Title: **APX and Fe-superoxide dismutase families differently response to stress and senescence in legumes**

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**Research Highlights**

- Two differentially localized sub-families of FeSODs are expressed in determinate nodule-forming legumes  
- Cytosolic and plastidial FeSODs showed very different patterns of response to stress and senescence.  
- Both subfamilies are expressed in nodules, but also in roots and leaves  
- Cytosolic FeSOD is the main protein in legumes and increases its contents and activity with ageing, especially in leaves.
Graphical abstract

Soybean / Cowpea
6 stress conditions
and Ageing

Immunoblot (3 Ab)
In-gel assays

Different subfamilies
with clearly
different expression
patterns

MnSODs

Plastidial CuZnSODs

Cytosolic CuZnSODs

Plastidial FeSODs

Cytosolic FeSODs

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Different subfamilies
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Title: Two Fe-superoxide dismutase families differently response to stress and senescence in legumes

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Abstract

Three main families of SODs may be distinguished in plants, according to the metal in the active center: CuZnSODs, MnSOD, and FeSOD. CuZnSODs have shown two sub-families localized either in plant cell cytosol or in plastids, the MnSOD family is essentially restricted to mitochondria, while FeSOD enzyme family has been typically localized into the plastid. Herein, we describe, based on a phylogenetic tree and experimental data, the existence of two FeSOD sub-families: a plastidial localised sub-family, which is universal to plants; and a cytosolic localised FeSOD sub-family observed in determinate-forming nodule legumes. Anti-cytosolic FeSOD (cyt_FeSOD) antibodies have been employed, together with a novel antibody raised against plastidial FeSOD (p_FeSOD). Stress conditions, such as nitrate excess or drought, markedly increased cyt_FeSOD contents in soybean tissues. Also, cyt_FeSOD content and activity increased with age in both soybean and cowpea plants, while cyt_CuZnSOD isozyme was predominant during early stages. P_FeSOD in leaves decreased with most of the stresses applied but this isozyme clearly increased with abscisic acid in roots. The great differences observed for p_FeSOD and cyt_FeSOD contents in response to stress and aging in plant tissues reveals a distinct functionality, and confirm the existence of two immunologically differentiated FeSOD sub-families. The in-gel FeSOD activity patterns showed a good correlation to cyt_FeSOD contents but not to those of p_FeSOD. This fact remarkably indicates that the cyt_FeSOD is the main active FeSOD in soybean and cowpea tissues. The diversity of functions associated to the complexity of FeSOD isoenzymes depending of the location is discussed.

Keywords:
Abiotic stress, antioxidant, ascorbate peroxidase, cowpea, iron superoxide dismutase, reactive oxygen species, soybean.
Abbreviations:

APX, ascorbate peroxidase; cyt_APX, cytosolic ascorbate peroxidase; cyt_FeSOD, cytosolic FeSOD; p_FeSOD, plastidial FeSOD; PVP-10, polyvinylpyrrolidone-10; ROS, reactive oxygen species; SOD, superoxide dismutase; Vu_FeSOD, cytosolic Fe-SOD from cowpea (Vigna unguiculata).
INTRODUCTION

Reactive oxygen species (ROS) are continually generated by the cell metabolism in different cellular compartments in plants (Apel and Hirt 2004). Indeed, ROS production increases during certain physiological processes, including respiration, photosynthesis or biological N₂ fixation (Dalton 1995). Although ROS are eminently harmful, at low levels they are known to act as important signalling molecules that facilitate an array of essential biological process, such as growth and development, seed germination, programmed cell death, etc. (Dowling and Simmons 2009). Two systems are, therefore, needed to control the intracellular concentration of ROS: one that allows an accurate signalling function and another with the capacity to remove any excess of these species (Marino et al. 2009). Superoxide dismutases (SODs) are a primary antioxidant barrier against ROS due to their ability to dismutate the superoxide anion into hydrogen peroxide, which is subsequently eliminated in a catalytic process involving enzymes, such as the ascorbate peroxidase (APX), present in photosynthetic organisms ranging from *Euglena* to higher plants (Ishikawa and Shigeoka 2008). The expression of both enzymes, SOD and APXs, has been shown to increase concomitantly under stress conditions such as drought, high salt concentration, ozone, high CO₂ and ionizing radiation (Bowler et al. 1992, Bowler et al. 1994, Ishikawa and Shigeoka 2008).

