

# Alfalfa nodules elicited by a flavodoxin-overexpressing *Ensifer meliloti* strain display nitrogen-fixing activity with enhanced tolerance to salinity stress

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**Abstract** Nitrogen fixation by legumes is very sensitive to salinity stress, which can severely reduce the productivity of legume crops and their soil-enriching capacity. Salinity is known to cause oxidative stress in the nodule by generating reactive oxygen species (ROS). Flavodoxins are involved in the response to oxidative stress in bacteria and cyanobacteria. Prevention of ROS production by flavodoxin overexpression in bacteroids might lead to a protective effect on nodule functioning under salinity stress. Tolerance to salinity stress was evaluated in alfalfa nodules elicited by an *Ensifer meliloti* strain that overexpressed a cyanobacterial flavodoxin compared with nodules produced by the wild-type bacteria. Nitrogen fixation, antioxidant and carbon metabolism enzyme activities were determined. The decline in nitrogenase activity associated to salinity stress was significantly less in flavodoxin-expressing than in wild-type nodules. We detected small but significant changes in nodule antioxidant metabolism involving the ascorbate–glutathione cycle enzymes and metabolites, as well as differences in activity of the carbon metabolism enzyme sucrose synthase, and an atypical starch accumulation pattern in flavodoxin-containing nodules. Salt-induced structural and ultrastructural alterations were examined in detail in alfalfa wild-type

nodules by light and electron microscopy and compared to flavodoxin-containing nodules. Flavodoxin reduced salt-induced structural damage, which primarily affected young infected tissues and not fully differentiated bacteroids. The results indicate that overexpression of flavodoxin in bacteroids has a protective effect on the function and structure of alfalfa nodules subjected to salinity stress conditions. Putative protection mechanisms are discussed.

**Keywords** *Ensifer (Sinorhizobium) meliloti* · Flavodoxin · Alfalfa · Nodule · Oxidative stress · Salinity stress

## Abbreviations

|       |                                 |
|-------|---------------------------------|
| APX   | Ascorbate peroxidase            |
| ARA   | Acetylene reduction assay       |
| DHAR  | Dehydroascorbate reductase      |
| dpi   | Days post-inoculation           |
| GR    | Glutathione reductase           |
| GSH   | Reduced glutathione             |
| GSSG  | Oxidized glutathione            |
| LPS   | Lipopolysaccharides             |
| MDHAR | Monodehydroascorbate reductase  |
| PEPC  | Phosphoenolpyruvate carboxylase |
| ROS   | Reactive oxygen species         |
| SS    | Sucrose synthase                |

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## Introduction

Legumes constitute the primary source of fixed nitrogen in arid and semiarid areas (Zahran 1999), where increasing soil salinity represents a major constraint to agriculture. Salinity stress affects legume yield adversely, mostly in nitrogen-deficient soils, as symbiotic nitrogen fixation is

more sensitive to salinity stress than the host legume or the free-living rhizobia (Zahran 1999; Coba de la Peña et al. 2003; Coba de la Peña and Pueyo 2012). In legume nodules, saline conditions induce a decrease in nitrogen-fixing activity, leghemoglobin levels, photosynthate availability and nodule respiratory rate, as well as an increase in oxygen diffusion resistance (Zahran 1999; Swaraj and Bishnoi 1999; Coba de la Peña et al. 2003; Coba de la Peña and Pueyo 2012 and references therein). Structural and ultrastructural alterations indicative of nodule senescence are observed in saline conditions (James et al. 1993; Serraj et al. 1995; Fernández-Pascual et al. 1996; Verdoy et al. 2004, 2006; Borucki and Sujkowska 2008). Salinity stress induces nodule senescence by enhancing production of toxic reactive oxygen species (ROS) and lowering of antioxidant defences (Swaraj and Bishnoi 1999; Puppo et al. 2005). Nodules are rich in leghemoglobin and other proteins related to nitrogen fixation, which can undergo autoxidation and generate ROS (Marino et al. 2009). ROS induce protein degradation, originating protein radicals and catalytic iron, which induce lipid peroxidation with generation of hydroxyl radicals and glutathione oxidation with generation of superoxide and oxygen peroxide (Chang et al. 2009; Marino et al. 2009 and references therein). Hydroxyl radicals can damage sugars, lipids, proteins and DNA.

Several antioxidant defences protect nodule structure and physiology against ROS-induced oxidative damage (Becana et al. 2000, 2010; Matamoros et al. 2003; Puppo et al. 2005; Marino et al. 2009). These antioxidant systems can be divided into two categories. The first comprises enzymes that react with ROS and maintain them at low levels, including superoxide dismutase (catalyses the dismutation of superoxide anion radical to  $H_2O_2$  and  $O_2$ ), catalase (mediates the cleavage of  $H_2O_2$  evolving  $O_2$ ) and peroxidase (reduces  $H_2O_2$  to  $H_2O$  using several reductants). The second category includes the ascorbate–glutathione pathway (including the enzymes ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase and glutathione reductase in concerted action), which regenerates the oxidized antioxidants, resulting in the detoxification of  $H_2O_2$  at the expense of NAD(P)H. This pathway requires a continuous supply of ascorbate and reduced glutathione, which can also scavenge superoxide and hydrogen peroxide (Noctor and Foyer 1998; Becana et al. 2000; Marino et al. 2009 and references therein).

Flavodoxins are electron carrier flavoproteins found in prokaryotes and some eukaryotic algae (Erdner et al. 1999). They have a flavin mononucleotide (FMN) group that acts as a redox centre, transferring electrons at low potentials (Pueyo et al. 1991; Pueyo and Gómez-Moreno 1991). The FMN cofactor of flavodoxin can exist in three redox states, oxidized, one-electron-reduced semiquinone and the two-electron-reduced hydroquinone. This property confers high

versatility to flavodoxins in electron transport systems (Simonsen and Tollin 1980; McIver et al. 1998). To date, flavodoxin has not been described in plants, as flavodoxin-encoding genes were probably lost during the transition of algae to plants (Zurbriggen et al. 2007). Flavodoxin is present as a constitutive or inducible protein in several microorganisms (Klugkist et al. 1986). In cyanobacteria and enterobacteria, flavodoxin levels increase several-fold following exposure to various superoxide-propagating compounds (Zheng et al. 1999; Yousef et al. 2003; Singh et al. 2004). Tognetti et al. (2006, 2007a, b) showed that transgenic tobacco plants expressing a cyanobacterial flavodoxin have increased tolerance to multiple types of stress. These effects seem to be due to the capacity of flavodoxin to mediate electron transfer and to react with ROS, facilitating ROS detoxification and protecting against oxidative damage. Transgenic creeping bentgrass (*Agrostis stolonifera*) plants expressing flavodoxin also display enhanced tolerance to drought (Li et al. 2011).

Prevention of ROS production in the nodule and reduction of oxidative stress could lead to a protective effect on nodule structure and function in salinity stress conditions. We recently reported that overexpression of a cyanobacterial flavodoxin in the bacteroids leads to amelioration of the oxidative balance, delay in natural nodule senescence (Redondo et al. 2009) and enhanced tolerance to cadmium stress (Shvaleva et al. 2010), as well as tolerance of nitrogen-fixing activity to salinity stress in transgenic flavodoxin-expressing *Medicago truncatula* plants (Coba de la Peña et al. 2010). Here, we evaluated the effects of salinity stress on alfalfa nodules elicited by flavodoxin-overexpressing *Ensifer* (formerly *Sinorhizobium*) *meliloti* compared with nodules elicited by the wild-type strain. The effects of flavodoxin overexpression on nitrogen fixation, nodule structure and ultrastructure, ROS-scavenging enzymes and carbon metabolism enzymes were examined in alfalfa nodules subjected to salinity stress.

