxide dismutase (SOD) isozyme activity following paraquat 1 μ M supply in nondenaturing 15% polyacrylamide gels. All lanes were loaded with 40 μ g of protein.

In vitro H₂O₂ and diamide effects on SS.

To monitor a likely direct ROS involvement in SS activity modulation at the post-translational level, nodule cell-lysates were incubated with increasing concentrations of H₂O₂ for up to 30 min (Fig. 4A). H₂O₂ already significantly inhibited SS activity after 1 min of incubation. Furthermore, this inhibition was dependant on H₂O₂ concentrations. On the other hand, H₂O₂ had virtually no effect on AI, the alternative sucrose-cleaving enzyme (Fig. 4B).

Nodule cell-lysates also were incubated with diamide, a thiol oxidizing agent, to assess whether the effect was H₂O₂ specific. Diamide effect was even more severe than H₂O₂ treatment, also provoking a striking SS decline at 0.1- to 0.5-mM concentrations in the first 5 min of incubation (Fig. 4). The highest diamide concentration applied (0.5 mM) nearly completely inhibited SS activity after 15 min of incubation (Fig. 5).

To investigate whether the effect of the oxidizing agents on SS occurs through a reversible oxidation, several reducing agents (dithiothreitol [DTT], *N*-acetyl-L-cysteine [NAC], ASC, and GSH) were applied at 5 and 10 mM to nontreated (control) and 5 mM H₂O₂ pretreated cell-lysates (Table 1). None of the reducing agents had a significant effect on control-lysate SS activity (data not shown). GSH, ASC, and NAC did not restore SS activity inhibition provoked by H₂O₂. However, 10 min of incubation with DTT completely restored SS activity in both H₂O₂- and diamide-treated nodule cell-lysates (Table 2).

Fig. 2. Apparent nitrogenase activity (ANA) for the 96-h period following paraquat (PQ) supply. Mean $\pm$ standard error ($n = 8$). NFW denotes nodule fresh weight. An asterisk (*) represents significant differences ($P \leq 0.05$) between controls and PQ. Inner panel shows nitrogenase Western blotting in control and PQ-treated plants at 96 h of treatment.

Table 1. *PsSus1*, *PsSus2*, *PsSus3*, and *PsSUT* gene relative expression under control conditions and following paraquat (PQ) supply

Time	Expression (%) ^a			
	<i>PsSus1</i>	<i>PsSus2</i>	<i>PsSus3</i>	<i>PsSUT</i>
Initial control (0 h)	1.56 $\pm$ 0.24 (98)	0.034 $\pm$ 0.012 (2)	2.44 $\times 10^{-5} \pm 0.05 \times 10^{-5}$ (0.0015)	6.7 $\times 10^{-3} \pm 0.5 \times 10^{-3}$
PQ 24 h	*0.75 $\pm$ 0.18 (94)	0.044 $\pm$ 0.023 (5)	3.29 $\times 10^{-5} \pm 0.05 \times 10^{-5}$ (0.0041)	4.6 $\times 10^{-3} \pm 0.5 \times 10^{-3}$
PQ 48 h	*0.68 $\pm$ 0.13 (97)	0.018 $\pm$ 0.003 (3)	*7.78 $\times 10^{-6} \pm 0.01 \times 10^{-6}$ (0.0011)	6.9 $\times 10^{-3} \pm 0.6 \times 10^{-3}$
PQ 72 h	*0.59 $\pm$ 0.15 (94)	0.034 $\pm$ 0.008 (5)	*7.12 $\times 10^{-6} \pm 0.01 \times 10^{-6}$ (0.0011)	5.2 $\times 10^{-3} \pm 0.6 \times 10^{-3}$
PQ 96 h	*0.45 $\pm$ 0.07 (90)	0.050 $\pm$ 0.007 (10)	*3.70 $\times 10^{-6} \pm 0.01 \times 10^{-6}$ (0.0007)	7.5 $\times 10^{-3} \pm 1.9 \times 10^{-3}$
Final control (96 h)	1.89 $\pm$ 0.51 (98)	0.031 $\pm$ 0.008 (2)	2.25 $\times 10^{-5} \pm 0.83 \times 10^{-5}$ (0.0012)	9.7 $\times 10^{-3} \pm 2.1 \times 10^{-3}$

^a The relative expression ratio was calculated as the ratio of normalized gene expression of the gene of interest against a *Pisum sativum* gene that encodes a translational elongation factor 1- α . Results are mean $\pm$ standard error ($n = 3$). An asterisk (*) represents significant differences between treated plants and the corresponding control. The relative contribution (%) of each gene to the overall *PsSus* gene expression is shown within parentheses.

DISCUSSION

Cellular redox state alteration provokes SS downregulation.