Three families of SODs, namely copper-zinc (CuZnSOD), manganese (MnSOD) and iron (FeSOD) are found in plant tissues, all of which have different molecular properties, including differential sensibility to inhibitors (Bowler et al. 1992). While two families of CuZnSODs are clearly established in the cytosol and the chloroplasts of plants (Kanematsu and Asada 1991; Bowler et al. 1992), CuZnSOD activities have also been detected in extracellular apoplast from different plant tissues (Karpinska et al. 2001) and in peroxisomes and glyoxysomes (Pastori and del Rio 1997; Corpas et al. 1998). MnSOD is a constitutive antioxidant enzyme in all mitochondria and also in peroxisomes of pea (Pastori and del Rio 1997). FeSOD is probably the least studied of the three SOD-isozymes in plants. Animals and fungi lack FeSOD, as this enzyme has a photosynthetic prokaryote origin. FeSOD have long been thought to be
located essentially in the plastid organelle within the plants or algi (Van Camp et al. 1990, Bowler et al. 1994, Karpinski et al. 1997) but some exceptions occurs, as a non-chloroplastic FeSOD isozyme located into the mitochondria in wheat (Khanna-chopra and Sinha 1988, Srivalli and Khanna-Chopra 2001), as well as, in the nucleus of *Sesbania rostrata* in association with chromatin (Rubio et al. 2009). In legume nodules, an original group of FeSODs with cytosolic localization (cyt_FeSODs), has been detected in soybean and cowpea plants and its function was associated, among others, to the nitrogen fixation process (Moran et al. 2003). Accordingly, a FeSOD isozyme from *Anabaena* has been found to be induced during heterocyst differentiation and *N* fixation process (Li et al. 2002), whereas in dinoflagellates FeSOD protects against metal toxicity (Okamoto et al. 2001). All this indicates that FeSOD proteins are more widely distributed in plant tissues than it was initially thought.

Furthermore, the genomic of FeSOD in plants also show complexity. Several copies of FeSOD gene may be present in the genome of plants such as *Arabidopsis thaliana*, which contain at least three different genes for FeSOD (Kliebenstein et al. 1998), while a multigene family is observed in the alfalfa genome (Rubio et al. 2009). Soybean and cowpea belong to the group of legumes of tropical and subtropical origin which form nodules with a determinate growing pattern. However, in indeterminate nodules, where the meristem continually grows, such as those of pea or the genus *Medicago*, cyt_FeSODs of plant origin have not been found yet. Recently, our group has evidenced the importance in pea plants of a member of the Fe/MnSOD family from the rhizobial symbiont into the cytosol of plant cells and other subcellular localizations as nucleus within nodule cells. These localizations emphasize the important role that FeSOD have in the management and signalling of reactive oxygen species (ROS) for the proper functioning of the plant-bacteria symbiosis (Asensio et al. 2011).

On the other hand, plastidial FeSODs (p_FeSOD) are widespread in legumes, from where we cloned and sequenced a partial cDNA containing a chloroplastic transit peptide from a cDNA pea library (Moran et al. 2003). The p_FeSOD are known to be part of the antioxidant response against abiotic
stress in plastids and chloroplasts (Gómez et al. 2004, Ariz et al. 2010). However, the members of both FeSOD groups, cytosolic and plastidial, have been never studied together in higher plants. Herein we present a comparative study of the contents and activities of plastidial and cytosolic FeSOD isoenzymes in different tissues of the legumes soybean (Glycine max) and cowpea (Vigna unguiculata). Soybean is a legume of agronomic interest with a good antioxidant system, while cowpea, because of the more simplified genome, lack the multiplicity of FeSODs isoforms observed in soybean. We show evidence for the existence of two FeSOD sub-families in legume plants with remarked different functions.
2. RESULTS

2.1. Two FeSOD sub-families in legumes.

While plant FeSOD are reportedly to be localized in chloroplasts and plastids, a cDNA gene coding for a FeSOD with cytosolic localisation was characterized from the legume cowpea (Vigna unguiculata; Moran et al. 2003).

To elucidate the relationships within FeSODs and with other SODs in legumes, we have constructed a more detailed phylogenetic tree based on the known SODs protein sequences (Fig. 1). Three branches can be clearly observed in the tree, one for each family of SODs, Mn-, CuZn- or Fe- containing SODs. In the case of CuZnSODs and FeSODs two different sub-families, cytosolic and plastidial, are observed. The two well established sub-families of Cu-ZnSODs can be observed (Fig. 1). It is remarkable that while the cytosolic FeSOD-type genes are only reported in determinate nodules forming legumes as cowpea, soybean or Lotus, the plastidial FeSOD sub-family is formed also by members of the indeterminated-nodule forming legumes as pea or alfalfa.

In order to follow the expression of the cytosolic FeSODs, a characterized antibody against cyt_FeSOD of cowpea plants (Moran et al. 2003) has been used. We have also raised in rabbit a novel anti-p_FeSOD based on Psat4 cDNA from pea which has been reported to exhibit a chloroplast transit peptide (Fig. 1) (Moran et al. 2003). A 15-mer peptide (200-214: VNPLATEEDKKLVVL) was selected from its exposed position, the distance to the dimmer interface, the predicted immunogenicity and its remarkable differences to other SODs present in the legumes (Fig. S1). In order to check the specificity of the antibody to recognize plastidial FeSODs, the anti-p_FeSOD was tested against leaf extracts from legumes and other species. Thus, pea and spinach plants both showed bands at 36 and 38 kDa, whereas the determinate-nodule forming legumes (cowpea and soybean) showed only one band at a molecular weight around 25 kDa (Fig. 2). Wheat extracts exhibited only one band at approximately 30 kDa. Anti-p_FeSOD did not cross-react with 500 ng of cowpea cytosolic FeSOD protein (Vu_FeSOD) used as negative control (Fig. 2, lane 6). In order to check the different size of the cyt_FeSOD and p_FeSOD, a sample of either soybean or cowpea leaves was
loaded in a SDS-PAGE into two parallel wells. Both wells were transferred to a PVDF membrane, which was cut and probed each with either anti-cyt_FeSOD or anti-p_FeSOD. Although, both FeSOD isozymes showed similar molecular weights for the monomer subunit, the immuno-reactive bands migrated at different height in the gel for both soybean or cowpea samples, pointing out that the antibodies are recognizing different proteins (Fig. S2).