## Materials and methods

### Bacterial strains

Nodulation-competent rifampicin-resistant *Ensifer meliloti* 2011 (formerly *Sinorhizobium meliloti*; Casse et al. 1979) was isolated from Vincent medium (Vincent 1970) plates containing 100  $\mu\text{g}/\text{mL}$  rifampicin. The sequence of *Anabaena variabilis* flavodoxin gene was obtained from Fillat et al. (1991); the gene was PCR-amplified using the pTrc99a-Fld plasmid as template, as described (Redondo et al. 2009). *A. variabilis* flavodoxin was cloned into the tetracycline-resistant expression plasmid pFAJ1709 (Dombrecht et al. 2001) that contains loci for plasmid

stability during symbiosis, and *S. meliloti* cells were transformed with the resulting plasmid pFAJ1709-Fld (Redondo et al. 2009). *A. variabilis* flavodoxin was detected in transformed bacteria and bacteroids by PCR amplification and by Western blot using a polyclonal rabbit antibody raised against *A. variabilis* flavodoxin (Fillat et al. 1991). No PCR product was obtained for bacteria bearing the pFAJ1079 plasmid or for untransformed bacteria (Redondo et al. 2009). Bacteria carrying the expression plasmid pFAJ1709 are referred to as wild-type (WT) bacteria; nodules elicited by these bacteria are referred to as WT nodules.

#### Plant material and growth conditions

*Medicago sativa* cv. Aragon R-1 (Rocalba) seeds were germinated as described (Coba de la Peña et al. 2008). Seedlings were transferred to growth pouches (CYG Seed Germination Pouches, Mega International, Minneapolis, MN, USA) containing nitrogen-free nutrient solution (Hoagland and Arnon 1938) and incubated in growth chamber conditions ( $180 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ , 23 °C, 16/8 h photoperiod). Bacteria were inoculated 2 days after sowing with WT *E. meliloti*, transconjugant *E. meliloti* bearing plasmid pFAJ1709, or transconjugant *E. meliloti* bearing plasmid pFAJ1709-Fld, grown to exponential phase. At 18 days post-inoculation (dpi), plants were subjected to salinity stress by adding 100 mM NaCl to the nutrient solution. Control plants were maintained in a NaCl-free nutrient solution.

#### Nitrogenase activity

Nitrogenase activity was determined by the acetylene reduction assay (ARA) as described (Shvaleyeva et al. 2010). Although use of a “closed” system for measuring acetylene reduction can lead to an acetylene-induced decline in nitrogenase activity (Minchin et al. 1983), it is appropriate for comparative measurements, especially when assay time is short.

#### Antioxidant enzymes

To determine enzyme activity, nodules (200 mg/mL buffer) were ground in a mortar with liquid nitrogen and homogenized in 50 mM K-phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 1 % (w/v) PVP-10 and 0.1 % (v/v) Triton X-100. The homogenate was centrifuged ( $12,000 \times g$ , 4 °C, 30 min) and the supernatant used for the activity assay for all antioxidant enzymes. Three separate experiments were performed, with 6–15 replicates.

Catalase (EC 1.11.1.6) activity was assayed spectrophotometrically by monitoring  $\text{H}_2\text{O}_2$  decomposition at 240 nm (Aebi 1984) (1 min, 25 °C). Superoxide dismutase

(SOD, EC 1.15.1.1) activity was determined spectrophotometrically by monitoring inhibition of the cytochrome c reduction rate in the presence of the xanthine–xanthine oxidase system at 550 nm (McCord and Fridovich 1969) (1 min, 25 °C). One unit of activity was defined as the amount of enzyme that inhibits the rate of cyt c reduction by 50 % at 25 °C. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed by monitoring disappearance of ascorbate at 290 nm (Asada 1984) (1 min, 25 °C). APX-independent ascorbate oxidation was quantified in control reactions with the addition of 1 mM KCN, of 0.5 mM *p*-chloromercuriphenylsulphonic acid and of boiled extract. Glutathione reductase (GR, EC 1.6.4.2) activity was assayed by monitoring NADPH oxidation at 340 nm (Dalton et al. 1986) (1 min, 25 °C). Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) activity was assayed by monitoring NADH oxidation at 340 nm (Dalton et al. 1992) (1 min, 25 °C). MDHAR-independent NADH oxidation was quantified. Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was assayed by following the generation of ascorbate at 265 nm (Nakano and Asada 1987) (1 min, 25 °C). A correction factor of 0.98 was applied in the assessment of enzyme activity to compensate for GSSG absorbance (Hernández-Jiménez et al. 2002). The precise reaction conditions for each enzymatic determination have been described (Redondo et al. 2009).

#### Glutathione determination

Reduced glutathione (GSH) and total glutathione (GSH + GSSG) were measured by the glutathione recycling assay (Anderson 1985) modified by Floreani et al. (1997). GSH induces reduction of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), resulting in 2-nitro-5-thiobenzoic acid (TNB) and GSSG. GSSG is recycled to GSH in the presence of GSSG reductase and NADPH. TNB generation was monitored spectrophotometrically at 412 nm (1 min, 25 °C) (Redondo et al. 2009).

#### Carbon metabolism enzymes

Sucrose synthase (SS, EC 2.4.1.13) activity was measured spectrophotometrically by monitoring  $\text{NAD}^+$  reduction at 340 nm (Morell and Copeland 1985). Reaction mixture volume was 1 mL and included 50  $\mu\text{L}$  nodule extract (45–70  $\mu\text{g}$  total protein), 100 mM sucrose, 2 mM UDP, 1.5 mM  $\text{NAD}^+$  and 250  $\mu\text{g}$  UDPG dehydrogenase in 100 mM bicine–KOH (pH 8.5). Nodule extracts were prepared in 50 mM K-phosphate buffer (pH 8.0) containing 1 mM EDTA, 20 % (v/v) ethylene glycol and 33 % (w/fresh weight) PVP. The homogenate was centrifuged ( $12,000 \times g$ , 4 °C, 30 min) and the supernatant used for the activity assay. Phosphoenolpyruvate carboxylase (PEPC,

EC 4.1.1.31) activity was assayed spectrophotometrically by monitoring NADH oxidation at 340 nm (Vandercammen et al. 1989) as reported (Verdoy et al. 2006).

Light microscopy, transmission electron microscopy and low-temperature scanning electron microscopy

Nodules were collected from plants treated with 0 and 100  $\mu\text{M}$  NaCl for 3 days. For light and transmission electron microscopy analysis, nodules were fixed immediately under vacuum at 4 °C in a solution of 5 % glutaraldehyde, 4 % paraformaldehyde in 100 mM Na-cacodylate buffer (pH 7.4) containing sucrose (25 mg/mL), as described (Redondo et al. 2009). After 2 h, fixative was replaced by fresh solution and fixation continued (1.5 h, 4 °C). Specimens were washed in the sucrose-cacodylate buffer three times (1 h each, 4 °C), and post-fixed with a solution of 1 % osmium tetroxide in the same buffer (5 h, 4 °C). After two washes with cacodylate buffer (5 min each, 4 °C), nodules were dehydrated in an ethanol series in water (30, 50, 70 %; 10 min each, 4 °C). Dehydration continued with 1 % uranyl acetate in 70 % ethanol (24 h, 4 °C), 90 % ethanol (10 min, 4 °C), 96 % ethanol (30 min, 4 °C), and 100 % ethanol (2 h, 4 °C) with one change after the first hour. Infiltration in London Resin White was carried out with mixtures of resin:ethanol (v:v) of 1:3, 1:1, and 3:1 (3, 16 and 3 h, respectively) with gentle shaking, then in pure resin (48 h, 4 °C). After one change of resin (overnight, 4 °C with shaking), specimens were placed in gelatine capsules, filled with resin, and allowed to polymerize (24 h, 60 °C).