PQ, a compound that exacerbates O_2^- and H_2O_2 production associated with the electron transport systems, was applied at a very low concentration (1 μM) to the nutrient solution of nodulated plants. This is actually $1/100$ of the concentrations commonly used for leaf treatments (Iturbe-Ormaetxe et al. 1998). This concentration is low enough to avoid PQ translocation to shoots because it did not provoke any effect on photosynthesis or plant shoot biomass for 96 h of application (Marino et al. 2006). However, it was sufficient to provoke changes in the cellular redox state of nodules, as shown by changes in the ASC/DHA balance (this article; Marino et al. 2006) and the enzymatic antioxidant defense (Fig. 1). Indeed, SOD activity staining of nondenaturing gels revealed the three types of SOD that can be found in plants. In order of increasing mobility, these corresponded to the MnSOD, FeSOD, and two CuZnSOD isozymes. The isozyme composition of SOD in our extracts (low CuZnSOD activity, high FeSOD activity) agrees with prior reports in legume nodules (Rubio et al. 2001;

2004). SODs not only are part of the enzyme antioxidant defense of plants but it has been shown that their action is a source of H_2O_2 (Rubio et al. 2004).

This alteration of the cellular redox balance provoked a significant decline of NF within 96 h after the onset of treatment (Fig. 2) that occurred despite no apparent changes in the content of bacteroid nitrogenase (Fig. 2, inset). Indeed, nodular NF fixation responses to most environmental constraints are more dependent on an adequate supply of carbon and oxygen to bacteroids than on direct changes in the nitrogenase activity (Arrese-Igor et al. 1999; Hunt and Layzell 1993; Serraj et al. 1999).

From the full array of enzyme activities of carbon and nitrogen metabolism, SS was the one that responded most quickly to the presence of PQ, with a significant decline of its activity within 3 days (Fig. 3B) which even precedes the one of NF (Fig. 2). SS downregulation in response to drought and other abiotic stresses before any change occurs in other enzyme activities has been reported previously, limiting carbon supply for bacteroid respiration (Gálvez et al. 2005). Thus, SS has been proposed to play a key role in regulating the NF process under stress situations (Arrese-Igor et al. 1999; Gálvez et al.

Fig. 3. **A**, Overall *PsSus* (*PsSus1* + *PsSus2* + *PsSus3*) gene expression measured by reverse-transcription polymerase chain reaction. **B**, Sucrose synthase (SS) activity of pea nodules treated with 1 μM paraquat (PQ) during 96 h and **C**, SS content measured by Western blotting analysis. Mean $\pm$ standard error ($n = 3$, A and C; $n = 6$, B). An asterisk (*) represents significant differences ($P \leq 0.05$) between controls and PQ. C 0 h and C 96 h denote initial and final control, respectively.

2005; Gordon et al. 1997). We have hypothesized on a likely role of ROS in controlling SS downregulation and, therefore, nitrogen-fixation inhibition under environmental constraints (Marino et al. 2006), because it has been widely reported that ROS overproduction occurs in nodules under several environmental stresses (Becana et al. 2000). Moreover, the involvement of H_2O_2 has been described on several plant signal transduction pathways (Mittler 2002). Therefore, the present study was designed to further investigate whether the cellular redox state is involved in the signal transduction pathway leading to SS downregulation under environmental constraints.

The use of real-time reverse-transcription polymerase chain reaction (RT-PCR) allowed quantification of the relative contribution of the expression of several genes to the overall SS expression (Table 1), showing that *PsSus1*, the prevailing form, represents 98% of the overall expression, whereas the expression of *PsSus3*, that was not detected in nodules by RNA gel-blot analysis of either total RNA samples or poly(A⁺) samples, although described as present in pea nodules samples (Barratt et al. 2001), represented less than 0.002%. The results are consistent with either the early description that *rug4* mutants of pea have nodule SS activity reduced by 85% and, despite containing

Fig. 4. A, Sucrose synthase and **B**, alkaline invertase relative activities exposed to different H_2O_2 concentrations. Assays were performed during a maximum of 30 min of oxidizing agent exposition. Mean $\pm$ standard error ($n = 5$).

Fig. 5. Sucrose synthase relative activity exposed to different diamide concentrations. Assays were performed during a maximum of 30 min of oxidizing agent exposition. Mean $\pm$ standard error ($n = 5$).

metabolically active bacteroids, the N content and $\delta^{15}\text{N}$ values of these plants in the field indicated that they were inefficient in NF (Craig et al. 1999) or the recent report that antisense-repression of the *M. truncatula MtSus1* leads to a handicapped NF (Baier et al. 2007), showing that this is the crucial form for nodule functioning, at least in indeterminate nodules. In *Lotus japonicus*, using TILLING mutants, it recently has been shown that the highly expressed gene in nodules (*LjSUS3*) accounts for only 60% of the total nodule *SUS* transcripts and, therefore, the two more prevailing SS genes (*LjSUS1* and *LjSUS3*) had to be knocked out concomitantly to obtain severe effects on NF (Horst et al. 2007). This fact, together with the enhanced expression in nodules of the alkaline or neutral invertase gene, *LjINV1* (Flemetakis et al. 2006), not described so far in other species, may explain some particular features of nodule carbon metabolism in *L. japonicus* nodules (López et al. 2007).

