Peroxisomes are required for *in vivo* nitric oxide (NO) accumulation in the cytosol following salinity stress of Arabidopsis plants

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Abstract

Peroxisomes are unique organelles involved in multiple cellular metabolic pathways. Nitric oxide (NO) is a free radical active in many physiological functions under normal and stress conditions. Using Arabidopsis thaliana wild-type and mutants expressing green fluorescent protein (GFP) through the addition of peroxisomal targeting signal 1 (PTS1), which enables peroxisomes to be visualized in vivo, this study analyzes the temporal and cell distribution of NO during the development of 3, 5, 8, and 11-day-old Arabidopsis seedlings and shows that Arabidopsis peroxisomes accumulate NO in vivo. Pharmacological analyses using nitric oxide synthase (NOS) inhibitors detected the presence of putative calcium-dependent NOS activity. Furthermore, peroxins PEX12 and PEX13 appear to be involved in transporting the putative NOS protein to peroxisomes, since pex12 and pex13 mutants, which are defective in PTS1- and PTS2-dependent protein transport to peroxisomes, registered lower NO content. Additionally, we show that under salinity stress (100 mM NaCl), peroxisomes are required for NO accumulation in the cytosol, thereby participating in the generation of peroxynitrite (ONOO−) and in increasing protein tyrosine nitration which is a marker of nitrosative stress.

Keywords - Nitric oxide, nitric oxide synthase, peroxisomes, peroxins, peroxynitrite, protein tyrosine nitration, salinity.
**Introduction**

Peroxisomes are single membrane-bound organelles whose basic enzymatic constituents are catalase and H$_2$O$_2$-producing flavin oxidases as their basic enzymatic and are found in virtually all eukaryotic cell types (Corpas et al., 2001; Hayashi and Nishimura, 2006; Reumann et al., 2007; Pracharoenwattana and Smith, 2008; Palma et al., 2009). These oxidative organelles are characterized by metabolic plasticity, as their enzymatic content can vary according to the organism, cell/tissue-type, and environmental conditions (Mullen et al., 2001; Hayashi and Nishimura, 2003; Corpas et al., 2009a). In higher plants, peroxisomes contain a complex battery of antioxidative enzymes such as catalase, superoxide dismutase, the components of the ascorbate-glutathione cycle, and the NADP-dehydrogenases of the pentose-phosphate pathway (Corpas et al., 2009a). The generation of superoxide radicals has also been reported in the matrices and membranes of peroxisomes (López-Huertas et al., 1999; del Río et al., 2006). All these findings point to the important role played by peroxisomes in the cellular metabolism of reactive oxygen species (ROS) (Corpas et al., 2001, 2009a; del Río et al., 2006).

Nitric oxide (NO) is a free radical involved in many physiological functions under normal and stress conditions in both animal and plant cells (Corpas et al., 2007; 2008; Arasimowicz & Floryszak-Wieczorek, 2007; Neill et al., 2008). Unlike animal systems, knowledge of NO generation and subcellular location in plants remains largely elusive, and the data is sometimes contradictory and ambiguous (Zemojtel et al., 2006; Jasid et al., 2006; Gas et al., 2008). In previous studies, we detected L-arginine-dependent nitric oxide synthase (NOS) activity in isolated pea-leaf peroxisomes (Barroso et al. 1999). In a later study, using electron paramagnetic resonance (EPR) techniques, we demonstrated the presence of NO in these types of peroxisomes (Corpas et al., 2004). However, several issues, such as whether NO is released into the cytosol and the physiological function of this free radical, remain unresolved.

In this study, we provide the first in vivo demonstration that *Arabidopsis* peroxisomes are essential for NO accumulation in the cytosol, thus participating in the generation of nitrosative stress under salinity conditions. In addition, using *Arabidopsis* mutants of PEX13 and PEX12, we also suggest that these peroxins are involved in importing into peroxisomes the enzyme responsible for NO generation.
RESULTS

Localization of NO during the development of Arabidopsis seedlings

The visualization of endogenous NO in primary roots and cotyledons from 3, 5, 8, and 11-day-old Arabidopsis seedlings was carried out through confocal laser scanning microscopy (CLSM) using 4-aminomethyl-2′,7′-difluorofluorescein diacetate (DAF-FM DA) as a fluorescent probe (Fig. 1, panels A to H). In cotyledons, an intense green fluorescence caused by NO was observed in the epidermal cells. This fluorescence was more intense in 8-day-old seedlings (panel E) and 11-day-old trichome seedlings (panel G). The orange colour corresponds to autofluorescence. On the other hand, in primary roots, the green fluorescence was present throughout the entire apical root regardless of length of development (Fig. 1 panels B, D, F and H). Figure 2 shows a high magnification image of root cells, where green spherical spots resembling peroxisomes inside the cell can be observed.