Serial semithin (1  $\mu\text{m}$ ) and ultrathin (70–90 nm) sections were cut with a Reichert Ultracut S ultramicrotome (Leica, Vienna, Austria) fitted with a diamond knife. Semithin sections for LM were stained with 1 % (w/v) toluidine blue in aqueous 1 % sodium borate for direct observation with a Zeiss Axiophot photomicroscope (Carl Zeiss, Oberkochen, Germany). Ultrathin sections for TEM were contrasted with lead citrate. TEM observations were performed with a STEM LEO 910 electron microscope (Carl Zeiss) at accelerating voltage of 80 kV, equipped with a Gatan Bioscan 792 digital camera (Pleasanton, CA). At least five nodules from three different plants of each treatment, and more than three sections per nodule were analyzed.

Alternatively, fresh nodules were processed as described (Verdoy et al. 2006) by low-temperature scanning electron microscopy (LTSEM). Fractured surfaces were observed with a DSM 960 Zeiss scanning electron microscope (Zeiss, Jena, Germany) at  $-135$  °C.

Statistical analyses

Data were analysed with the SPSS v. 19 software package (SPSS Inc., Chicago, IL, USA) by two-way analysis of

variance (ANOVA,  $P \leq 0.05$ ), to assess the effect of two factors: bacterial strain and salt stress and to test for possible interaction. When significant interaction was detected, one-way ANOVA analysis ( $P \leq 0.05$ ) was separately performed for each factor.

## Results

Nitrogenase activity decline due to salinity stress is significantly lower in flavodoxin-expressing nodules

Nodulation kinetics, nodule number and weight were unaffected by flavodoxin expression, as no significant differences were observed in these parameters between plants inoculated with flavodoxin-expressing or WT bacteria in the presence of 0, 25, 50 or 100 mM NaCl (not shown).

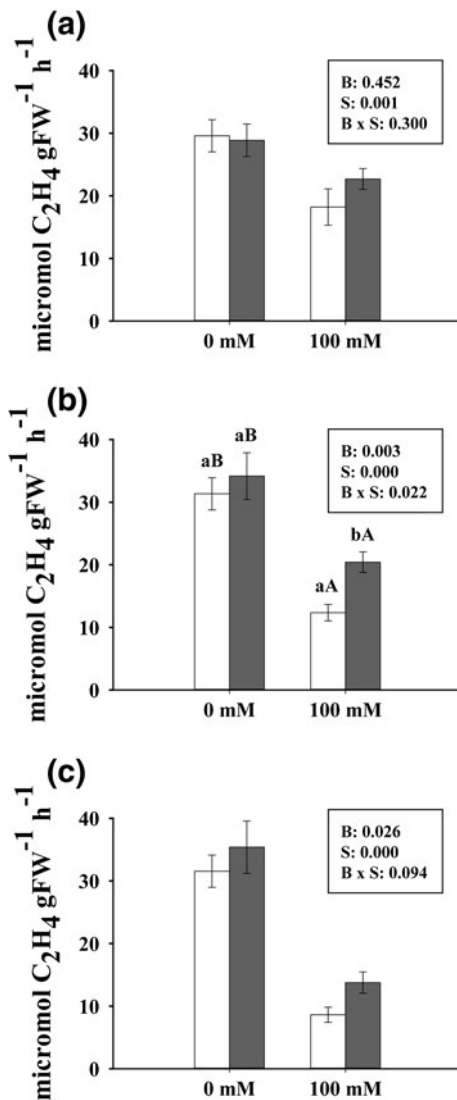
To estimate the protective effect of flavodoxin during salinity stress, nitrogen-fixing activity was measured in *M. sativa* plants nodulated by flavodoxin-expressing or WT bacteria, unstressed or subjected to salinity stress (100 mM NaCl) for 2, 3 and 4 days (Fig. 1). Two-way ANOVA indicated salinity stress induced a significant decrease in nitrogenase activity in nodules after 2 days, independently of the type of bacteria, which had no significant effect at this time (Fig. 1a). After 3 days of stress, both bacterial strain and salinity stress had a significant effect on nitrogenase activity and a significant interaction between the two factors was detected. One-way ANOVA indicated that nitrogenase activity decreased significantly in both types of nodules. This decrease was approximately 60 % for nodules elicited by WT bacteria and only 40 % for flavodoxin-expressing nodules. The nitrogen-fixing activity of flavodoxin-expressing nodules was significantly higher than that of nodules elicited by WT rhizobia in plants subjected to salinity stress for 3 days (Fig. 1b). Both factors had also a significant effect on nitrogenase activity after 4 days of stress, although no significant interaction was detected at this time (Fig. 1c).

We measured several parameters related to plant growth, including fresh and dry weight of aerial parts, and fresh weight and number of nodules, and nitrogen content, and found no significant differences between plants inoculated with flavodoxin-expressing or WT bacteria (not shown).

Flavodoxin overexpression reduces salinity stress-induced damage to alfalfa nodule structure and ultrastructure

Alfalfa nodules have been very well characterized at the structural and ultrastructural levels (Vasse et al. 1990). Here, we follow their nomenclature for nodule zones and





**Fig. 1** Nitrogenase activity per gram of fresh weight of nodule, measured by the acetylene reduction assay (ARA) in *Medicago sativa* nodules elicited by wild-type (white bars) or flavodoxin-expressing *S. meliloti* (grey bars) after 2 days (a), 3 days (b) and 4 days (c) treatment with 0 mM (Control) or 100 mM NaCl (NaCl). Values are mean  $\pm$  SE of three experiments ( $n = 10$  per experiment).  $P$  values corresponding to two-way ANOVA for the factors bacterial strain ( $B$ ), salinity stress ( $S$ ) and interaction between both ( $B \times S$ ) are displayed. In the case of significant interaction (3-day treatment), one-way ANOVA ( $P \leq 0.05$ ) was separately performed for each factor. For each bacterial strain, different capital letters indicate significant differences between salinity treatments; for each salinity treatment, different lower case letters indicate significant differences between bacterial strains

bacteroid types. Structural and ultrastructural features of alfalfa nodules induced by flavodoxin-expressing rhizobia have been described; the main difference compared to WT nodules is atypical starch accumulation in cells of zone III (Redondo et al. 2009; Shvaleva et al. 2010).