PQ supply led to a very rapid SS expression downregulation because, within 24 h of its application, the overall *PsSus* expression sharply declined (Fig. 3A), mostly due to the effect on *PsSus1* (Table 1). These results are in agreement with those of Gordon and associates (1997), who reported SS gene downregulation under moderate water stress conditions, using Northern blot analysis, and the parallel in nodule metabolism behavior toward water stress and PQ-generated oxidative stress (Marino et al. 2006). This downregulation of SS expression leads to a decline of SS content (Fig. 3C) and SS activity (Fig. 3B) which, in turn, precedes NF inhibition (Fig. 2), which has been shown to occur under water-deficit conditions, mediated by an accumulation of sucrose and a depletion of malate (Gálvez et al. 2005). Thus, taken together, these data reveal the involvement of cellular redox alteration on the in vivo response of SS to oxidative-generating stresses that lead to the inhibition of NF decline because of a decrease in nodule carbon flux.

SS is in vitro regulated by a fast and reversible oxidation.

The in vitro experiments performed with pea nodule cell-lysates incubated with either H_2O_2 or diamide (a thiol oxidizing agent) showed a very fast inhibition of SS activity (Figs. 4 and 5). The fact that not only H_2O_2 but also diamide affects SS activity indicates that the cellular redox state, probably through a change in the redox state of thiol groups, is involved in a direct SS inhibition independently of *PsSus1* gene expression downregulation. It has been shown that PQ led to an altered cellular redox state in pea nodules (this article; Marino et al. 2006, 2007a).

The interconversion of thiols to a disulfide commonly has been reported to provide a mechanism for the regulation of enzyme catalytic activity. In the case of chloroplast phosphoribulokinase, a disulfide can physically block the enzyme active site (Brandes et al. 1996) or, in the case of fructose-1,6-bisphosphate phosphatase, disulfide reduction produces a small structural shift that affects the catalytic properties of the enzyme (Chiadmi et al. 1999). Another possibility could be the reaction of a cysteine with a thiol-containing molecule, such as oxidized glutathione, a reaction that is known as glutathionylation (Buchanan and Balmer 2005).

It has been reported that SS activity can be modulated by redox agents, suggesting the involvement of thiol groups in the catalytic process (Pontis et al. 1981). Furthermore, Röhrig and associates (2004) proposed that the cysteine sulfhydryl group of peptide A, a peptide encoded by the ENOD40 gene, binds SS by a disulfide bond formation. This bond would increase sucrose cleavage activity, indicating that SS activity is able to be modified by a disulfide covalent bond formation. These authors suggest that peptide A could be a mechanism of protection against a potential inactivation of SS provoked by its oxidation. In agreement with this hypothesis, in the present study,

we observed that a thiol-oxidizing agent provokes SS cleavage activity decline, which was reversible after incubation with DTT, a compound commonly used to reduce disulfide bonds.

Concluding remarks.

The results shown in this article reveal that the cellular redox state may regulate nodular SS functioning by two mechanisms. First, downregulating SS gene expression leads to a decline of SS activity. This SS downregulation leads to N_2 fixation impairment, as has been reported previously in nodulated legumes subjected to several environmental constraints (Gálvez et al. 2005; González et al. 1995, 1998; Gordon et al. 1997). Second, SS is also in vitro regulated through a fast and reversible SS protein oxidation. This fact is particularly important because the in vitro measurement of maximum potential SS activity from nodules subjected to environmental constraints leading to oxidative stress may largely overestimate the capacity of SS in in vivo conditions. It remains to be determined that the in vitro effects shown in this study may actually operate in in vivo conditions. H_2O_2 concentrations used in the present study are commonly used for in vitro experiments (Romero-Puertas et al. 2002); however, in situ nodule H_2O_2 concentration is a controversial issue. Indeed, H_2O_2 concentration can be largely different in the different nodule zones and localized, elevated H_2O_2 concentrations have been described, particularly associated with high SOD activities in nodules (Rubio et al. 2004). The occurrence of these two different mechanisms to control SS activity marks the importance of this enzyme for nodule performance and reinforces the suggestion that, under environmental constraints, the plant exerts a strict control on nodule carbon economy, a mechanism that may have been subjected to evolutionary selection in conditions where plants face survival rather than maximize growth (Arrese-Igor et al. 1999). However, given the variation in nodule metabolism between species and, particularly, between determinate and indeterminate nodules, the extent to which the regulatory mechanisms proposed for sucrose synthase in pea nodules apply more generally remains to be determined.

MATERIALS AND METHODS

Growth conditions.

Pea seed (*P. sativum* L. cv. Sugar-snap, kindly provided by Bonduelle SA, Milagro, Spain) were surface sterilized (Labhili

Table 2. Sucrose synthase reversible inhibition in H_2O_2 and diamide pretreated cell-lysates by reducing agents

Cell-lysates ^a	SS activity ^b	Relative activity (%) ^c
Control cell-lysates	0.17 ± 0.01	100
H_2O_2 5 mM-treated cell-lysates	0.12 ± 0.01*	70
+ GSH 5 mM	0.14 ± 0.01	84
+ GSH 10mM	0.14 ± 0.01	81
+ DTT 5 mM	0.16 ± 0.01#	98
+ DTT 10 mM	0.17 ± 0.01#	100
+ ASC 5 mM	0.13 ± 0.01	79
+ ASC 10 mM	0.12 ± 0.00*	72
+ NAC 5 mM	0.13 ± 0.01	79
+ NAC 10 mM	0.11 ± 0.01*	68
Diamide 0.3 mM-treated cell-lysates	0.09 ± 0.01*	56
+ DTT 10 mM	0.17 ± 0.01#	100

^a GSH = reduced glutathione, DTT = dithiothreitol, ASC = ascorbate, and NAC = N-acetyl-L-cysteine.