2.2. p_FeSOD and cyt_FeSOD under stress conditions in soybean

We have studied the response of both Fe-SOD sub-families when plants were submitted to different stress conditions and natural senescence (aging). The senescence condition was assayed in both cowpea and soybean species, while we decided to focus the stress conditions only in one specie, soybean, because of its agronomic importance. Single bands were detected by immunoblot around 25-28 kDa for p cyt_FeSOD, and FeSOD proteins (Figs. 3). In the case of the FeSOD a differential content pattern and response was found for both anti-cyt_FeSOD and the anti-p_FeSOD antibodies. The cyt_FeSOD content was markedly affected by the stress conditions (Fig. 3). Almost all the stress conditions increased cyt_FeSOD content relative to their controls. Excess nitrate increased the cyt_FeSOD content in leaves, in nodules and, especially in roots, whereas drought induced the highest content in leaves and, to a lesser extent, in roots. Darkness induced the cyt_FeSOD signal in all three organs, whereas salt stress only induced such a signal in leaves and nodules. The induction of cyt_FeSOD content by abscisic acid was notable in all organs tested (Fig. 3).

An immune response to anti-p_FeSOD found in control tissues of three organs studied (Fig. 3), show that this protein is not only restricted in chloroplast but also in other plastids. While most stress conditions generate a decrease in the immunoreactive signal of p_FeSOD in leaves, it disappeared completely under drought stress. To the contrary, high irradiance induced higher contents (Fig. 3). Both nodules and roots were mostly unaffected by these conditions, except for darkness stress in nodules, which resulted in a lower expression of the protein. An induction of p_FeSOD content by abscisic acid was observed in nodules, and to a greater extent in roots (Fig. 3).
As the cytosolic localization for a FeSOD is certainly original, we have studied if the contents and activities of the cyt_SOD may relate in some way to the function of APX in the cytosol. The contents of cyt_APX in soybean tissues were studied by western immunoblot performed with anti-cyt_APX antibody (Dalton 1995). The immunoblot signal for the cytosolic APX protein in leaf, nodule and root samples showed small changes under the different conditions tested (Fig. S3). Contents of cyt_APX showed slight higher contents in leaves under high irradiance and drought, in nodules under darkness, high irradiance, nitrate excess or ABA, and in roots when the plants were subjected to darkness or high irradiance. If we compare the contents of soybean cyt_APX during the response to stresses we may appreciate a certain degree of correlation to nodule cyt_FeSODs (Fig. S3 and Fig. 3), while it showed fully divergent from the response obtained in p_FeSOD contents for the tested tissues and stresses (Fig. 3).

2.3. Effect of ageing on soybean and cowpea cyt_FeSOD and p_FeSOD

Leaves, nodules and roots from soybean and cowpea exhibited notable differences in cyt_FeSOD contents and to a lesser extent in p_FeSOD during ontogeny (Fig 4). Thus, the western immunoblot indicated an increase in cyt_FeSOD content in soybean and cowpea plants with natural senescence, increasing from practically undetectable in young samples of all three organs to a much higher response in older tissues, being particularly notable the increase in plant leaves (Fig. 4).

In soybean organs the highest cyt_FeSOD content was found in leaves. The contents of p_FeSOD in soybean showed a less clear pattern, as the content of this isozyme was both age- and organ-dependent. Thus, the p_FeSOD content was notably higher in roots than in the other organs and stable for all developmental stages (Fig. 4), whereas p_FeSOD content in leaves decreased with maturity and senescence. The contents of p_FeSOD remained low and invariable in all three stages in soybean nodules.
In cowpea plants, western immunoblot also showed the highest cyt_FeSOD content in leaves as shown for soybean especially during senescence (Fig. 4). In contrast, p_FeSOD showed low difference in their contents during the ageing process in the three organs, showing only a decrease in mature roots, and a new band at 20 kDa in mature leaves which has been observed in some other replicates and may correspond to protein degradation.