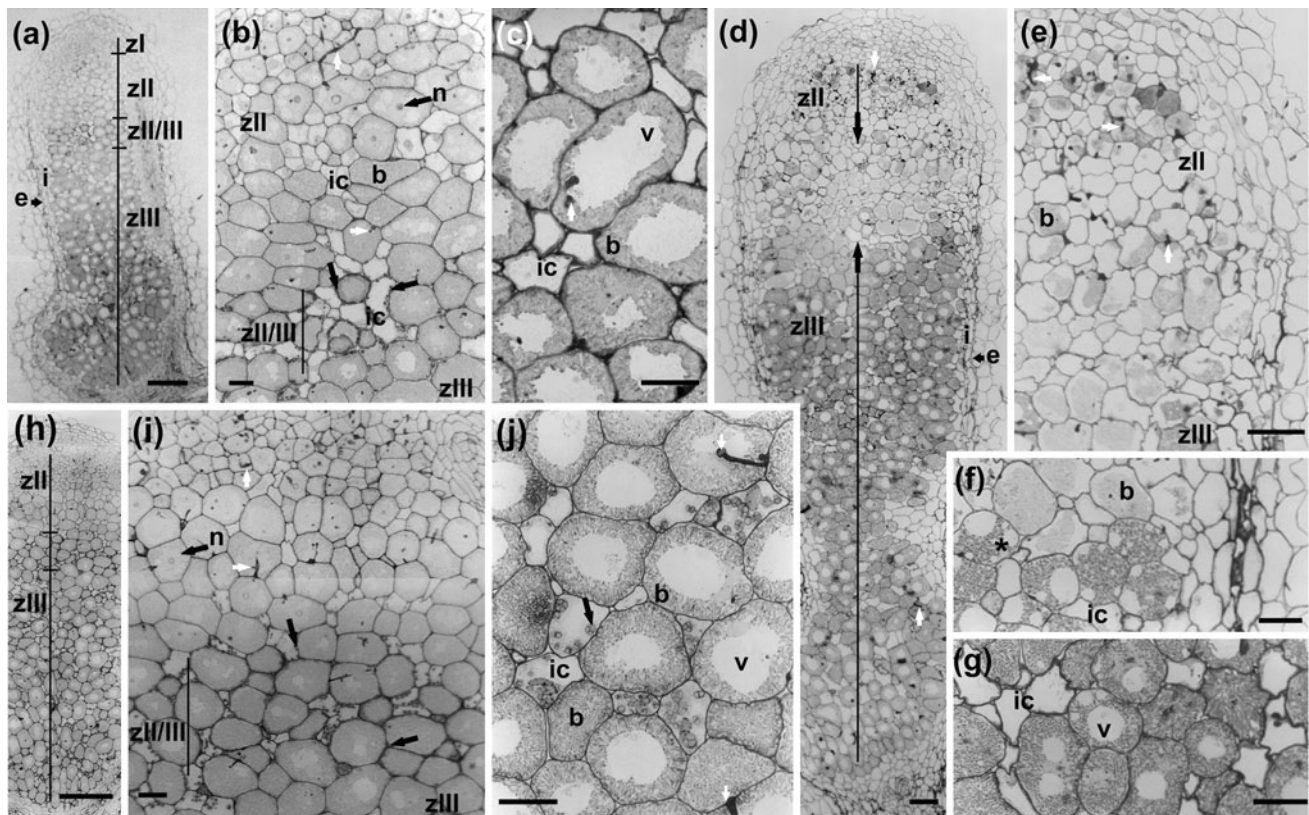
To assess the protective effect of flavodoxin expression, structural alterations related to salinity stress were analysed

in alfalfa nodules; 21-dpi nodules elicited by WT or flavodoxin-expressing bacteria were compared after 3 days of salinity stress (100 mM NaCl). Nodules were examined by light microscopy (Fig. 2), transmission electron microscopy (Fig. 3) and low-temperature scanning electron microscopy (Fig. 4).

In plants subjected to salinity stress, WT nodules showed severe alterations in cell structure in the cortex and central tissues (Fig. 2d–g) compared with non-stressed WT nodules (Fig. 2a–c) or with salt-treated flavodoxin-expressing nodules (Fig. 2h–j). Inner cortex cells appeared collapsed (Fig. 2d) and central zone cells lost their natural turgidity and became irregular in shape. These alterations were especially evident in cells of zone II (invasion zone) and zone III (nitrogen-fixing zone). The invasion zone II displayed many infection threads and altered cells with cytoplasm residues containing numerous bacteria (Fig. 2e). Nuclei and some other organelles were not distinguishable. Cell layers from proximal zone II to distal zone III showed severe symptoms of cytological damage. The characteristic interzone II/III of the elongated indeterminate nodules, with visible amyloplasts (Vasse et al. 1990), was not recognizable (Fig. 2d, e). Cells presented shrinkage and irregular shape, and reduced intercellular spaces were observed (Fig. 2e). In central zone III, infected cells had numerous vacuoles (Fig. 2f) instead of the typical single central vacuole found in non-stressed nodules (Fig. 2c). Cells of proximal zone III (Fig. 2g) were clearly less altered than those of distal zone III.

Following NaCl treatment, flavodoxin-expressing nodules (Fig. 2h–j) were less affected than WT nodules (Fig. 2d–g). The characteristic longitudinal organization of the central tissue of alfalfa nodules (Vasse et al. 1990) was distinguishable by light microscopy (Fig. 2h). There were no appreciable differences compared to non-stressed flavodoxin-containing nodules (Redondo et al. 2009) at light microscopy. The cortex was unaffected by salinity stress and the morphology of cells in the central tissues was normal (Fig. 2h–j). The characteristic starch accumulation in zone III, attributable to flavodoxin expression (Redondo et al. 2009), was also found (Fig. 2j).

In accordance with the light microscopy data, the electron microscopy study showed ultrastructural differences between WT and flavodoxin-expressing nodules from salt-treated plants (Figs. 3, 4). No differences were found in nodule ultrastructure between WT and flavodoxin-containing nodules in untreated plants other than the starch accumulation in zone III attributable to flavodoxin (Fig. 4a, c). In the distal part of zone II in WT nodules subjected to salt treatment, host cells had numerous vesicles, denoting high vacuolization and plasmolysis (Fig. 3b). Symbiosomes recently released from the infection threads with type 1 bacteroids showed abnormal enlarged peribacteroid



**Fig. 2** Effect of salinity stress on the structure of nodules elicited by wild-type (a–g) and flavodoxin-expressing (h–j) rhizobia. **a** Longitudinal section of a WT nodule from an untreated plant showing the outer cortex, endodermis (e), inner cortex (i), meristem (zI), interzone II/III (zII/III) and nitrogen-fixing zone (zIII). **b** Detail of **a** showing zone II containing abundant infection threads (white arrow), infected cells harbouring bacteroids (b) and interstitial cells (ic), and interzone II/III with abundant starch granules (black arrows) in infected and interstitial cells. **c** Detail of infected and interstitial cells of zone III, showing a central vacuole (v). **d** Section of a WT nodule subjected to salt treatment (100 mM NaCl, 3 days) showing structural alterations of the inner cortex and the distal part of the central tissues. **e** Detail of

**d** showing cytological alterations in zone II. Note that interzone II/III is not distinguishable and cells show remnant cytoplasm harbouring bacteroids (b). **f** Image of distal zone III cells showing infected cells with remnant cytoplasm and infected cells with abundant vacuoles (asterisk). **g** Detail of proximal zone III showing infected cells with fewer symptoms of cytological damage compared to cells in **f**. **h** Longitudinal section of a flavodoxin-nodule after salt treatment. Note the normal indeterminate nodule zonation. **i** Detail of zone II and interzone II/III. **j** Detail of infected and interstitial cells of zone III, showing normal structure and characteristic starch accumulation in flavodoxin-expressing nodules. Bars **a, h** 200  $\mu$ m; **b, e–g, i** 30  $\mu$ m; **c, j** 20  $\mu$ m; **d** 60  $\mu$ m

spaces (Fig. 3a). Although some apparently unaltered type 1 and type 2 bacteroids were found in zone II, many bacteroids had heterogeneous cytosol with numerous vesicle-like structures and were surrounded by a wide peribacteroid space (Fig. 3b, c). In cells of interzone II/III, some normal symbiosomes harbouring type 3 bacteroids coexisted with many altered symbiosomes with wide peribacteroid spaces and bacteroids containing vesicles close to the membrane (Fig. 3d, e). The absence of plastids with normal starch granule content in interzone II/III cells (Vasse et al. 1990; Redondo et al. 2009) observed by light microscopy was confirmed by electron microscopy. Most plastids contained no starch granules, and only a small number had small granules (Fig. 3d, e). In distal zone III, cells were seriously damaged and bacteroids and other cell organelles were hardly recognizable (Fig. 3f). In central zone III, vesiculation and damaged bacteroids were also

visible (Fig. 4b). Vacuole content was altered in stressed nodules (Fig. 4b) compared to control nodules (Fig. 4a); wide electron-dense areas were found, rather than a regular pattern of electron-dense lines. In proximal zone III, cells were less affected and the most severe symptom of symbiosome alteration was the presence of abundant internal vesicles in type IV bacteroids (Fig. 3g). Cell organelles were recognizable, but mitochondria were irregular in shape and had lost their membrane integrity (Fig. 3g).