^b Sucrose synthase (SS) activity measured in $\mu\text{mol NADH min}^{-1} \text{mg}^{-1}$ of protein. Mean ± standard error ($n = 5$). An asterisk (*) represents significant difference compared with control cell-lysates for $P \leq 0.05$ and a hash (#) compared with H_2O_2 - or diamide-treated cell-lysates.

^c Relative activity was calculated taken as 100% the activity of control (nontreated) cell-lysates.

et al. 1995) and inoculated with *Rhizobium leguminosarum* bv. *viciae* NLV8. Plants were grown in a 1:1 (vol/vol) perlite/vermiculite mixture in a controlled environment chamber (day and night temperatures of 22 and 18°C, respectively; 70% relative humidity; 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (photosynthetic photon flux); and a 15-h photoperiod), with an N-free nutrient solution (Rigaud and Puppo 1975).

PQ treatment.

Four-week-old plants grown as exposed above were transferred to 1-liter hydroponic tanks 4 days prior to the beginning of treatment under the same environmental conditions. Plants were divided randomly into two sets, which were exposed to 0 or 1 μM PQ (Sigma-Aldrich) for 96 h. Nodules were harvested at 0, 24, 48, 72, and 96 h. After harvesting, nodules were frozen immediately in liquid nitrogen and stored at -80°C for analytical determinations.

Extraction and determination of low molecular mass antioxidants.

ASC, DHA, GSH, and GSSG were extracted from frozen nodules and analyzed by high-performance capillary electrophoresis in a Beckman Coulter PACE system 5500 (Beckman Instruments, Fullerton, CA, U.S.A.) associated with a diode array detector, as described by Zabalza and associates (2007).

SOD activity staining.

The isozyme pattern of SODs was analyzed by activity staining following electrophoresis on 15% nondenaturing polyacrylamide gels. The activity stain is based on the inhibition of nitroblue tetrazolium reduction by superoxide radicals generated photochemically (Beauchamp and Fridovich 1971). Identification of isozymes was based on the differential inhibition of SOD activity on gels preincubated with 3 mM KCN or 5 mM H_2O_2 for 1 h (Rubio et al. 2001).

NF measurements.

NF was determined as apparent nitrogenase activity. Plant root systems were sealed and H_2 evolution was measured in an open flow-through system under $\text{N}_2:\text{O}_2$ (79:21%) in accordance with Witty and Minchin (1998) using an electrochemical H_2 sensor (Qubit System Inc., Kingston, Canada). The H_2 sensor was calibrated with high purity gases (Praxair, Madrid) employing a gas mixer (Air Liquid, Madrid) flowing at the same rate as the sampling system (500 ml min^{-1}).

Extraction and assay of enzymes activities.

Nodules were homogenized in a mortar and pestle with 50 mM morpholinepropanesulfonic acid (MOPS), pH 7; 20% polyvinyl-pyrrolidone; 10 mM DTT; 10 mM 2-mercaptoethanol; 1 mM EDTA; 20 mM KCl; and 5 mM MgCl_2 at 0 to 2°C (5 ml/g of fresh weight). The homogenate was centrifuged for 30 min at $20,000 \times g$, 4°C .

Aliquots of the supernatants were retained for determination of plant fraction protein (Bradford 1976) and for PEPC (EC 4.1.1.31) activity. The rest of the supernatant was desalted by low-speed centrifugation ($180 \times g$, 1 min) through Bio Gel

P6DG columns (Bio-Rad, Hercules, CA, U.S.A.) equilibrated with 50 mM MOPS, pH 7; 20 mM KCl; and 5 mM MgCl_2 . Desalted extracts were used to assay SS (EC 2.4.1.13), AI (EC 3.2.1.26), UDPGPP (EC 2.7.7.9), MDH (EC 1.1.1.37), GOGAT (EC 1.4.1.14), AAT (EC 2.6.1.1), and ICDH (EC 1.1.1.42). All activities were measured within the linear range at 30°C , as described elsewhere (Marino et al. 2006).

In vitro enzyme activities assay.

To determine the effect of exogenous oxidizing agents on SS and AI activities, desalted nodule cell-lysates were incubated previously at ambient temperature during 0 to 30 min with H_2O_2 (0, 1, 5, and 20 mM) or diamide (0, 0.1, 0.3, and 0.5 mM). Cell-lysates were used instead of purified SS, because of the loss in activity following purification (data not shown) (Ross and Davies 1992).

For SS activity recovery assays, nodule cell-lysates were pretreated with 5 mM H_2O_2 for 5 min prior to a 10-min incubation with several reducing agents, DTT, NAC, ASC, and GSH, to 5 and 10 mM. A similar assay was performed in cell-lysates pretreated with 0.3 mM diamide prior to 10 mM DTT and 10 min of incubation. Incubations with reducing agents were done with nonpretreated cell-lysates (control). Enzymes used for the coupled SS activity assay were not affected by the oxidizing and reducing agent incubations used in the present study.