Location of NO in root peroxisomes (GFP-PTS1)

Figure 3A shows in vivo CLSM visualization of peroxisomes in the root tips of transgenic Arabidopsis plants expressing the green fluorescent protein (GFP) through the addition of peroxisomal targeting signal 1 (PTS1) (Mano et al., 2002). The peroxisomes appeared in the form of spherical spots in all root tip cells. Figure 3B shows the same field analyzed using the diaminorhodamine-4M acetoxymethyl ester (DAR-4M AM) fluorescence probe, which also enabled NO to be detected (Kojima et al., 2001; Tun et al., 2006, 2008). An intense red fluorescence was found in spherical spots with a pattern similar to that of the GFP-PTS1. Figure 3C shows the linear-correlation spots obtained using Leica software, indicating that most of these spots coincide. Figure 3D contains a merged image of the overlap of panels A and B, showing a virtually complete overlap of the two punctuate patterns, indicating that NO was present in peroxisomes. The location of NO in two Arabidopsis mutants with an aberrant peroxisome morphology (apm) (Mano et al., 2006) was analysed to gain more knowledge about the transport of the protein assumed to be responsible for NO generation in peroxisomes. Figures 3E and 3G show the green fluorescent patterns corresponding to GFP-PTS1 in apm2/pex13 and apm4/pex12 mutants, respectively. The peroxisomes were detected in the form of spherical spots in all cells. However, GFP fluorescence was also detected in the cytosol, indicating that the targeting of PTS1-containing proteins in apm2/4 mutants was impaired. Figures 3F and 3H show the
location of NO in the same fields as those depicted in panels E and G, respectively. Supplemental Fig. 1 shows the overlap of panel D with the bright-field image of the *Arabidopsis* root tip.

Figure 4 shows the change in the fluorescence intensity of NO in panels B, F, and H of Fig. 3. In the mutants characterized by defective protein transport to peroxisomes, NO production was significantly lower (-39% for *apm2* and -43% for *apm4*), suggesting that the protein responsible for releasing NO into peroxisomes is adversely affected in these mutants.

Several controls were used to confirm the specific findings of NO detection using the new fluorescence probe DAR-AM AM in *Arabidopsis* seedling expressing GFP-PTS1. Figure 5, panels E to H, show the images of the same tip root of *Arabidopsis* seedlings preincubated with 100 µM sodium nitroprusside (SNP), a NO donor used as positive control. Under these conditions, a NO-dependent increase in DAR-AM AM fluorescence (red colour) was observed compared with the control seedlings (Fig. 5, panel B), showing the specificity of this fluorescence probe in relation to NO, as previously reported for *Arabidopsis* wild type (Tun et al., 2006, 2008). On the other hand, when the seedlings were preincubated with 200 µM cPTIO (a NO scavenger), the red fluorescence was greatly reduced (Fig. 5 panel J) without affecting GFP-PTS1 detection (Fig. 5 panel I). The quantification of NO showed that SNP increases fluorescence intensity 1.9-fold and that the cPTIO reduced NO approximately 5.8-fold (Fig. 5M).

Similarly, supplemental Fig. 2 shows the staining of *Arabidopsis apm2/pex13* (panels A to H) and *apm4/pex12* (panels I to P) mutants with DAR-AM AM preincubated with 100 µM sodium nitroprusside (SNP), a NO donor used as positive control. The quantification of the fluorescence intensity of NO showed that SNP increases fluorescence 1.9-fold in *apm2/pex13* and 1.6-fold in *apm4/pex12* (Supplemental Fig. 2Q). It is therefore possible to conclude that DAR-AM AM uptake and staining in these mutants were not affected by the use of the NO donor.

A pharmacological approach was also used to evaluate the putative involvement of a nitric oxide synthase (NOS) activity in the release of NO into peroxisomes. The preincubation of *Arabidopsis* seedlings with 5 mM L-NAME (Fig. 6, panels E to H) and 2 mM aminoguanidine (Fig. 6, panels I to L), two well-known inhibitors of animal NOS, produced a sharp reduction in NO detected by DAR-AM AM when compared to the control (Fig. 6, panels A to D), which suggests that a L-arginine-dependent NOS
activity is involved in the production of the NO detected. Furthermore, when the Arabidopsis seedlings were preincubated with 0.5 mM EGTA (Fig. 5, panels M to P) as a calcium chelator, NO content was also significantly reduced, indicating that NO generation was calcium dependent.

Nitric oxide in Arabidopsis root peroxisomes under salinity stress

To study the potential physiological function of NO released into peroxisomes, its production was studied under abiotic stress conditions. Previous studies have shown that Arabidopsis seedlings grown with 100 mM NaCl underwent salinity stress (Sun et al., 2007; Nishizawa et al., 2008). Figure 7A shows Arabidopsis seedlings grown with 100 mM NaCl, which significantly reduced root length by 24% (Fig. 7B). When NO generation was analyzed by CLSM, using both DAF-FM DA (Fig. 7, panels C and D) and DAR-AM AM (Fig. 7, panels E and F) as fluorescence probes, a significant increase in NO production was observed in roots under salt stress. On the other hand, the fluorescence intensity of NO under salinity conditions, as detected by DAF-FM DA and DAR-AM AM, increased 6.4-fold (Fig 7G) and 6.3-fold (Fig. 7H) respectively, indicating that both these fluorescence probes are useful tools.