In salt-treated nodules elicited by flavodoxin-expressing rhizobia, symptoms of ultrastructural damage were less pronounced than in WT nodules. Bacteroids at different developmental stages were recognizable in their characteristic nodule zones (Fig. 3h–n). Infected cells in zone II showed normal features, with type 1 bacteroids recently released from the infection threads, and unaffected type 2 bacteroids and organelles (Fig. 3h, i). Normal amyloplasts,

mitochondria and type 3 bacteroids were found in zone II/III (Fig. 3j). No significant differences were observed between distal (Fig. 3k) and proximal (Fig. 3i–n) zone III. Cells showed the characteristic high starch accumulation of the flavodoxin-expressing nodules (Figs. 3k, i, j, 4d). Most infected cells harboured unaltered type 4 bacteroids (Fig. 3k–n). Altered symbiosomes also coexisted with normal symbiosomes in some zone III cells (Fig. 3m). There were no significant differences in vacuoles between salt-treated and untreated nodules (Fig. 4c, d). Numbers of cells with altered type 4 bacteroids (approximately 30–40 vs 80–90 % in WT nodules) and numbers of altered bacteroids per cell were always much lower in flavodoxin-expressing than in WT nodules.

Flavodoxin overexpression ameliorates the oxidative balance in nodules subjected to salinity stress

Enzymatic activities of several antioxidant enzymes were determined in nodules of 21-dpi *M. sativa* plants inoculated with flavodoxin-expressing bacteria and compared to WT nodules, untreated or subjected to 100 mM NaCl for 3 days.

Salinity stress had a significant effect on superoxide dismutase (SOD) activity, inducing a decrease in both types of nodules. No significant effect of the bacterial strain was observed for SOD activity (Fig. 5a). Catalase activity increased in nodules subjected to salinity stress (Fig. 5b), and a significant effect of the bacterial strain was observed independently of salinity stress, with lower activity values in nodules inoculated with flavodoxin-expressing bacteria. No interaction between salinity and bacterial strain was detected for these two enzymes.

We determined the activity of the ascorbate–glutathione cycle enzymes ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Fig. 5c–f). Salinity stress induced a significant decrease of all these enzymatic activities. For APX and GR activities, a significant effect of both salinity stress and bacterial strain was observed with interaction between both factors. One-way ANOVA indicated that both enzymatic activities significantly decreased upon salinity stress, and they were significantly higher in flavodoxin-expressing nodules compared to nodules infected by WT bacteria. For DHAR activity, there was a significant effect of both factors, with higher values in flavodoxin-expressing nodules; no interaction between factors was observed.

Reduced (GSH) and oxidized (GSSG) glutathione were quantified. Salinity stress had a significant effect on GSH concentration, and a decrease of GSH levels was observed in both types of nodules following salinity stress (Fig. 6a). Bacterial strain had also a significant effect independent of

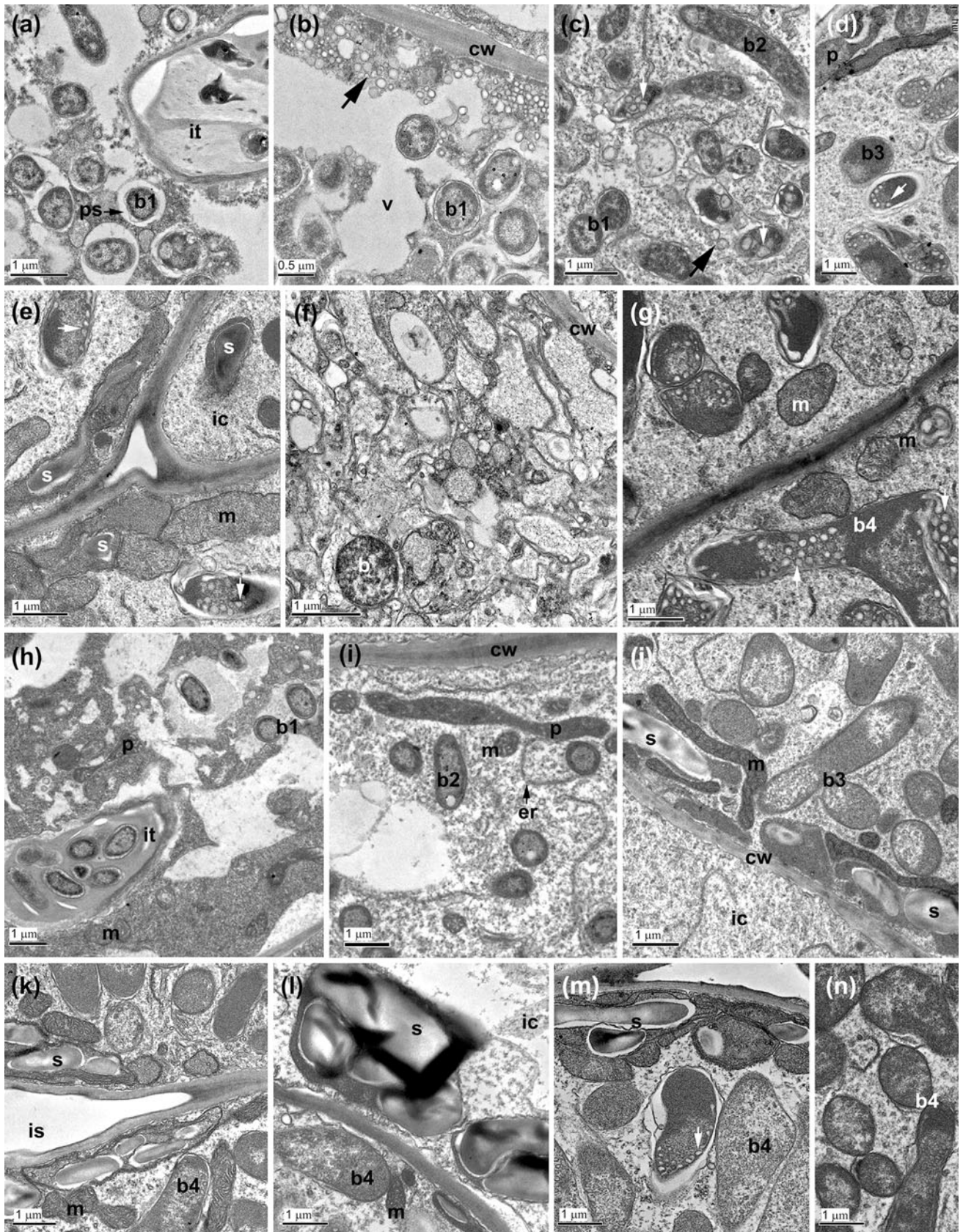
salinity stress, and higher GSH levels were observed in flavodoxin-containing than in WT nodules. Both factors had a significant effect on GSSG levels, and there was interaction between them. One-way ANOVA indicated that salinity stress induced a significant increase of GSSG levels in both types of nodules and this increase was significantly higher for nodules infected by the WT bacteria (Fig. 6b). There was also a significant effect and interaction between both factors for the GSH/GSSG ratio, a parameter indicative of the oxidative balance in the nodule. This ratio significantly decreased in both types of nodules when subjected to salinity stress (Fig. 6c), and was significantly higher in flavodoxin-containing nodules subjected to salinity stress compared with nodules infected by WT bacteria.

Carbon metabolism was also studied in both nodule types. SS and phosphoenolpyruvate carboxylase (PEPC) activities were determined. Salinity treatment and bacterial strain both had a significant effect on SS activity; a decrease of activity upon salinity stress was observed, and values were higher in flavodoxin-containing nodules independent of the salinity treatment (Fig. 7a). PEPC activity decreased significantly with salinity stress (Fig. 7b), but differences between nodule types were not significant.