2.4. Effect of ageing on soybean and cowpea total SOD activities

Four different SOD isoenzymatic activities were found in soybean and cowpea tissues (Fig. 5 and Fig. 6). However, most of the SOD isoenzymes of soybean split in several bands of similar mobility. In order to assign a SOD family to the different activities found in gel, a series of inhibition experiments were performed using KCN and H$_2$O$_2$. As described previously (Moran et al. 2003) KCN inhibited only CuZnSOD activities and H$_2$O$_2$ inhibited both CuZnSOD and FeSOD isozymes, whereas MnSOD was not affected for any inhibited treatment. According to previous work, and the inhibition experiment (Fig. 5), the activities observed in soybean samples corresponded, from top to bottom, to MnSOD, FeSOD, cyt_CuZnSOD and p_CuZnSOD (Fig. 5 and 6). For MnSOD two bands showed up, three bands for FeSOD, four bands for cyt_CuZnSOD and two activity bands for p_CuZnSOD. In soybean, MnSOD remained almost constant irrespective of ageing and organ with the exception of young and mature roots which showed somewhat lower contents (Fig. 5). FeSOD activity matched perfectly with the cyt_FeSOD content pattern shown by western blot (Fig. 4), including age-related changes.

In contrast with the soybean multiple bands patterns (Fig. 5), cowpea tissues showed a well-defined \textit{in-gel} isoenzyme pattern and provides a better comparison of the SOD isoenzymatic contents and activities (Fig. 4 and Fig. 6). These activities have been named previously for cowpea (Moran et al. 2003) as it is marked in Fig. 6. Thus, the \textit{in-gel} FeSOD activity observed for cowpea samples (Fig. 6) matched the cyt_FeSOD content detected by western blot (Fig.
4), increasing its activity with the ageing, as shown for soybean tissues. Furthermore, both CuZnSOD activities changed drastically, being higher in young tissues than in mature or senescent samples in both cases, showing a profile opposite to that found for FeSOD activity. However, putative MnSOD activity was modified only slightly by ageing, with the exception of an increase in mature and older nodules.

3. DISCUSSION

3.1. Specificity of the novel anti-p_FeSOD antibody against plastidial FeSODs.

Three main branches corresponding to the three isoenzymatic groups of SODs are depicted in the phylogenetic tree among different species of legumes. The tree shows one family of MnSOD, two subfamilies within CuZnSODs the plastidial and cystolic, and also two subfamilies of FeSODs. This branching provides new evidence for the classification of FeSODs into two different subfamilies. The structural and phylogenetic relationships among FeSODs and MnSODs are manifested by the closer proximity respect to the unrelated CuZnSOD. The high number of *Glycine max* SOD genes, which contains two sequences for almost every subfamily, evidences the high diversity and complexity of the SOD isozyme system function in legumes. This complexity has also been observed in the *FeSOD* gene copy number in Southern blots performed with *fesod* cDNA as a probe on alfalfa plants (Rubio et al. 2009).

The plastidial FeSODs share structural similarities with other members of the Fe/MnSOD family, such as cyt_FeSOD (Muñoz et al. 2005). However, anti-cyt_FeSOD antibody did not recognize plastidial FeSOD in either pea, or alfalfa plants (Moran et al. 2003), which indicates that important differences in the structure are present among cytosolic and plastidial FeSODs. In this study, we have produced a single epitope antibody against a pea chloroplastic FeSOD protein (Acc. #. Q7XHK3; Moran et al. 2003), which detected one band in both legumes soybean and cowpea, and in wheat. In pea plants, we detect a strong band at approx. 38 kDa, and a minor band at higher size (Fig. 2). These results agree with previous work by Gómez et al. (2004) who identified two FeSODs
isozymes in chloroplasts, one stromal and one thylakoidal, also being the stromal the smaller of both FeSOD isozymes (Gómez et al. 2004). Also, two faint bands can be seen in the non-leguminous spinach plants (Fig. 2).

In our work, the immunoblots with anti-cyt_FeSOD and anti-p_FeSOD antibodies have showed that two FeSOD proteins with different molecular weights are present in plant legume tissues under stress conditions (Figs. 3 and S2). Cyt_FeSOD has a known MW of 28.6 KDa per subunit. The p_FeSOD has showed a molecular weight slightly above the cyt_FeSOD, in the proximity to 30-32 KDa. (Fig. S2)

The lack of reactivity with high quantities of Vu_FeSOD protein (Fig. 2, lane 6), indicates a high specificity of this antibody to the p-FeSOD family. The recognition of two bands in pea indicates a good sensitivity and specificity as p_FeSOD is present in plant in low amounts. Plastidial FeSODs analysis by western immunoblot has previously required purification of chloroplasts, thylakoids or stroma as a prior step, thus loading at least 100 μg of protein from purified chloroplastic samples (Gómez et al. 2004). Interestingly, along the present work p_FeSOD was found in all three organs tested, confirming a role of p_FeSOD in non-green tissues.

3.2. APX and FeSOD contents under stress conditions in soybean plants

An increase in cytosolic APX expression levels under excess of light, high salt, paraquat and drought has been described previously, although this increase did not result in significant increases in protein content, as detected by western immunoblot or peroxidase activity (Mittler and Zilinskas 1992, Karpinski et al. 1997, Mittler 2002). In accordance with this mentioned works, no important changes in APX contents were detected in our experiments by immunoblot techniques, except for small increases under darkness and high irradiance stress in root samples. A relation of cyt_APX protein contents with cyt_FeSOD contents may be taking place in soybean nodules, where the high activity of APX suggests that APX provides an essential protective action in processes related to nitrogen fixation (Dalton 1995). The unparalleled response of cyt_FeSOD and p_FeSOD contents in other tissues respect to APX contents reveals a complex functionality of FeSOD isozymes. It is also noteworthy that
our experiment was performed after seven day of stress conditions, where a short-term stress response may be overlooked.