Mutants expressing GFP-PTS1 were used to evaluate if peroxisomes are a potential source of NO in Arabidopsis under salinity conditions, Figure 8 shows NO location and production in Arabidopsis roots expressing GFP-PTS1 under salinity stress (100 mM NaCl). Figures 8A and C show that peroxisomes are subcellular compartments where NO is produced mainly in the roots of control (0 mM NaCl) plants, as both punctuate patterns are seen to overlap in the merged image (Fig. 8E). Under salinity conditions (100 mM NaCl), the pattern of peroxisomes did not appreciably change compared with control (0 mM NaCl) plants; however, NO production increased very significantly (4.8 fold) under salinity conditions (Fig. 8G). The distribution of fluorescence detected in both peroxisomes and cytosol also varied considerably (Figs. 8D and F). This suggests that NO is released from the peroxisomes into the cytosol under salinity stress. To corroborate this hypothesis, the effect of salinity stress was studied in apm4/pex12 mutants, where the import of proteins into the peroxisomal matrix is affected, including probably the protein that generates NO and showed a low NO content (see Fig. 3, panel H). In figures 9A and B, the pattern of peroxisomes appears in the form of green spots in the roots of control and stressed plants, with a slight increase in the number of peroxisomes being observed under
salinity stress. Figure 9C shows the location of NO (red colour) in the same root area of Fig. 9A (0 mM NaCl) where NO was almost totally absent. Under salinity stress (Fig. 9D), NO slightly increased. Figures 9E and F show the merged images of control and stressed roots, respectively, where it can be clearly observed that NO is present in cytosol, indicating that the peroxisomal protein responsible for NO generation was not imported into the peroxisomes. Figure 9J shows the relative quantities of NO production in apm4/pex12 mutants under control and salinity conditions, where NO increased 1.4-fold, a lower increase than that the observed in parent plants expressing GFP-PTS1 (Fig. 8G). This suggests that peroxisomes are the main source of NO under normal and stress conditions in *Arabidopsis* roots.

The reaction of NO with the superoxide radical (O$_2^-$) generates peroxynitrite (ONOO$^-$), which has been shown to mediate an increase in the tyrosine nitration of proteins under stress conditions in animal cells (Radi, 2004; Szabó et al., 2007). To evaluate this possible correlation between peroxynitrite and protein nitration in *Arabidopsis* under salinity conditions, peroxynitrite was analyzed in roots by CLSM using the fluorescence probe 3'-({p-aminophenyl} fluorescein (APF) (Chaki et al., 2009a,b), while the presence of tyrosine nitration was studied by immunoblot analysis using a well-characterized antibody against 3-nitrotyrosine (Valderrama et al., 2007; Corpas et al., 2008b; Chaki et al., 2009a,b). Figure 10A shows the location of ONOO$^-$ in the control roots of *Arabidopsis* wild-type seedlings and ONOO$^-$ significantly increased in roots under salinity stress (Fig. 10B). Figure 10C shows that the fluorescence intensity of ONOO$^-$ increased 5.6-fold under salinity conditions. On the other hand, Figure 10D depicts the immunoblot analysis of protein tyrosine nitration in *Arabidopsis* roots using an antibody against 3-nitrotyrosine (NO$_2$-Tyr) and the corresponding silver-stained SDS (10%) gel. Thus, in control roots, a three-immunoreactive-band pattern with molecular masses of 78, 67, and 55 kDa, respectively, was observed. This resembled the pattern observed in plant roots subject to salinity conditions but with an additional band of 42 kDa and an intensification of the 55-kDa immunoreactive band.

**Discussion**

Peroxisomes are cell compartments involved in many physiological functions such as lipid mobilization, photorespiration, and hormone biosynthesis under normal and stress conditions (del Río et al., 1992; Hayashi and Nishimura, 2003; Reumann, 2004; Pracharoenwattana and Smith, 2008; Corpas et al., 2009a). Nitric oxide (NO), a free...
radical generated in animal and plant cells, has attracted the attention of many researchers due to its involvement in various physiological processes such as seed germination, plant development, and senescence (Leshem, 1996; Corpas et al., 2004, 2006) as well abiotic and biotic stress (Corpas et al., 2007, 2008; Besson-Bard et al., 2008; Neill et al., 2008; Chaki et al. 2009). However, several key questions, such as how NO is produced and its subcellular location in plants, are still a subject of debate (Corpas et al., 2009b). On the basis of previous experimental data, we had hypothesized that pea-leaf peroxisomes could be a potential source of NO in plant cells (Corpas et al., 2001). This study therefore aims to demonstrate the presence of NO in the peroxisomes of other plant species (using Arabidopsis as a model for plant analysis), to determine whether peroxisomal NO is released into the cytosol, and to analyze its physiological function. To achieve these objectives, we used a combination of biochemical, genetic, and cell- biology tools.