## Discussion

The decline in nitrogenase activity following salinity stress was significantly delayed in flavodoxin-containing nodules with respect to those elicited by WT bacteria. Flavodoxin can replace ferredoxin in the photosynthetic electron transport from photosystem I to  $\text{NADP}^+$  and in nitrogenase reduction (Sandmann et al. 1990). Blanco et al. (2011) have shown that cyanobacterial flavodoxin expression can complement ferredoxin deficiency in transgenic ferredoxin-deficient tobacco lines. In alfalfa nodules, flavodoxin might increase effectiveness of the nitrogenase complex by compensating the ferredoxin decline that occurs under osmotic stress (Zimmermann et al. 2004; Tognetti et al. 2006). Flavodoxin might also confer tolerance to salinity stress in the nodule by preserving the ROS detoxification systems. Salinity stress can induce nodule senescence through ROS generation (Swaraj and Bishnoi 1999; Puppo et al. 2005), and the presence of flavodoxin appears to have a facilitating effect on ROS detoxification. Tognetti et al. (2006) showed that oxidative damage decreased in stressed tobacco plants harbouring a functional cyanobacterial flavodoxin when exposed to oxidants as compared to wild-type plants. We recently reported that alfalfa nodules elicited by flavodoxin-overexpressing rhizobia show delayed nodule senescence (Redondo et al. 2009) and







**Fig. 3** Transmission electron micrographs showing nodules elicited by wild-type (a–g) and flavodoxin-expressing (h–n) rhizobia. Plants were treated with NaCl (100 mM) for 3 days. **a, b** Images of zone II of a wild-type nodule showing cells with infection thread (*it*), symbiosomes with large peribacteroid spaces (*sp*) harbouring type 1 bacteroids (*b1*), vacuole (*v*) and cell wall (*cw*). Note the high vesicle proliferation (*black arrow*) in cytoplasm. **c** Cell in proximal zone II displaying bacteroids type 1 and type 2 (*b2*) and altered bacteroids with vesicle-like structures (*white arrow*). **d, e** Cells of interzone II/III showing normal type 3 bacteroid (*b3*), altered symbiosomes with vesicles (*white arrow*) and abnormal mitochondria (*m*). Plastids without starch (*p*) and small starch granules (*s*) in infected and interstitial (*ic*) cells. **f** Image showing an affected infected cell of distal zone III in which only a bacteroid (*b*) is recognizable. **g** Cells of proximal zone III showing altered mitochondria and type 4 bacteroid (*b4*) with abundant vesicles. **h, i** Images of zone II of a flavodoxin-expressing nodule showing infection thread and normal ultrastructures of type 1 and type 2 bacteroids, mitochondria, plastid, vacuole and endoplasmic reticulum (*er*). **j** Interzone II/III displaying normal mitochondria, amyloplasts with starch granules and type 3 bacteroids. **k** Cells of distal zone III showing apparently unaffected mitochondria, type 4 bacteroid and amyloplasts with the starch content attributable to the flavodoxin expression. **l–n** Cells of proximal zone III showing characteristic starch accumulation in the flavodoxin-containing nodules and the type 4 bacteroid ultrastructure. Note the presence of an altered bacteroid

enhanced tolerance to cadmium toxicity (Shvaleva et al. 2010), due to flavodoxin-induced changes in the antioxidant metabolism.

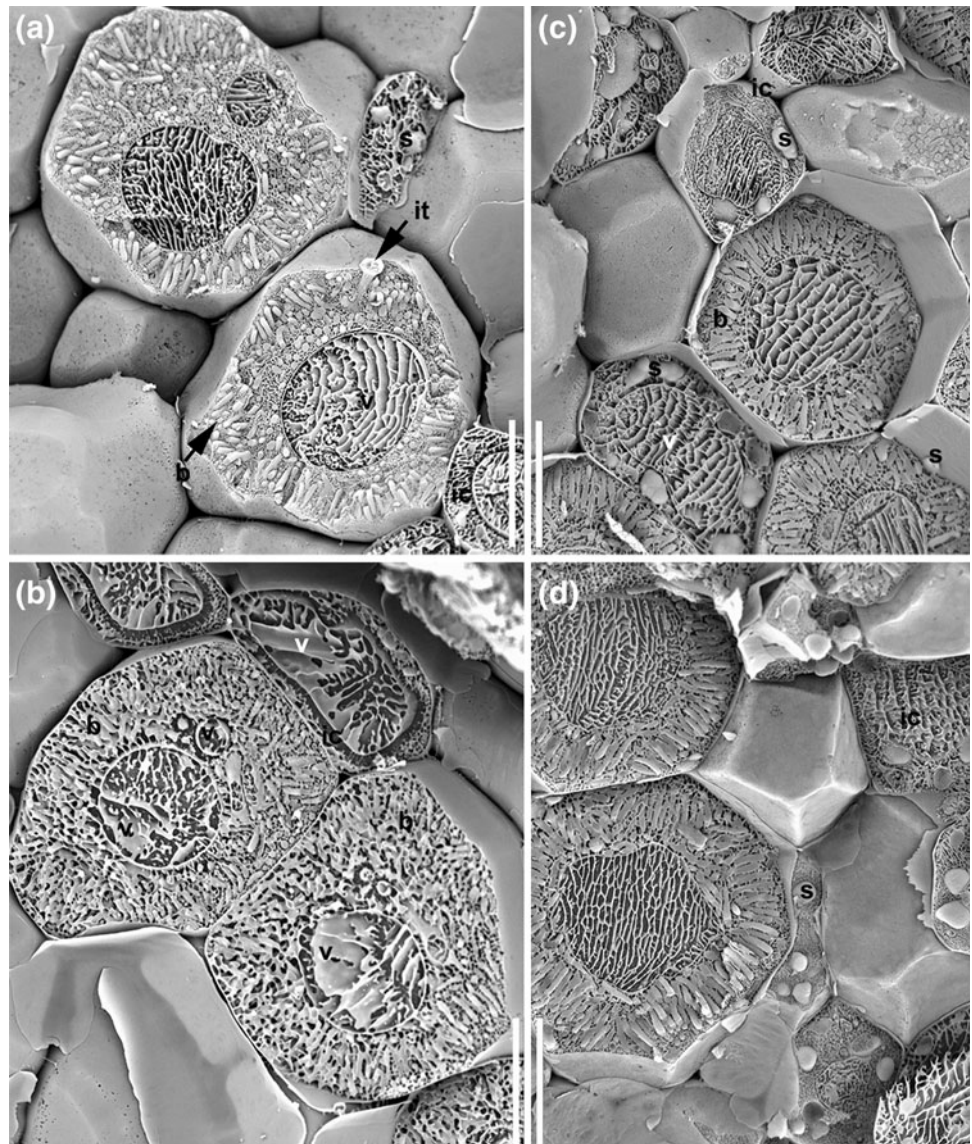
Superoxide dismutase and catalase are the first line of defence against ROS in the nodule (Matamoros et al. 2003; Marino et al. 2009). Flavodoxin expression nonetheless did not have a protective effect on SOD and catalase activities under salinity stress. The ascorbate–glutathione cycle acts as an alternative H<sub>2</sub>O<sub>2</sub> scavenging system and is important for maintaining nitrogen fixation in nodules (Matamoros et al. 2003). A decrease in activity of enzymes of the ascorbate–glutathione cycle has been observed in legume nodules subjected to salinity stress (Tejera et al. 2004; Jebara et al. 2005). We found that activity of these enzymes decreased in alfalfa nodules under saline conditions. Flavodoxin expression had a protective effect on APX and GR activities, which were significantly higher in flavodoxin-containing than in WT-stressed nodules. Salinity stress induces a decrease in GSH levels in the nodule (Comba et al. 1998); increased GSSG levels and a decreased GSH/GSSG ratio have been reported in dark- and nitrate-stressed nodules (Matamoros et al. 1999; Hernández-Jiménez et al. 2002). GSH levels were significantly higher in flavodoxin-expressing nodules than in WT nodules, independently of salinity stress. In saline conditions, flavodoxin-containing nodules showed significantly lower GSSG levels and a higher GSH/GSSG ratio than WT nodules. Increased effectiveness of the ascorbate–glutathione cycle might have a protective effect on nodule structure and function under salinity stress.