Immunoblotting.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to Laemmli (1970) with a 1-mm-thick 12% acrylamide (wt/vol) resolving gel and a 4.6% (wt/vol) stacking gel in a vertical electrophoresis cell (Mini-Protein III; Bio-Rad) at 150 V during 60 min. Gels were electroblotted onto PVDF membrane during 75 min at 100 V in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Blots were blocked in 5% (wt/vol) skim milk in 20 mM Tris-buffered saline at 4°C overnight. The primary antibody was anti-SS (1:5000, vol/vol) or anti-nitrogenase (1:1000, vol/vol). The secondary antibody was goat anti-rabbit horseradish peroxidase conjugate (1:50000, vol/vol; Sigma-Aldrich, St. Louis). Immunoreactive bands were visualized with a highly sensitive chemiluminescent substrate for peroxidase detection (Amersham Pharmacia, Uppsala, Sweden).

Real-time RT-PCR.

The RNA was extracted using the RNeasy plant mini kit (Qiagen, Hilden, Germany). The RNA preparations were concentrated to $1 \mu\text{g} \mu\text{l}^{-1}$ using Microcon30 columns (Millipore, Schwalbach, Germany) and stored at -80°C until use. The integrity of total RNA was checked on agarose gels and its quantity as well as purity was determined spectrophotometrically. Real-time RT-PCR was carried out with the QuantiTect SYBR Green RT-PCR kit (Qiagen) using gene-specific primers. For RT-PCR, 50 ng of DNaseI-treated total RNA were used in a total volume of 25 μl . Reactions were run on the Opticon real-time PCR cycler (MJ Research, Waltham, MA, U.S.A.) and quantification was performed with the Opticon

Table 3. Primer sequences used for real-time reverse-transcription polymerase chain reaction

Gene	Source	ID ^a	Primer A	Primer B	Product length (bp)
Sucrose synthase <i>PsSus1</i>	Craig et al. 1999	AJ012080	cagctcttattggatctctt	cttgatccaaaatgtaacaa	195
Sucrose synthase <i>PsSus2</i>	Buchner et al. 1998	AJ001071	agttacaagtgtatcccgatt	tggtcatggctattagatcag	233
Sucrose synthase <i>PsSus3</i>	Barrat et al. 2001	AJ311496	tggtacattttggatcaagt	tttctgttctaagggaactc	223
Sucrose transporter <i>PsSUT</i>	Tegeder et al. 1999	AF109922	ttaactctcttcctcggttc	gagacaaacatctgtggtatg	175
Elongation factor 1- α	Hohnjec et al. 2003	X96555	caactctacttgatgctcttg	accagtaggacaaaagttac	196

^a GenBank accession number.

Monitor analysis software (version 1.08). Every reaction was set up in two technical replicates. The program used was as follows: reverse transcription (50°C for 30 min), polymerase activation (95°C for 15 min), amplification and quantification cycles repeated 44 times (94°C for 15 s, 45°C for 30 s, and 72°C for 30 s with a single fluorescence measurement), and melting curve (40 to 95°C with one fluorescence read every 1°C). A relative expression ratio was calculated as the ratio of normalized gene expression of the gene of interest against a *P. sativum* gene that encodes a translational elongation factor 1- α (showing the highest homology to the constitutively expressed *MtTefa* gene, TC106470 (Medicago Gene Index). The following formula was used: relative expression ratio of the gene of interest is $2^{-\Delta CT}$, with $\Delta CT = CT_{\text{gene}} - CT_{\text{Tefa}}$. The gene-specific primers used for RT-PCR are listed in Table 3. All primers had a calculated melting temperature of $53 \pm 0.2^\circ\text{C}$, amplification products were not larger than 233 bp, and the primer sequences were unique in the TIGR *M. truncatula* Gene Index.

Statistical analysis.

Results were examined by two-way analysis of variance, using Fisher's protected least significant difference tests between means. All results discussed in this study were significant at $P \leq 0.05$ between treatments at a given day.