Moreover, the fact that the expression profiles obtained for anti-cyt_FeSOD and anti-p_FeSOD were highly differential among them confirmed that no cross-reaction of proteins occurs and evidenced that different function and regulatory mechanisms may be taking place (Figs. 3 and 4). Both findings confirm the existence of two FeSOD sub-families with different regulatory roles and specific functions. Darkness is a stress known to importantly contribute to senescence in cowpea and soybean plants and it also increased the contents of cyt-FeSOD compare to control in all organs (Fig. 3). This is a novel finding for cyt_FeSODs, as FeSOD are usually involved in high irradiance protection during the water-water cycle (Soitamo et al. 2008). Different conditions may lead to higher superoxide production under dark stress; i.e., mitochondrial respiration activity, which replaces chloroplastic activity as a cellular energy source under such conditions. This would affect all the tissues of the plant and may explain the results observed. Also, we cannot exclude that the destruction of cellular integrity and Lb protein, with concomitant onset of oxidative stress may be taking place in nodules (Moran et al. 2003). In contrast, p_FeSOD decreases its activity under dark conditions (Fig 3) probably related to a lesser total antioxidant content in plastids. A low induction of the chloroplastic FeSOD gene under dark conditions has been reported for *Arabidopsis thaliana* (Soitamo et al. 2008), although this result was not checked by immunoblot analysis.

In our study, high irradiance slightly increase p_FeSOD amount in leaves and roots (Fig. 3). Plastidial FeSOD in pea plants is also known to be induced by high irradiance conditions (Ariz et al. 2010). These findings are in accordance with the chloroplastic location of this enzyme, where superoxide generation is linked to the photosystem II activity. High irradiation therefore increases chloroplastic activity, and hence superoxide generation leads to higher antioxidant demand (Ariz et al. 2010). High irradiance also slightly increased the cyt_FeSOD isozyme content in roots of soybean plants (Fig. 3). In contrast to p_FeSOD, the role of cyt_FeSOD seems not related to protection of the photosynthesis system in chloroplast of leaves, although it is likely that
both sub-families are involved in the response to darkness and high irradiance stresses.

Drought, which has already been described to induce severe oxidative stress and a notable induction of SODs in pea plants (Moran et al. 1994, Gogorcena et al. 1995, Iturbe-Ormaetxe et al. 1998), proved to be a strong inducer of cyt_FeSOD protein in leaves and roots, although p_FeSOD seems to be unaffected by this stress except in leaves where the signal disappears completely. Both drought and darkness have been reported to result in an increased concentration of catalytic metals, which can then participate in Fenton-like reactions to generate ROS (Becana and Klucas 1990, Moran et al. 1994, Iturbe-Ormaetxe et al. 1998). However, the clearance of the p_FeSOD in leaves probably is due to the fact that photosynthesis is progressively impeded during drought stress mainly because of decreasing stomatal conductance, which results in a decreasing CO₂ and O₂ availability (Pieruschka et al. 2010).

ABA is known to be involved in stress responses mediated by oxidative damage (Beligni and Lamattina 1999b, Beligni and Lamattina 1999a) and to induce the production of NO (Lu et al. 2009). In our study, ABA produced an increase in cyt_FeSOD protein levels in all organs and in p_FeSOD in roots. Moreover, it has been demonstrated that ABA can protect plants from drought stress by inducing NO-mediated antioxidant response and hence induce SOD enzymes (Lu et al. 2009). As excess nitrate produced an induction in nitrate reductase, an enzyme which has been reported to be an NO generator (Rockel et al. 2002), it is not surprising that excess nitrate increased cyt:FeSOD in all organs tested (Fig. 3) as it was the strongest inductor in root an nodules, where it also increases the p_FeSOD expression levels (Fig. 3).

3.3. FeSOD contents and activities in soybean and cowpea samples during natural senescence

The use of KCN and H₂O₂ inhibitors allowed us to make the isozyme assignation of the SOD bands within the soybean samples (Fig. 5), and thus determine that MnSOD, FeSOD and CuZnSOD are present in both soybean and cowpea. It is notable that most isozyme activities split into several bands,
with similar mobilities, for the soybean samples. Previous reports have shown a
certain degree of multiplicity of FeSODs proteins may be observed (Sevilla et al.
1994), and also similar findings have already been reported for other soybean
varieties, as well as for alfalfa (Rubio et al. 2009). However, only one band was
found by SDS-PAGE and immunoblot in soybean (Fig 3) tissues, thus
indicating that the smearing effect is due to small molecular weigh post-
transcriptional modifications. It is quite remarkable the splitting in different
bands of activity for all the families of SODs in soybean samples (Fig 5). In
other experiment performed in our lab with recombinant cyt_FeSOD which was
incubated with different types of phenolic compounds such as tannic or gallic
acid a similar splitting was originated (unpublished results), pointing out that this
could be attributed to the higher contents in phenolic and tannin compounds
within the soybean plants either free or as glucosides (Moran et al., 1998). In
contrast, SOD multiplicity is not observed in cowpea samples (Fig. 6) and the
SODs isozymes can be easily identified as their number and mobility agreed
well with the previously described SOD activities (Moran et al. 2003). The data
obtained from the induced senescence experiments in both species clearly
demonstrated that cyt_FeSOD isoenzymes increase with the tissue age (Fig. 4),
the p_FeSOD protein content seems constitutive (Fig. 3), whereas
cyt_CuZnSOD plays a role in the earlier stages of the tissues (Figs. 5 and 6).