Different lines of evidence demonstrate that antioxidants produced by bacteria play an important role in the maintenance of symbiosis. SOD activity-defective and GSH synthesis-deficient rhizobia have been reported to induce premature nodule senescence (Santos et al. 2000; Muglia et al. 2008). We have previously reported that flavodoxin-expressing bacteroids induce changes in the nodule antioxidant metabolism leading to a delay in nodule senescence (Redondo et al. 2009). In the present work, our results suggest that the redox balance in flavodoxin-expressing bacteroids does have a direct effect on the nodule host cell that translates into an improved tolerance to saline stress.

The carbon metabolism enzymes SS and PEPC are essential for nitrogen fixation (King et al. 1986; Rosendahl et al. 1990; Gordon et al. 1999). Salt treatment induces specific PEPC activity in pea nodules (Delgado et al. 1993) and increased PEPC activity in *M. truncatula*, *Cicer arietinum* and *Lotus japonicus* nodules (Soussi et al. 1998; Verdoy et al. 2006; López et al. 2008). We also found increased PEPC activity in salt-stressed alfalfa nodules; flavodoxin expression apparently had no influence on this activity.

Sucrose synthase activity is required for the establishment and maintenance of efficient nitrogen-fixing symbiosis, at least in indeterminate nodules (Baier et al. 2007; Marino et al. 2008). SS activity and SS mRNA transcripts decrease in nodules subjected to several types of stress, including salinity stress (Fernández-Pascual et al. 1996; Gordon et al. 1997; Verdoy et al. 2006; López et al. 2008; Ben Salah et al. 2009). Differences in the SS activity of nodules from several legume species or lines have been related to the grade of sensitivity to salinity (López et al. 2008; Ben Salah et al. 2009). We observed a decline of SS activity in both types of nodules following salinity stress; two-way ANOVA indicated that flavodoxin does not exert a protective effect on SS activity. However, SS activity was significantly higher in flavodoxin-expressing nodules compared to WT nodules independent of salinity stress. This relatively elevated SS activity could be partly responsible for maintaining nitrogen fixation under stress, and might be related to the amelioration of the oxidative balance observed in flavodoxin-containing nodules. In indeterminate pea nodules, regulation of nodule SS by the cellular redox state at both the transcriptional and post-transcriptional level has been described (Marino et al. 2008). In nodules of roots treated with paraquat (a compound that provokes ROS overproduction), SS gene expression was downregulated and led to a decrease in SS levels and activity that preceded inhibition of nitrogen-fixing activity (Marino et al. 2008). In non-stressed plants, flavodoxin-containing nodules displayed a more positive redox balance and higher SS activity than WT nodules.

**Fig. 4** STEM micrographs showing cells of the nitrogen-fixing zone III of nodules elicited by wild-type (**a, b**) and flavodoxin-expressing (**c, d**) rhizobia. Plants were treated with 0 mM NaCl (**a, c**) or 100 mM NaCl (**b, d**) for 3 days. Nodules overexpressing flavodoxin showed high starch accumulation (**c, d**). Note the ultrastructural damage in bacteroids and vacuoles in salt-stressed WT nodule cells (**b**). *b* bacteroid, *ic* interstitial cell, *it* infection thread, *v* vacuole, *s* starch. Bars 20  $\mu\text{m}$

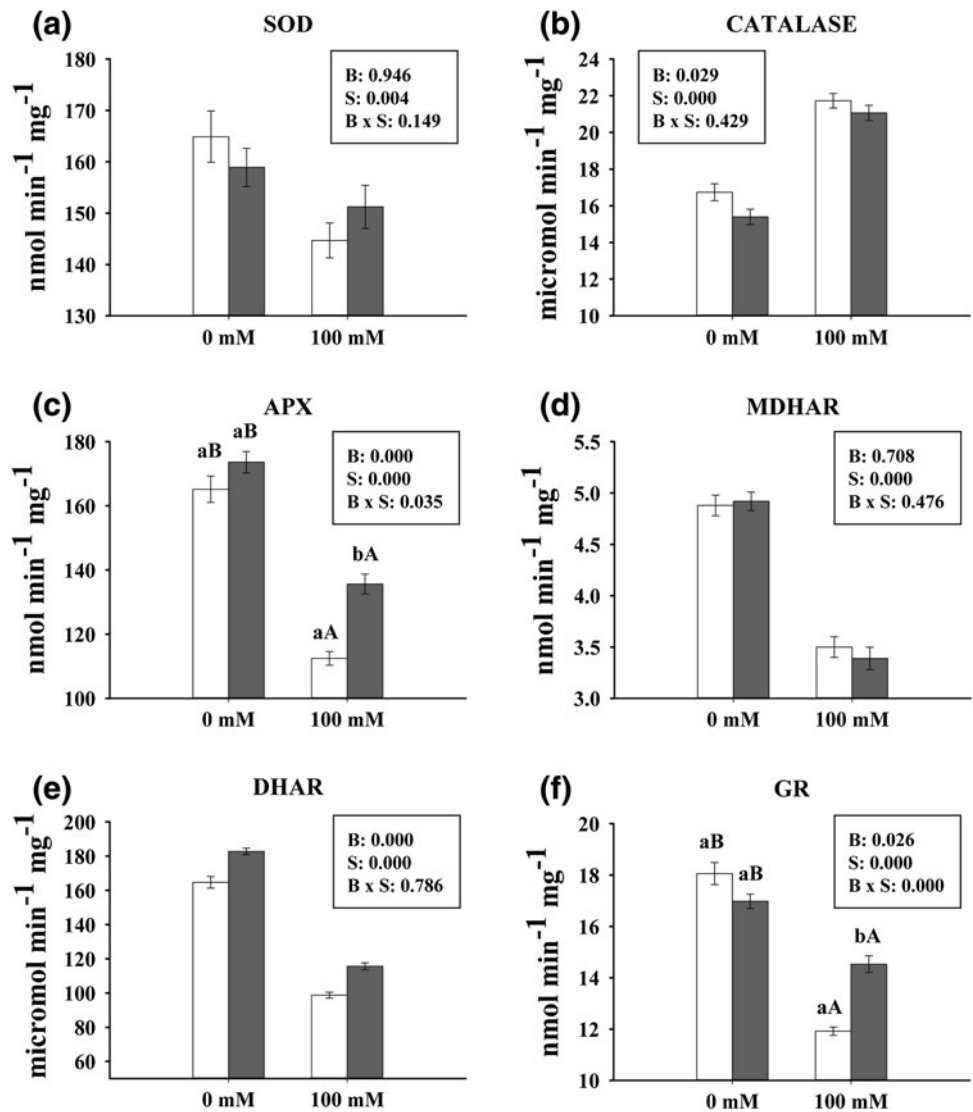


Sucrose synthase activity could contribute to the unusual starch accumulation in cells of flavodoxin-expressing nodules, a feature that is maintained under salt as well as cadmium stress (Shvaleva et al. 2010). Although variations in starch accumulation are characteristic of different nodule development stages and stress conditions (Vasse et al. 1990; Lucas et al. 1998), starch metabolism has barely been studied in nodules. SS might have a role in nodule starch synthesis, as reported in certain other tissues (Zrenner et al. 1995; Baroja-Fernandez et al. 2009). Our results and the observation that starch levels decrease in parallel with the decline of SS activity in *L. albus* and *M. truncatula* nodules in response to salinity stress (Fernández-Pascual et al. 1996; López et al. 2008) support this hypothesis. Based on our data, it is tempting to conjecture that in alfalfa nodules formed by flavodoxin-expressing rhizobia, amelioration of the oxidative balance

provokes an increase in SS activity that, by maintaining the glycolytic flux into the bacteroids, might lead to starch accumulation and to increased tolerance to oxidative stress.