Green fluorescent protein (GFP), from the jellyfish Aequorea victoria and its variants has become a highly effective tool to study the subcellular location of many proteins and specifically plant peroxisomes (Hayashi et al., 2000; Mano et al., 2002, 2006; Letterrier et al., 2005; Reumann et al., 2007). Thus, the use of Arabidopsis mutants expressing GFP fused with peroxisomal targeting signal 1 (PTS1) has enabled us to visualize the peroxisomes in vivo. Simultaneous visualization of this GFP-PTS1 using the available fluorescent probes for NO such as DAF-2 DA and DAF-FM DA (Corpas et al., 2006) was not possible due to the overlap of the excitation and emission wavelengths. However, relatively new fluorescent probes for detecting NO, such as DAR-AM AM (Kojima et al., 2001), which have been successfully tested in plant cells (Tun et al. 2006, 2008), have enabled us to resolve this technical problem, as the excitation and emission wavelengths of neither GFP or DAR-AM overlap. The only drawback is that DAR-AM AM provides a less intense NO signal than DAF- FM DA.

NO is present in cotyledons and roots during the development of Arabidopsis seedlings.

Arabidopsis thaliana, a small flowering plant commonly used as a model in plant biology, offers important advantages for basic research in genetics and molecular biology. This plant has been used in many plant studies to determine the involvement of NO in seed germination (Beligni & Lamattina, 2000; Batak et al., 2002; Bethke et al., 2006), biotic stress (Delledone et al., 1998, 2001), abiotic stress (Mackerness et al.,
2001; Huang et al. 2004), programmed cell death (Clarke et al., 2000; Zhang et al., 2003), stomatal closure (Desikan et al., 2004; Garcia-Mata et al., 2004), iron metabolism (Murgia et al., 2002; Perazzolli et al., 2004), flowering (He et al., 2004; Simpson, 2005), and the protein S-nitrosylation (Lindermayr et al., 2005; Feechan et al., 2005) among others. Using the DAF-FM DA as a fluorescence probe, our results provide a detailed picture of the overall distribution of NO during the early development of 3 to 11-day-old Arabidopsis seedlings. Thus, primary roots were observed to have high NO content as compared with cotyledons, which resembles the level detected in Arabidopsis seedlings with a different development period and using different fluorescence probes for NO (DAF-2 DA or DAR-AM DA) (Tu et al., 2006, 2008; Kolbert et al., 2007).

**NO is generated in Arabidopsis root peroxisomes**

Confocal laser scanning microscopy (CLSM) analysis of Arabidopsis mutants expressing the GFP-PTS1 demonstrated that NO is present in Arabidopsis peroxisomes in vivo (Fig. 3 A to D). The presence of NO has been shown only in isolated pea-leaf peroxisomes by using the EPR technique (Corpas et al., 2004). It has also been suggested that the small spots, similar to peroxisomes, detected using CLSM, could be a source of NO involved in growth regulation and re-orientation of Lilium longiflorum pollen tubes (Prado et al., 2004). These new findings based on a different approach confirm that NO is present in the root peroxisomes of Arabidopsis, indicating that the presence of NO in peroxisomes could be a general feature of plant cells.