It is very remarkably, that FeSOD in-gel activity (Fig. 5 and 6) are
paralleled to the cyt_FeSOD protein content, thus evidencing that cyt_FeSOD is
the most active of FeSOD enzymes in mature and senescent samples for all
organs in both species. No match to activity bands could be assigned to the
bands resulting from the western blot performed with anti-p_FeSOD neither in
soybean nor cowpea extracts. Indeed, while some reasonable doubts arise from
the in-gel SOD assay for soybean tissues, no FeSODs other than cytosolic
SOD were detected in the in-gel assay for cowpea tissues, which may be
attributed by the low p_FeSOD contents and activities reported in legumes
(Gómez et al. 2004, Ariz et al. 2010). These data demonstrate that the
cyt_FeSOD is the main FeSOD protein in determinate forming nodules plants
as soybean and cowpea.
We do not exclude the possibility that new genes may be present in chloroplast which can account for tylakoidal isozymes, as they have been found in pea plants (Gómez et al. 2004). We have shown here, that two faint bands are present in pea and spinach, and also some previous report showed that citrus plants showed two chloroplastic isoenzymes (Sevilla et al. 1984). The complexity related to location and regulation is supported by other studies. In olive trees SODs are expressed differentially depending on cell type and are most abundant in palisade mesophyll cells (Corpas et al. 2006) and in cyt_FeSOD enzymes were found in the nucleus in association with the chromatin (Rubio et al. 2009, Asensio et al. 2011).

A subjacent question to answer is why cytosolic FeSOD genes are in legumes and which role can be played. We have shown that Fe-containing SOD in the cytosol is not exclusive to those legumes that form determinate nodules since pea nodules also showed a cambialistic SOD (Asensio et al. 2011), which plays a critical role during the symbiosis (Santos et al. 2000). Legumes display the ability to establish symbiosis with rizobia, and exhibit a high content of Fe due to the presence of the cytosolic protein leghemoglobin in the nodules. It has been shown that cyt_FeSOD content of nodules co-relates with Lb content in young and mature stages of the cowpea plants (Moran et al., 2003), however, the presence in non-infected cells (Moran et al., 2003; this work) evidences that the function is not exclusively related to the physiology of the nitrogen fixation. It has been proposed that cambialistic SOD is required in diverse sub-cellular localization of pea nodules, including the cytosol and nuclei to effectively establish a functional symbiosis where it plays not only an antioxidant function but also an essential role in modulating gene activity and/or signalling during interaction between legume and rizobia (Asensio et al. 2011). Thereby, the existence of cyt_FeSOD in legumes may have been acquired in other tissues increasing the plasticity and complexity of the plant response to stimuli.

5. MATERIAL AND METHODS

5.1. Plant growth and harvesting
Nodulated cowpea (Vigna unguiculata Walp. cv California blackeye no.5) x Bradyrhizobium sp. [Vigna] strain 32H1) and soybean (Glycine max cv. Oxumi x Bradyrhizobium japonicum UPM792) plants were grown in pots containing 2:1 (v/v) perlite:vermiculite (three plants per pot) under controlled-environment conditions (day length: 15 h; photosynthetic photon flux density: 400 µmol photons m\(^{-2}\) s\(^{-1}\); day-night regime: 18/22°C and 60/70% relative humidity). Plants were watered three times a week, alternating between distilled water and nitrate-free nutrient solution (Rigaud and Puppo 1975). To study the effect of ageing on FeSOD and APX, young, mature and senescent leaf, nodule and root samples were harvested 24, 46 and 83 days after seed germination of cowpea and soybean. To study the effect of stress on the expression of these antioxidant enzymes in soybean, 37-day-old plants were subjected to one of the following treatments: continuous darkness, high irradiance (600 µmol photon · s\(^{-1}\) · m\(^{-2}\)) (Ariz et al., 2010), 10 mM KNO\(_3\), 0.1 M NaCl, drought (by withholding irrigation) (Moran et al., 2003) or 40 µM ABA (Feria et al., 2008). All treatments were applied for 7 days in the growth chamber, then samples were harvested, weighted and frozen in liquid N\(_2\) at -80ºC until analyses. All chemicals not otherwise indicated were from Sigma-Aldrich (St. Louis, MO, USA).