Alfalfa nodules showed structural and ultrastructural modifications when subjected to salinity stress. The ultrastructural alteration of bacteroids and other cell organelles is a gradual process in nodules subjected to salinity, and variations in biochemical parameters such as enzymatic activities, precede detectable ultrastructural damage. A similar situation has been described in nodules subjected to different stresses or during nodule aging (de Lorenzo et al. 1990; Matamoros et al. 1999; Redondo et al. 2009). The effects of salinity on nodule structure and ultrastructure have been studied in pea, lupin and soybean. Alterations in the cortex (related mainly to the oxygen diffusion barrier) and in the nitrogen-fixing zone in nodules in saline conditions have been described (James et al. 1993; Serraj et al.

**Fig. 5** Antioxidant enzymes superoxide dismutase (a) and catalase (b), and ascorbate–glutathione cycle enzymes ascorbate peroxidase (c), monodehydroascorbate reductase (d), dehydroascorbate reductase (e) and glutathione reductase (f) activities in WT (white bars) and flavodoxin-expressing (grey bars) nodules, after 3 days treatment with 0 or 100 mM NaCl. Values are mean ± SE of three experiments ( $n = 10$  per experiment).  $P$  values corresponding to two-way ANOVA for the factors bacterial strain ( $B$ ), salinity stress ( $S$ ) and interaction between both ( $B \times S$ ) are displayed. In the case of significant interaction (APX and GR), one-way ANOVA ( $P \leq 0.05$ ) was separately performed for each factor. For each bacterial strain, different capital letters indicate significant differences between salinity treatments; for each salinity treatment, different lower case letters indicate significant differences between bacterial strains



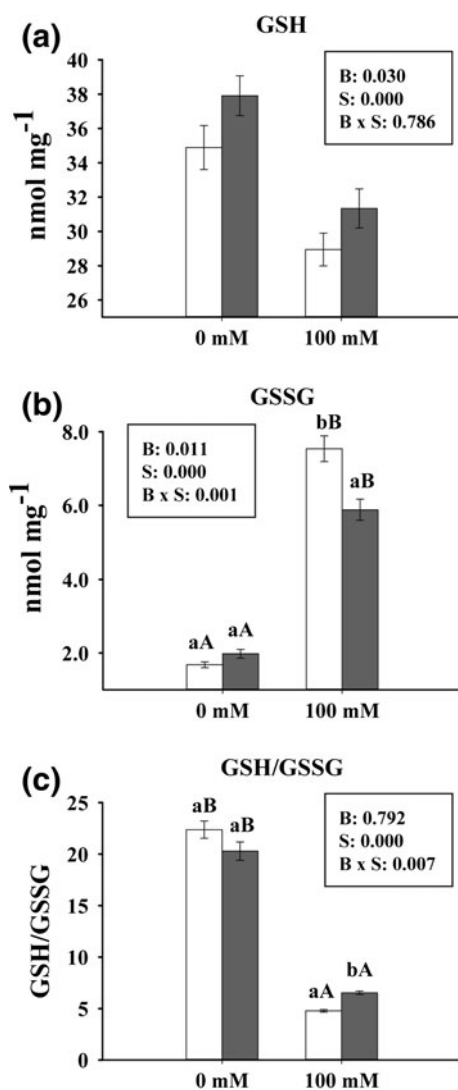
1995; Fernández-Pascual et al. 1996; Verdoy et al. 2004, 2006; Borucki and Sujkowska 2008). To our knowledge, however, this is the first study to describe in detail the effects of salinity stress on the different nodule zones and their bacteroid types in indeterminate nodules.

In WT nodules, young tissues (zones I, II and distal zone III) showed a greater degree of structural alterations than mature tissues. In salt-stressed nodules, abnormal infection threads and accumulation of bacteria were found in cells of zone II, suggesting alterations in rhizobial release, probably due to partial or total stop of meristematic activity in zone I. Cells in zone II and interzone II/III were extremely damaged; interzone II/III appeared most sensitive to salinity stress. Bacteroid differentiation seemed to be severely affected in zone II. Our results suggest that not fully differentiated bacteroids (types 1, 2 and 3) are more susceptible to salinity stress than mature bacteroids (type 4). Rhizobial cell wall components such as

lipopolysaccharides (LPS) might be responsible. LPS changes have been detected during bacteroid differentiation (Kannenberg and Carlson 2001); in addition, rhizobial LPS changes are reported in response to salinity stress (Lloret et al. 1995). Whether the LPS of type 1, 2 and 3 bacteroids from zone II to distal zone III are more sensitive to salinity stress than the LPS of type 4 bacteroid remains to be determined. In our experimental conditions, NaCl led to degeneration of the symbiosome, characterised mainly by the presence of vesicle-like structures in the rhizobial cytosol, which fused with the bacterial membrane in all types of bacteroids. Salt-stressed nodules nonetheless did not show the symbiosome fusion observed following cadmium stress (Shvaleva et al. 2010) or during natural senescence of nodules (Hernández-Jiménez et al. 2002; Redondo et al. 2009).

In nodules induced by flavodoxin-expressing bacteria, salt-induced alterations in structure and ultrastructure were

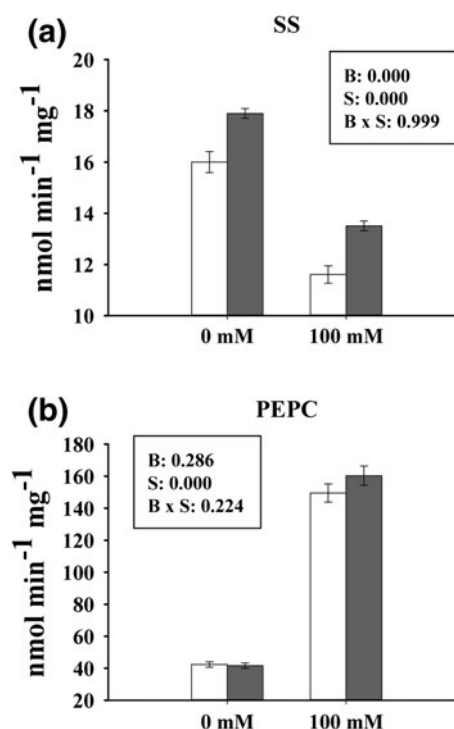




**Fig. 6** Determination of reduced glutathione (a), oxidized glutathione (b), and GSH/GSSG ratio (c) in WT (white bars) and flavodoxin-expressing (grey bars) nodules, after 3 days treatment with 0 or 100 mM NaCl. Values are mean  $\pm$  SE of three experiments ( $n = 10$  per experiment).  $P$  values corresponding to two-way ANOVA for the factors bacterial strain ( $B$ ), salinity stress ( $S$ ) and interaction between both ( $B \times S$ ) are displayed. In the case of significant interaction (GSSG and GSH/GSSG ratio), one-way ANOVA ( $P \leq 0.05$ ) was separately performed for each factor. For each bacterial strain, different capital letters indicate significant differences between salinity treatments; for each salinity treatment, different lower case letters indicate significant differences between bacterial strains

much less severe than those in nodules elicited by WT rhizobia. These results, taken together with the positive effect on the nodule redox balance and on the nitrogen-fixing activity, strongly suggest that overexpression of flavodoxin provides enhanced tolerance to salinity stress in alfalfa nodules.

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**Fig. 7** Nodule carbon metabolism enzymes sucrose synthase (a) and phosphoenol pyruvate carboxylase (b) activities in WT (white bars) and flavodoxin-expressing (grey bars) nodules, after 3 days treatment with 0 and 100 mM NaCl. Values are mean  $\pm$  SE of three experiments ( $n = 10$  per experiment).  $P$  values corresponding to two-way ANOVA for the factors bacterial strain ( $B$ ), salinity stress ( $S$ ) and interaction between both ( $B \times S$ ) are displayed

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