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LITERATURE CITED

- Alesandrini, F., Mathis, R., Van de Sype, G., Hérouart, D., and Puppo, A. 2003. Possible roles for a cysteine protease and hydrogen peroxide in soybean nodule development and senescence. *New Phytol.* 158:131-138.
- Apel, K., and Hirt, H. 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55:373-399.
- Arrese-Igor, C., González, E. M., Gordon, A. J., Minchin, F. R., Gálvez, L., Royuela, M., Cabrerizo, P. M., and Aparicio-Tejo, P. M. 1999. Sucrose synthase and nodule nitrogen fixation under drought and other environmental stresses. *Symbiosis* 27:189-212.
- Baier, M. C., Barsch, A., Küster, H., and Hohnjec, N. 2007. Antisense-repression of the *Medicago truncatula* nodule-enhanced sucrose synthase leads to a handicapped nitrogen fixation mirrored by specific alterations in the symbiotic transcriptome and metabolome. *Plant Physiol.* 145:1600-1618.
- Barratt, D. H. P., Barber, L., Kruger, N. J., Smith, A. M., Wang, T. L., and Martin, C. 2001. Multiple, distinct isoforms of sucrose synthase in pea. *Plant Physiol.* 127:655-664.
- Beauchamp, C., and Fridovich, I. 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* 44:276-287.
- Becana, M., Dalton, D. A., Moran, J. F., Iturbe-Ormaetxe, I., Matamoros, M. A., and Rubio, M. C. 2000. Reactive oxygen species and antioxidants in legume nodules. *Physiol. Plant.* 109:372-381.
- Beers, E. P., and McDowell, J. M. 2001. Regulation and execution of programmed cell death in response to pathogens, stress and developmental cues. *Curr. Opin. Plant Biol.* 4:561-567.
- Bieniaswska, Z., Barratt, D. H. P., Garlick, A. P., Thole, V., Kruger, N. J., Martin, C., Zrenner, R., and Smith, A. M. 2007. Analysis of the sucrose synthase gene family in *Arabidopsis*. *Plant J.* 49:810-828.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72:248-254.
- Brandes, H. K., Larimer, F. W., and Hartman, F. C. 1996. The molecular pathway for the regulation of phosphoribulokinase by thioredoxin f. *J. Biol. Chem.* 271:3333-3335.
- Buchanan, B. B., and Balmer, Y. 2005. Redox regulation: A broadening horizon. *Annu. Rev. Plant Biol.* 56:187-220.
- Buchner, P., Poret, M., and Rochat, C. 1998. Cloning and characterization of a cDNA (accession no. AJ001071) encoding a second sucrose synthase gene in pea (*Pisum sativum* L.). *Plant Physiol.* 117:719-719.
- Chiadmi, M., Navaza, A., Miginiac-Maslow, M., Jacquot, J. P., and Cherfils, J. 1999. Redox signalling in the chloroplast: Structure of oxidized pea fructose-1,6-bisphosphate phosphatase. *EMBO (Eur. Mol. Biol. Organ.) J.* 18:6809-6815.
- Copeland, L., Vella, J. and Hong, Z. Q. 1989. Enzymes of carbohydrate metabolism in soybean nodules. *Phytochemistry* 28:57-61.
- Craig, J., Barratt, P., Tatge, H., Dejardin, A., Handley, L., Gardner, C. D., Barber, L., Wang, T., Hedley, C., Martin, C., and Smith, A. M. 1999. Mutations at the *rug4* locus alter the carbon and nitrogen metabolism of pea plants through an effect on sucrose synthase. *Plant J.* 17:353-362.
- Déjardin, A., Sokolov, L. N., and Kleczkowski, L. A. 1999. Sugar/osmoticum levels modulate differential abscisic acid-independent expression of two stress-responsive sucrose synthase genes in *Arabidopsis*. *Biochem. J.* 344:503-509.
- Desikan, R., Mackerness, S. A. H., Hancock, J. T., and Neill, S. J. 2001. Regulation of the *Arabidopsis* transcriptome by oxidative stress. *Plant Physiol.* 127:159-172.
- Duncan, K. A., and Huber, S. C. 2007. Sucrose synthase oligomerization and F-actin association is regulated by sucrose concentration and phosphorylation. *Plant Cell Physiol.* 48:1612-1623.