5.2. Anti-p_FeSOD production

The Gene 3D software from Pôle bioinformatique Lyonnaise (Combet et al. 2002) was applied to a Pisum sativum chloroplastic FeSOD gene sequence (Q7XHK3; (Moran et al. 2003) for structural modelling of the FeSOD protein by comparison with a 3D cowpea FeSOD structural model (Muñoz et al. 2005). A synthetic peptide (VNPLATEEDKKLVVL) was selected based on its exposed position in the pea p_FeSOD protein (Fig. S1) its predicted antigenic properties, and its differences with other SOD proteins possibly present. The anti-pea plastidial FeSOD antibody (anti-p_FeSOD) was raised in rabbit using this peptide as antigen. The IgG antibody fraction used in the experiments was produced by affinity purification with a column containing the synthetic peptide.

5.3. Protein immunoblot assays and in-gel SOD activity
Plant samples were homogenized at 4°C in a mortar in 50% (w/v) SOD extraction buffer containing 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, 0.1% (v/v) Triton X-100 and 1% pvp-10 (w/v). The extract was filtered through Miracloth and centrifuged at 20,000 x g at 4°C for 30 min. Protein immunoblots or a total SOD activity assay were performed immediately after extraction. For immunoblot assays, the extracts were normalized for protein concentration (30 to 50 µg of protein per lane, depending on the experiment) and loaded onto 12% (w/v) SDS-PAGE gels, then electroblotted onto polyvinylidene difluoride membranes (Bio-Rad). Coomasie staining SDS-PAGE gels were performed in order to ensure equal loading. The primary antibody and concentration used were anti-cyt_FeSOD (1:2,000, v/v), anti-p_FeSOD (1:100 v/v) or anti-APX (1:2000, v/v). Anti-cyt_FeSOD IgG was produced as described in Moran et al. (2003), while anti-cyt_APX was a kind gift from Dr. David Dalton (Dalton 1995). The secondary antibodies employed in the assays were goat anti-rabbit IgG alkaline phosphatase conjugate (1:20,000, v/v; Sigma-Aldrich) or horseradish peroxidase conjugate (1:20,000, v/v; Sigma-Aldrich). Immunoreactive bands were visualized with the NBT system, Sigmafast 3,3’-Diaminobenzidine Tablets (Sigma-Aldrich) or with a chemiluminescent substrate (SuperSignal West Pico, Pierce, Rockford, IL, USA). Controls were run with rabbit pre-immune serum to check for nonspecific adsorption of primary antibody. Recombinant cowpea FeSOD was produced and purified as described previously (Moran et al. 2003). Protein concentration was calculated using the Bradford protein assay (Bio-Rad). Freshly prepared samples were used when performing the anti-p_FeSOD immunoblot as the freeze-thaw cycle resulted in band smearing. Cowpea and soybean extracts were separated in 15% (w/v) native-PAGE gels and total SOD activity bands visualized (Beauchamp and Fridovich 1971) with the modifications indicated (Moran et al. 2003). All protein immunoblots and the in-gel SOD activity assay were performed at least three times in order to ensure the consistency of the results.

Acknowledgements Bradyrhizobium japonicum UPM792 was kindly donated by Dr. Tomás Ruiz Argueso. Anti-cyt_APX antibody was a kind gift from Dr. David Dalton. This work was supported by the Spanish MICINN (grants no. AGL2007-64432/AGR and AGL2010-16167) and by the Government of

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Figure 1. Phylogenic tree of the superoxide dismutase protein families from legumes species. The tree was constructed using ClustalX2 and visualized using Dendroscope software. Boxed proteins were used for antibody generation (Psat4 for p_FeSOD, Vung1 for cyt_FeSOD). The abbreviations and UniProtKB accession numbers for plant SODs are as follows: Glycine max: Gmax1 (A5JVZ7), Gmax2 (C6TNA5), Gmax3 (Q7M1R5), Gmax4 (C6TFH5), Gmax5 (C6T360), Gmax6(P28759), Gmax7 (Q71UA1), Gmax8 (C6T8W6), Gmax9.
Lotus japonicus: Ljap1 (Q56VR0), Ljap2 (Q56VR6), Ljap3 (Q53D71), Ljap4 (Q53D73). Medicago sativa: Msat1 (Q8H1L8), Msat2 (O65198), Msat3 (Q8W596). Pisum sativum: Psat1 (P27084), Psat2 (Q02610), Psat3 (P11964), Psat4 (Q7XHK3). Vigna unguiculata: Vung1 (Q9M7R2).

Figure 2. Two immunoblots for leguminous and non-leguminous leaf samples using anti-p_FeSOD antibody. Lanes, 1: pea; 2: wheat; 3: spinach; 4: cowpea; 5: soybean; 6: pure recombinant cytosolic FeSOD from cowpea; Molecular weight markers are indicated in the side lanes for each immunoblot. Left (1, 2, 3) 15% SDS-PAGE, right (4, 5, 6) 12% SDS-PAGE

Figure 3. Cytosolic FeSOD (left) and plastidial FeSOD (right) contents analyzed by immunoblot detection in soybean leaves, nodules and roots from plants subjected to different stresses. Lane 1: control plants; 2: darkness; 3: high irradiance; 4: excess nitrate; 5: salt stress; 6: drought; 7: abscisic acid. All lanes were loaded with 50 µg in a 12% SDS-PAGE. The primary antibody and concentration used were anti-cyt_FeSOD (1:2,000, v/v), anti-p_FeSOD (1:100 v/v). The secondary antibodies employed were goat anti-rabbit IgG alkaline phosphatase conjugate (1:20,000, v/v; for anti-cyt_FeSOD) or horseradish peroxidase conjugate (1:50,000, v/v; for p_FeSOD). Tables of densitometry show the percentage of band intensity quantification compare to the control.