**PEX12 and PEX13 appear to be involved in the peroxisomal import of the NO-generating protein.**

Peroxisomal proteins are selectively targeted for import from the cytosol post-translationally by either a peroxisomal targeting sequence (PTS) or protein–protein associations. So-called PEX genes are also involved in regulating peroxisomal biogenesis. At least 22 PEX genes have been identified in Arabidopsis (Nito et al., 2007), and it has been demonstrated that APM2 and APM4 encode proteins homologous to peroxins PEX13 and PEX12, respectively (Mano et al., 2006). PEX12 is an integral membrane protein containing a RING-finger domain that functions as an ubiquitin ligase, an essential component of a multi-protein complex for peroxisomal matrix protein import (Albertini et al., 2001; Mano et al., 2006). It has also been suggested that PEX13 as well as PEX14 and PEX17 are membrane-bound peroxins that act as a
docking complex to import proteins into the peroxisomal matrix (Mullen and Trelease, 2001). Thus, *Arabidopsis* mutant defects in the PEX13 gene cause loss of peroxisomal function due to misdistribution of peroxisomal matrix proteins in the cytosol (Mano et al., 2006). The Pex13 and Pex14 proteins also appear to operate stoichiometrically *in vivo*, acting as the ideal docking proteins for the receptor–cargo complexes (Azevedo and Schliebs, 2006). Furthermore, it has been demonstrated that *apm2/4* mutants have also disturbed the PTS2-dependent protein transport mechanism (Mano et al., 2006), indicating that the *apm2/4* mutants are characterized by defective targeting of both PTS1- and PTS2-containing proteins. Under our experimental conditions, we observed that NO production was lower in both *apm2/pex13* and *apm4/pex12* Arabidopsis mutants (Fig. 3), indicating that the peroxisomal transport of a protein responsible for NO generation in peroxisomes was affected. Additionally, it was demonstrated that peroxisomal NO generation was sensitive to animal NOS inhibitors and was calcium-dependent. This data is in line with the biochemical evidence on the presence of nitric oxide synthase (NOS) activity reported in pea-leaf peroxisomes (Barroso et al., 1999; Corpas et al. 2004), olive leaf (Valderrama et al., 2007) and sunflower hypocotyls (Chaki et al., 2009a). It is worth noting that some of the cofactors involved in the L-arginine-dependent NOS activity in plant peroxisomes have been been described in previous studies (Corpas et al., 2009b). Several NADP-dehydrogenases can produce NADPH (Corpas et al 1998, 1999), while the presence of calmodulin in plant peroxisomes has been also demonstrated (Yang and Poovaiah, 2002) being both essential elements for this activity. These results could be important, particularly given that inducible NOS (iNOS) has also been reported in peroxisomes of rat hepatocytes (Stolz et al., 2002; Loughran et al., 2005).

On the other hand, it has been established that Pex12 is not only required for peroxisome biogenesis but is also essential for plant development (Fan et al., 2005). This could plausibly be explained by Pex12’s effect on the import of the peroxisomal NO-generating protein given that NO is also involved in plant germination and development processes (Lamattina et al., 2003; Corpas et al., 2006).

**Peroxisomes release NO under salinity stress which is involved in the generation of peroxynitrite**

To investigate the possibility of peroxisomal NO release into cytosol and its physiological function, we analyzed NO production under salinity conditions in GFP-
PTS1 parent plants (Fig. 8) and *apm4*/Pex12 mutants (Fig. 9). It was demonstrated that under these stress conditions the generation of NO increased significantly both in peroxisomes and in the cytosol. However, this increase in NO was not found in *apm4* mutants exposed to salinity stress, suggesting that the peroxisomal NO-generating protein is involved in the process. These findings also suggest that NO could be released into the cytosol under salinity stress, which is a prerequisite for peroxynitrite generation, resulting in nitrosative stress, as indicated by the increase in protein tyrosin nitration (Fig. 10D). In a previous work involving olive-plant growth under salinity (200 mM NaCl) stress conditions, similar behaviour was observed, with an increase in the number and intensity of leaf proteins undergoing tyrosine nitration (Valderrama et al., 2007). In this context, it must be mentioned that a rise in protein tyrosine nitration is considered an indicator of peroxynitrite activity and a marker of pathological diseases and oxidative stress in animals (Ischiropoulos, 1998; Radi 2004; Szabó et al., 2007) and plants (Corpas et al., 2007, 2008; Chaki et al., 2009a; Corpas et al., 2009c). Under salinity stress conditions, peroxisomes, as a source of NO, can therefore be expected to play a significant role in increasing nitrated proteins in either the peroxisomes or the cytosol. On the other hand, the release of NO into the cytosol could be explained by a protection mechanism triggered by overproduction due to peroxisomal NOS activity. These findings suggest that the peroxisomes are required for NO accumulation into the cytosol, which is a prerequisite for the nitrosative stress process mediated by peroxynitrite observed in *Arabidopsis* under salinity conditions.

In summary, the data reported in this study provides the first experimental evidence that *Arabidopsis* nitric oxide (NO) production under normal and salinity stress depends on peroxisomal protein import genes Pex12 and Pex13, and generates nitrosative stress mediated by peroxynitrite overproduction. All this data constitutes a significant advance in our knowledge of the metabolism of NO in plant peroxisomes and their involvement in the response to abiotic stress. Furthermore, the importance of human peroxisomes in biomedicine as these organelles are associated with several important genetic diseases caused by peroxisomal dysfunction (Steinberg et al., 2006), suggests that significant advances in human health research could be made on the basis of these results. Although the identification of the peroxisomal protein responsible for NO production remains a major challenge, the involvement of specific peroxins (PEX12 and PEX13) could be a useful tool in its identification.
Material and Methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia and mutant (apm2 and apm4) seeds expressing GFP-PTS1 (Mano et al., 2006) were surface-sterilized for 5 min in 70% ethanol containing 0.1 % SDS, then placed for 20 min in sterile water containing 20% bleach and 0.1% SDS, and washed four times in sterile water. The seeds were sown for 2 days at 4°C in the dark for vernalization on the basal growth medium composed of 4.32 g/l commercial Murashige and Skoog medium (Sigma) with a pH of 5.5, containing 1% sucrose and 0.8% phyto-agar. The Petri plates containing the Arabidopsis seeds were then grown at 16 h light, 22°C/8 h dark, at 18 °C (long-day conditions) under a light intensity of 100 µE m⁻² s⁻¹. For the experiments with NaCl stress, 6-day-old seedlings, wild type, and apm4 mutants were transferred to MS medium plates both with and without 100 mM NaCl for another 7 days under long-day conditions (Sun et al., 2007).