- Durand, J. L., Sheehy, J. E., and Minchin, F. R. 1987. Nitrogenase activity, photosynthesis and nodule water potential in soybean plants experiencing water-deprivation. *J. Exp. Bot.* 38:311-321.
- Escuredo, P. R., Minchin, F. R., Gogorcena, Y., Iturbe-Ormaetxe, I., Klucas, R. V., and Becana, M. 1996. Involvement of activated oxygen in nitrate-induced senescence of pea root nodules. *Plant Physiol.* 110:1187-1195.
- Evans, P. J., Galesi, D., Mathieu, C., Hernandez, M. J., de Felipe, M. R., Halliwell, B., and Puppo, A. 1999. Oxidative stress occurs during soybean nodule senescence. *Planta* 208:73-79.
- Flemetakis, E., Efroze, R. C., Ott, T., Stedel, C., Aivalakis, G., Udvardi, M. K., and Katinakis, P. 2006. Spatial and temporal organization of sucrose metabolism in *Lotus japonicus* nitrogen-fixing nodules suggests a role for the elusive alkaline/neutral invertase. *Plant Mol. Biol.* 62:53-69.
- Foyer, C. H., and Noctor, G. 2005. Redox homeostasis and antioxidant signaling: A metabolic interface between stress perception and physiological responses. *Plant Cell* 17:1866-1875.
- Gálvez, L., González, E. M., and Arrese-Igor, C. 2005. Evidence for carbon flux shortage and strong carbon/nitrogen interactions in pea nodules at early stages of water stress. *J. Exp. Bot.* 56:2551-2561.
- González, E. M., Gordon, A. J., James, C. L., and Arrese-Igor, C. 1995. The role of sucrose synthase in the response of soybean nodules to drought. *J. Exp. Bot.* 46:1515-1523.
- González, E. M., Aparicio-Tejo, P. M., Gordon, A. J., Minchin, F. R., Royuela, M., and Arrese-Igor, C. 1998. Water-deficit effects on carbon and nitrogen metabolism of pea nodules. *J. Exp. Bot.* 49:1705-1714.
- González, E. M., Gálvez, L., and Arrese-Igor, C. 2001. Abscisic acid induces a decline in nitrogen fixation that involves leghaemoglobin, but is independent of sucrose synthase activity. *J. Exp. Bot.* 52:285-293.
- Gordon, A. J., Minchin, F. R., Skot, L., and James, C. L. 1997. Stress-induced declines in soybean N_2 fixation are related to nodule sucrose synthase activity. *Plant Physiol.* 114:937-946.
- Gordon, A. J., Minchin, F. R., James, C. L., and Komina, O. 1999. Sucrose synthase in legume nodules is essential for nitrogen fixation. *Plant Physiol.* 120:867-877.
- Hardin, S. C., Winter, H., and Huber, S. C. 2004. Phosphorylation of the amino terminus of maize sucrose synthase in relation to membrane association and enzyme activity. *Plant Physiol.* 134:1427-1438.
- Harrison, E. P. 1995. Sucrose metabolism in soybean and white clover root nodules. Ph.D. thesis, University College of Wales, Aberystwyth, UK.
- Hitz, W. D., Schmitt, M. R., Card, P. J., and Giaquinta, R. T. 1985. Transport and metabolism of 1'-fluorosucrose, a sucrose analog not subject to invertase hydrolysis. *Plant Physiol.* 77:291-295.
- Hohnjec, N., Becker, J. D., Puhler, A., Perlick, A. M., and Küster, H. 1999. Genomic organization and expression properties of the *MtSucS1* gene, which encodes a nodule-enhanced sucrose synthase in the model legume *Medicago truncatula*. *Mol. Gen. Genet.* 261:514-522.
- Hohnjec, N., Perlick, A. M., Puhler, A., and Küster, H. 2003. The *Medicago truncatula* sucrose synthase gene *MtSucS1* is activated both in the infected region of root nodules and in the cortex of roots colonized by arbuscular mycorrhizal fungi. *Mol. Plant-Microbe Interact.* 16:903-915.
- Horst, I., Welham, T., Kelly, S., Kaneko, T., Sato, S., Tabata, S., Parniske,