Figure 4. FeSOD content analysed by western immunoblot using antibodies against cytosolic FeSOD (left) and plastidial FeSOD (right) in soybean leaves, nodules and roots from plants subjected to different stresses. Lane 1: young leaves (24 days old); 2: mature leaves (46 days old); 3: senescent leaves (83 days old); 4: young nodules; 5: mature nodules; 6: senescent nodules; 7. young roots; 8. mature roots; 9. senescent roots. All lines were loaded with 50 µg in a 12% SDS-PAGE. The primary antibody and concentration used were anti-cyt_FeSOD (1:2,000, v/v), anti-p_FeSOD (1:100 v/v) The secondary antibodies employed in the assays were goat anti-rabbit IgG alkaline phosphatase conjugate (1:20,000, v/v; for anti-cyt_FeSOD) or horseradish peroxidase conjugate (1:50,000, v/v; for p_FeSOD). Tables of densitometry show the percentage of band intensity quantification with respect to the control.
**Figure 5.** (A) In-gel SOD activities during natural senescence of soybean plants. Plant tissues and developmental stages are as follows: 1. young leaves (24 days old); 2. mature leaves (46 days old); 3. senescent leaves (83 days old); 4. young nodules; 5. mature nodules; 6. senescent nodules; 7. young roots; 8. mature roots; 9. senescent roots. (B) Inhibition experiments using mature nodule extracts (from left to right): control without inhibitor, 1 h with KCN or 1 h with H₂O₂. All lines were loaded with 30 µg in a 15% SDS-PAGE gel.

**Figure 6.** In-gel SOD activity during natural senescence of cowpea plants. Plant tissues and developmental stages were as follows: 1. young leaves 24 days old; 2. mature leaves; 3. senescent leaves; 4. young nodules; 5. mature nodules; 6. senescent nodules; 7. young roots; 8. mature roots; 9. senescent roots. All lines were loaded with 30 µg in a 15% SDS-PAGE gel.

**Figure S1.** Left: Calculated 3D model for *Pisum sativum* plastidial FeSOD, based on the X-ray crystallographic structure of cowpea cytosolic FeSOD (Muñoz et al., 2005) using the GENO3D software package from *Pôle Bioinformatique Lyonnaise* (http://pbil.ibcp.fr) and Swiss-Pdb Viewer. Right: 3D model of the X-ray crystallographic structure of cowpea cytosolic FeSOD (PDB Entry: 1unf). Yellow ball represents the iron molecule position. The synthetic peptide used for production of the anti-p_FeSOD antibody is highlighted in red.

**Figure S2.** Comparison of the mobilities in SDS-PAGE gel of the cytosolic (A) and the chloroplastic FeSODs (B) from leaves of cowpea (left) and soybean (right). Two identical extracts were loaded in parallel lines and transferred to PVDF membrane. The lanes were cut separately, and the membranes were independently immunoblotted with either anti cyt-FeSOD, or anti pFeSOD antibody. Wells were loaded with 50 µg in a 12% SDS-PAGE gel. The primary antibody and concentration used were anti-cyt_FeSOD (1:2,000 v/v) or anti-p_FeSOD (1:100 v/v). The secondary antibodies employed in the assays were
goat anti-rabbit IgG alkaline phosphatise-conjugate (1:20,000 v/v, for anti-
cyt_FeSOD) or horseradish peroxidise-conjugate (1:50,000 v/v, for p_FeSOD).

Figure S3. APX content analysed by western immunoblot using anti-cytosolic
APX antibody in soybean leaves (A), nodules (B) and roots (C) under different
treatments. Lane 1: control plants; 2: darkness; 3: high irradiance; 4: excess
nitrate; 5: salt stress; 6: drought; 7: abscisic acid.
Figure 3

**Cytosolic FeSOD**

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**Plastidial FeSOD**

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### Cowpea

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Figure 6

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MnSOD
FeSOD
Cyt_CuZnSOD
P_CuZnSOD
Figure S1. Left: Calculated 3D model for Pisum sativum plastidial FeSOD, based on the X-ray crystallographic structure of cowpea cytosolic FeSOD (Muñoz et al., 2005) using the GENO3D software package from Pôle Bioinformatique Lyonnaise (http://pbil.ibcp.fr) and Swiss-Pdb Viewer. Right: 3D model of the X-ray crystallographic structure of cowpea cytosolic FeSOD (PDB Entry: 1unf). Yellow ball represents the Fe molecule position. The synthetic peptide used for production of the anti-p_FeSOD antibody is highlighted in red.
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