Crude extracts of plant tissues

Arabidopsis roots were frozen in liquid N₂ and ground in a mortar with a pestle. The powder was suspended in a homogenizing medium containing 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 10% (v/v) glycerol. Homogenates were centrifuged at 17,000 g for 30 min, and the supernatants were used for the assays.

SDS-PAGE and immuno-blot analysis

SDS-PAGE was carried out according to the Laemmli method (1970) in 10% acrylamide-slab gels. Gels were stained with silver according to the modifications described in detail by Jiang et al. (1994) for the Heukeshoven and Dernick method (1985). For Western blot analysis, proteins were electroblotted to PVDF membranes with a semi-dry Trans-Blot cell (BioRad). For immunodetection of nitrotyrosine, a rabbit polyclonal antibody against 3-nitrotyrosine (NO₂-Tyr) (Valderrama et al 2007) diluted 1:3,000 was used with an enhanced chemiluminescence kit (ECL-PLUS, Amersham Pharmacia Biotech), and immunoreactive bands were detected with a photographic film (Hyperfilm; Amersham Pharmacia Biotech).
Detection of nitric oxide (NO) in mutants expressing GFP-PTS1 using confocal laser scanning microscopy (CLSM).

Nitric oxide was detected using the fluorescent reagent diaminorhodamine-4M acetoxymethyl ester (DAR-AM AM, Calbiochem). *Arabidopsis* seedlings were incubated at 25°C for 1 h in darkness with 5 µM DAR-AM AM prepared in 100 mM potassium phosphate buffer (pH 7.4) (Tun et al., 2006, 2008). The samples were then washed twice in the same buffer for 15 min each and mounted in a microscope slide for examination with a confocal laser scanning microscope (Leica TCS SL) using standard filters and collection modalities for DAR-4M (excitation 543 nm; emission 575 nm) and GFP (excitation 495 nm; emission 515 nm). As controls, 14-day-old *Arabidopsis* seedlings expressing GFP-PTS1 were preincubated for 2h 30 min at 25 ºC with 100 µM sodium nitroprussiate (SNP) as NO donor, 200 µM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt (cPTIO) as NO scavenger, 5 mM N⁰-nitro-L-arginine methyl ester (L-NAME) as competitive inhibitor of animal NOSs and 2 mM aminoguanidine (AG) as general inhibitor of animal NOSs, and then incubated with 5 µM DAR-AM AM for 1 h at 25ºC to detect and visualize NO using CLSM.

Alternatively, NO was detected using the fluorescent reagent 10 µM 4-aminomethyl-2',7'-difluorofluorescein diacetate (DAF-FM DA, Calbiochem) prepared in 10 mM Tris-HCl (pH 7.4). These probes are highly specific for NO (Nakatsubo et al., 1998; Zhang et al., 2003; Corpas et al., 2006), which is detected using standard filters and collection modalities for DAF-2 green fluorescence (excitation 495 nm; emission 515 nm).

In all cases, the images obtained by CLSM from control and treated Arabidopsis seedlings were held constant during the course of the experiment in order to produce comparable data. The images were processed and analyzed using statistical Leica confocal software.

Detection of peroxynitrite (ONOO⁻) by CLSM

Peroxynitrite (ONOO⁻) was detected using the fluorescent reagent 3'-(p-aminophenyl) fluorescein (APF, Invitrogen). *Arabidopsis* seedlings were incubated at 25°C for 1 h in darkness with 10 µM APF prepared in 10 mM Tris-HCl (pH 7.4). Then, the samples were washed twice in the same buffer for 15 min each and mounted in a microscope.
slide for examination with CLSM using standard filters and collection modalities for APF fluorescence (excitation 495 nm; emission 515 nm).

**Other assays**
Protein concentration was determined with the Bio-Rad Protein Assay (Hercules, CA) using bovine serum albumin as standard. To estimate the statistical significance between means, the data was analyzed by Student’s *t* test.

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Figure Legends

Figure 1. Representative images illustrating the confocal laser scanning microscopy (CLSM) detection of endogenous NO (green colour) in Arabidopsis seedlings at different stages of development. Panels A and B: cotyledon and primary root of 3-day-old Arabidopsis seedling, respectively. Panels C and D: cotyledon and primary root of 5-day-old Arabidopsis seedling, respectively. Panels E and F: cotyledon and primary root of 8-day-old Arabidopsis seedling, respectively. Panels G and H: cotyledon and primary root of 11-day-old Arabidopsis seedling, respectively. Arabidopsis seedlings were incubated with 10 µM DAF-FM DA as fluorescent probe. The orange-yellow colour corresponds to the auto-fluorescence.