- M., and Wang, T. L. 2007. TILLING mutants of *Lotus japonicus* reveal that nitrogen assimilation and fixation can occur in the absence of nodule-enhanced sucrose synthase. *Plant Physiol.* 144:806-820.
- Hunt, S., and Layzell, D. B. 1993. Gas-exchange of legume nodules and the regulation of nitrogenase activity. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 44:483-511.
- Iturbe-Ormaetxe, I., Escuredo, P. R., Arrese-Igor, C., and Becana, M. 1998. Oxidative damage in pea plants exposed to water deficit or paraquat. *Plant Physiol.* 116:173-181.
- Komina, O., Zhou, Y., Sarath, G., and Chollet, R. 2002. In vivo and in vitro phosphorylation of membrane and soluble forms of soybean nodule sucrose synthase. *Plant Physiol.* 129:1664-1673.
- Labhili, M., Joudrier, P., and Gautier, M. F. 1995. Characterization of cDNAs encoding *Triticum durum* dehydrins and their expression patterns in cultivars that differ in drought tolerance. *Plant Sci.* 112:219-230.
- Ladrera, R., Marino, D., Larrainzar, E., González, E. M., and Arrese-Igor, C. 2007. Reduced carbon availability to bacteroids and elevated ureides in nodules, but not in shoots, are involved in the nitrogen fixation response to early drought in soybean. *Plant Physiol.* 145:539-546.
- Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of head of bacteriophage-T4. *Nature* 227:680-685.
- Larrainzar, E., Wienkoop, S., Ladrera, R., Weckwerth, W., Arrese-Igor, C., and González, E. M. 2007. *Medicago truncatula* root nodule proteome analysis reveals differential plant and bacteroid responses to drought stress. *Plant Physiol.* 144:1495-1507.
- Lodwig, E., and Poole, P. 2003. Metabolism of Rhizobium bacteroids. *Crit. Rev. Plant Sci.* 22:37-78.
- López, M., Herrera-Cervera, J. A., Iribarne, C., Tejera, N. A., and Lluch, C. 2007. Growth and nitrogen fixation in *Lotus japonicus* and *Medicago truncatula* under NaCl stress: Nodule carbon metabolism. *J. Plant Physiol.* 165:641-650.
- Marana, C., García-Olmedo, F., and Carbonero, P. 1990. Differential expression of two types of sucrose synthase-encoding genes in wheat in response to anaerobiosis, cold shock and light. *Gene* 88:167-172.
- Marino, D., González E. M., and Arrese-Igor C. 2006. Drought effects on carbon and nitrogen metabolism of pea nodules can be mimicked by paraquat: Evidence for the occurrence of two regulation pathways under oxidative stresses. *J. Exp. Bot.* 57:665-673.
- Marino, D., Frendo, P., González, E. M., Puppo, A., and Arrese-Igor, C. 2007a. NADPH recycling systems in oxidative stressed nodules: A key role for the NADP⁺-dependent isocitrate dehydrogenase. *Planta* 225:413-421.
- Marino, D., Frendo, P., Ladrera, R., Zabalza, A., Puppo, A., Arrese-Igor, C., and González, E. M. 2007b. Nitrogen fixation control under drought stress: Localized or systemic? *Plant Physiol.* 143:1968-1974.
- Martin, T., Frommer, W. B., Salanoubat, M., and Willmitzer, L. 1993. Expression of an *Arabidopsis* sucrose synthase gene indicates a role in metabolization of sucrose both during phloem loading and in sink organs. *Plant J.* 4:367-377.
- Minchin, F. R. 1997. Regulation of oxygen diffusion in legume nodules. *Soil. Biol. Biochem.* 29:881-888.
- Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* 7:405-410.
- Morell, M., and Copeland, L. 1984. Enzymes of sucrose breakdown in soybean nodules: Alkaline invertase. *Plant Physiol.* 74:1030-1034.
- Morell, M., and Copeland, L. 1985. Sucrose synthase of soybean nodules. *Plant Physiol.* 78:149-154.
- Neill, S., Desikan, R., and Hancock, J. 2002. Hydrogen peroxide signalling. *Curr. Opin. Plant Biol.* 5:388-395.
- Pei, Z. M., Murata, Y., Benning, G., Thomine, S., Klusener, B., Allen, G. J., Grill, E., and Schroeder, J. I. 2000. Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* 406:731-734.
- Pontis, H. G., Babio, J. R., and Salerno, G. 1981. Reversible unidirectional inhibition of sucrose synthase activity by disulfides. *Proc. Natl. Acad. Sci. Biol.* 78:6667-6669.
- Rigaud, J., and Puppo, A. 1975. Indole-3-acetic-acid catabolism by soybean bacteroids. *J. Gen. Microbiol.* 88:223-228.
- Röhrig, H., John, M., and Schmidt, J. 2004. Modification of soybean sucrose synthase by S-thiolation with ENOD40 peptide A. *Biochem. Biophys. Res. Commun.* 325:864-870.
- Romero-Puertas, M. C., Palma, J. M., Gómez, M., Del Río, L. A., and Sandalio, L. M. 2002. Cadmium causes the oxidative modification of proteins in pea plants. *Plant Cell Environ.* 25:677-686.
- Ross, H. A., and Davies, H. V. 1992. Purification and characterization of sucrose synthase from the cotyledons of *Vicia faba* L. *Plant Physiol.* 100:1008-1013.
- Rubio, M. C., Ramos, J., Webb, K. J., Minchin, F. R., Gonzalez, E., Arrese-Igor, C., and Becana, M. 2001. Expression studies of superoxide dismutases in nodules and leaves of transgenic alfalfa reveal abundance of iron-containing isozymes, posttranslational regulation, and compensation of isozyme activities. *Mol. Plant-Microbe Interact.* 14:1178-1188.
- Rubio, M. C., James, E. K., Clemente, M. R., Bucciarelli, B., Fedorova, M., Vance, C. P., and Becana, M. 2004. Localization of superoxide dismutases and hydrogen peroxide in legume root nodules. *Mol. Plant-Microbe Interact.* 17:294-305.
- Santos, R., Hérouart, D., Sigaud, S., Touati, D., and Puppo, A. 2001. Oxidative burst in alfalfa-*Sinorhizobium meliloti* symbiotic interaction. *Mol. Plant-Microbe Interact.* 14:86-89.
- Serraj, R., Sinclair, T. R., and Purcell, L. C. 1999. Symbiotic N₂ fixation response to drought. *J. Exp. Bot.* 50:143-155.
- Silvente, S., Camas, A., and Lara, M. 2003. Heterogeneity of sucrose synthase genes in bean (*Phaseolus vulgaris* L.): Evidence for a nodule-enhanced sucrose synthase gene. *J. Exp. Bot.* 54:749-755.
- Tegeder, M., Wang, X. D., Frommer, W. B., Offler, C. E., and Patrick, J. W. 1999. Sucrose transport into developing seeds of *Pisum sativum* L. *Plant J.* 18:151-161.
- Thummler, F., and Verma, D. P. S. 1987. Nodulin-100 of soybean is the subunit of sucrose synthase regulated by the availability of free heme in nodules. *J. Biol. Chem.* 262:14730-14736.
- Winter, H., Huber, J. L., and Huber, S. C. 1997. Membrane association of sucrose synthase: Changes during the graviresponse and possible control by protein phosphorylation. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 420:151-155.
- Witty, J. F., and Minchin, F. R. 1998. Methods for the continuous measurement of O₂ consumption and H₂ production by nodulated legume root systems. *J. Exp. Bot.* 49:1041-1047.
- Zabalza, A., Gaston, S., Sandalio, L. M., del Río, L. A., and Royuela, M. 2007. Oxidative stress is not related to the mode of action of herbicides that inhibit acetolactate synthase. *Environ. Exp. Bot.* 59:150-159.
- Zeng, Y., Wu, Y., Avigne, W. T., and Koch, K. E. 1998. Differential regulation of sugar-sensitive sucrose synthases by hypoxia and anoxia indicate complementary transcriptional and posttranscriptional responses. *Plant Physiol.* 116:1573-1583.

AUTHOR-RECOMMENDED INTERNET RESOURCE

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