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Figure 3. Representative images illustrating the CLSM in vivo detection of NO (red colour) and peroxisomes (green colour) in root tips of 5-day-old Arabidopsis seedlings of parent plants and mutants with aberrant peroxisome morphology (apm2 and apm4) expressing GFP-PTS1. Panel A: fluorescence punctuates (green) attributable to GFP-PTS1 indicating the localization of peroxisomes in parent plants. Panel B: fluorescence punctuates (red) attributable to NO detection in the same root area. Panel C: plot showing the linear correlation between the punctuate corresponding to GFP-1 and NO. Panel D: merged image of panels A and B showing colocalized fluorescence punctuates (yellow). Panel E: fluorescence punctuates (green) attributable to GFP-PTS1 in apm2/pex13 mutants. Panel F: fluorescence punctuates (red) attributable to NO detection in apm2/pex13 mutants. Panel G: fluorescence punctuates (green) attributable to GFP-PTS1 in apm4/pex12 mutants. Panel H: fluorescence punctuates (red) attributable to NO detection in apm4/pex12 mutants. Nitric oxide was detected with diaminorhodamine-4M acetoxymethyl ester (DAR-4M, excitation 543 nm; emission 575 nm) and peroxisomes with green fluorescence protein (GFP, excitation 495 nm; emission 515 nm). Arrows indicate representative punctuate spots corresponding to NO and peroxisome localization.
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**Figure 7. Effect of salinity on Arabidopsis seedling growth and NO content detected with DAF-FM DA and DAR-AM AM.** Appearance (panel A) and primary root length (panel B) of 13-day-old Arabidopsis seedling growth in MS medium supplemented and nonsupplemented with 100 mM NaCl. Results are the mean of three different experiments ± S.E.M. *Differences in relation to control values were significant at P < 0.05. Representative images illustrating the CLSM detection of NO (green or red colour) in primary roots of 6-day-old Arabidopsis wild-type seedlings exposed and nonexposed to 100 mM NaCl using DAF-FM DA (panels C and D) and DAR-AM AM (panels E and F) as fluorescent probes. Fluorescence intensity of NO was detected by DAF-FM DA (panel G) or DAR-AM AM (panel H). Nitric oxide was detected using 10 µM DAF-FM DA (green colour; excitation 495 nm; emission 515 nm) and DAR-AM AM (red colour; excitation 543 nm; emission 575 nm) as fluorescent probes. Fluorescence is expressed as arbitrary units (A.U.) using Leica confocal software.

**Figure 8. Representative images illustrating the CLSM in vivo detection of NO (red colour) in root tips of 6-day-old Arabidopsis seedlings expressing GFP-PTS1 (green colour) exposed to 100 mM NaCl for 7 days.** Panel A: fluorescence punctuates (green) attributable to GFP-PTS1 indicating the localization of peroxisomes in control (0 mM NaCl) plants. Panel B: fluorescence punctuates (green) attributable to GFP-PTS1 indicating the location of peroxisomes in Arabidopsis plants exposed to 100 mM NaCl. Panel C: fluorescence punctuates (red) attributable to NO detection in the same root area of panel A. Panel D: NO detection (red) in the same root area of panel B. Panel E: merged image of panels A and C showing colocalized fluorescence punctuates (yellow). Panel F: merged image of panels B and D showing colocalized fluorescence punctuates (yellow). Panel G: fluorescence intensity of NO from panels C and D. Nitric oxide was detected with diaminorhodamine-4M acetoxyethyl ester (DAR-4M, excitation 543 nm; emission 575 nm) and peroxisomes with green fluorescence protein (GFP, excitation 495 nm; emission 515 nm). Fluorescence is expressed as arbitrary units (A.U.) using Leica confocal software.
Figure 9. Representative images illustrating the CLSM in vivo detection of NO (red colour) in root tips of 6-day-old Arabidopsis apm4/pex12 mutant seedlings expressing GFP-PTS1 (green colour) exposed to 100 mM NaCl for 7 days. Panel A: fluorescence punctuates (green) attributable to GFP-PTS1 indicating the location of peroxisomes in control (0 mM NaCl) plants. Panel B: fluorescence punctuates (green) attributable to GFP-PTS1 indicating the location of peroxisomes in Arabidopsis apm4/pex12 mutants exposed to 100 mM NaCl. Panel C: fluorescence punctuates (red) attributable to NO detection in the same root area of panel A. Panel D: NO detection (red) in the same root area of panel B. Panel E: merged image of panels A and C showing colocalized fluorescence punctuates (yellow). Panel F: merged image of panels B and D showing colocalized fluorescence punctuates (yellow). Panel J: fluorescence intensity of NO from panels C and D. Nitric oxide was detected with diaminorhodamine-4M acetoxymethyl ester (DAR-4M, excitation 543 nm; emission 575 nm) and peroxisomes with green fluorescence protein (GFP, excitation 495 nm; emission 515 nm). Fluorescence is expressed as arbitrary units (A.U.) using Leica confocal software.

Figure 10. Representative images illustrating the CLSM in vivo detection of peroxynitrite (red colour) and immunoblot showing the protein tyrosine nitration in root tips of 6-day-old Arabidopsis wild-type seedlings exposed to 100 mM NaCl for 7 days. Panels A and B: root tips of control (0 mM NaCl) plants. Peroxynitrite (ONOO⁻) was detected using the fluorescent reagent 3’-(p-aminophenyl) fluorescein (APF, excitation 495 nm; emission 515 nm). Panel C: fluorescence intensity of ONOO⁻ observed in panels A and B. Panel D: representative immunoblot showing protein tyrosine nitration in the roots of 6-day-old Arabidopsis wild-type seedlings exposed to 100 mM NaCl for 7 days and the corresponding silver-stained gel. Root samples (5 µg of protein per lane) were subjected to SDS–PAGE (10%) and Western analysis using an antibody against 3-nitrotyrosine (NO₂-Tyr) (dilution 1:8,000). NO₂-BSA, commercial nitrated bovine serum albumin. Fluorescence is expressed as arbitrary units (A.U.) using Leica confocal software.
Supplemental Figures

Figure 1. Image showing overlap between panel D of figure 3 with the corresponding bright-field image of the Arabidopsis root tip. Green fluorescence punctuates are attributable to GFP-PTS1 indicating the localization of peroxisomes, red fluorescence is attributable to NO detection, and yellow fluorescence punctuates show the co-localization of NO in peroxisomes in the same root area.

Figure 2. Representative images illustrating the CLSM in vivo detection of NO (red colour) in the root tips of 14-day-old Arabidopsis apm2/pex13 (panels A to H) and apm4/pex12 (panels I to P) mutant seedlings expressing GFP-PTS1 (green colour) preincubated for 2h 30 min at 25 ºC with 100 µM SNP as NO donor. Arabidopsis apm2/pex13 seedlings were incubated only with DAR-4M AM as a fluorescent probe for NO (panels A to D) and pre-incubated for 2h 30 min at 25 ºC with 100 µM SNP as NO donor (panels E to H). Arabidopsis apm4/pex12 seedlings were incubated only with DAR-4M AM as fluorescent probe for NO (panels I to L) and pre-incubated for 2h 30 min at 25 ºC with 100 µM SNP as NO donor (panels M to P). 
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Figure 9
Figure 9. Representative images illustrating the CLSM in vivo detection of NO (red colour) in root tips of 6-day-old Arabidopsis apm4/pex12 mutant seedlings expressing GFP-PTS1 (green colour) exposed to 100 mM NaCl for 7 days. Panel A: fluorescence punctuates (green) attributable to GFP-PTS1 indicating the location of peroxisomes in control (0 mM NaCl) plants. Panel B: fluorescence punctuates (green) attributable to GFP-PTS1 indicating the location of peroxisomes in Arabidopsis apm4/pex12 mutants exposed to 100 mM NaCl. Panel C: fluorescence punctuates (red) attributable to NO detection in the same root area of panel A. Panel D: NO detection (red) in the same root area of panel B. Panel E: merged image of panels A and C showing colocalized fluorescence punctuates (yellow). Panel F: merged image of panels B and D showing colocalized fluorescence punctuates (yellow). Panel J: fluorescence intensity of NO from panels C and D. Nitric oxide was detected with diaminorhodamine-4M acetoxyethyl ester (DAR-4M, excitation 543 nm; emission 575 nm) and peroxisomes with green fluorescence protein (GFP, excitation 495 nm; emission 515 nm). Fluorescence is expressed as arbitrary units (A.U.) using Leica confocal software.
Figure 10. Representative images illustrating the CLSM in vivo detection of peroxynitrite (red colour) and immunoblot showing the protein tyrosine nitration in root tips of 6-day-old Arabidopsis wild-type seedlings exposed to 100 mM NaCl for 7 days. Panels A and B: root tips of control (0 mM NaCl) plants. Peroxynitrite (ONOO·) was detected using the fluorescent reagent 3’-(p-aminophenyl) fluorescein (APF, excitation 495 nm; emission 515 nm). Panel C: fluorescence intensity of ONOO· observed in panels A and B. Panel D: representative immunoblot showing protein tyrosine nitration in the roots of 6-day-old Arabidopsis wild-type seedlings exposed to 100 mM NaCl for 7 days and the corresponding silver-stained gel. Root samples (5 µg of protein per lane) were subjected to SDS–PAGE (10%) and Western analysis using an antibody against 3-nitrotyrosine (NO2-Tyr) (dilution 1:8,000). NO2-BSA, commercial nitrated bovine serum albumin. Fluorescence is expressed as arbitrary units (A.U.) using Leica confocal software.