Cellular Response of Pea Plants to Cadmium Toxicity: Cross Talk between Reactive Oxygen Species, Nitric Oxide, and Calcium[1][W][OA]

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Cadmium (Cd) toxicity has been widely studied in different plant species; however, the mechanism involved in its toxicity as well as the cell response against the metal have not been well established. In this work, using pea (*Pisum sativum*) plants, we studied the effect of Cd on antioxidants, reactive oxygen species (ROS), and nitric oxide (NO) metabolism of leaves using different cellular, molecular, and biochemical approaches. The growth of pea plants with 50 μM $CdCl_2$ affected differentially the expression of superoxide dismutase (SOD) isozymes at both transcriptional and posttranscriptional levels, giving rise to a SOD activity reduction. The copper/zinc-SOD down-regulation was apparently due to the calcium (Ca) deficiency induced by the heavy metal. In these circumstances, the overproduction of the ROS hydrogen peroxide and superoxide could be observed in vivo by confocal laser microscopy, mainly associated with vascular tissue, epidermis, and mesophyll cells, and the production of superoxide radicals was prevented by exogenous Ca. On the other hand, the NO synthase-dependent NO production was strongly depressed by Cd, and treatment with Ca prevented this effect. Under these conditions, the pathogen-related proteins PrP4A and chitinase and the heat shock protein 71.2, were up-regulated, probably to protect cells against damages induced by Cd. The regulation of these proteins could be mediated by jasmonic acid and ethylene, whose contents increased by Cd treatment. A model is proposed for the cellular response to long-term Cd exposure consisting of cross talk between Ca, ROS, and NO.

Cadmium (Cd) is a toxic element whose presence in the environment is mainly due to industrial processes and phosphate fertilizers and then is transferred to the food chain (Pinto et al., 2004). Cd is rapidly taken up by plant roots and can be loaded into the xylem for its transport into leaves. Most plants are sensitive to low Cd concentrations, which inhibit plant growth as a consequence of alterations in the photosynthesis rate and the uptake and distribution of macronutrients and micronutrients (Lozano-Rodriguez et al., 1997; Sandalio et al., 2001; Benavides et al., 2005). It is known that the content of polyvalent cations can be affected by the presence of Cd through competition for binding sites of proteins or transporters (Gussarson et al., 1996). Thus, Cd produced a decrease of calcium (Ca) content in different plant species (Gussarson et al., 1996; Sandalio et al., 2001). Ca is involved in the regulation of plant cell metabolism and signal transduction (Yang and Poovaiah, 2002; Rentel and Knight, 2004) and modulates cellular processes by binding proteins such as calmodulin (CaM), which in turn regulates the activity of target proteins (Roberts and Harmon, 1993).

Cd can be detoxified by phytochelatins, whose synthesis is induced by Cd and other metals and is accompanied by a decrease in the concentration of glutathione (Zenk, 1996). In addition, Cd produces disturbances in the plant antioxidant defenses, producing an oxidative stress (Somashekaraiah et al., 1992; Shaw, 1995; Gallego et al., 1996; Dixit et al., 2001; Sandalio et al., 2001; Schützendübel et al., 2001; Romero-Puertas et al., 2002, 2007; Rodríguez-Serrano et al., 2006). Recently, the cellular production of reactive oxygen species (ROS) in leaves from pea (*Pisum sativum*) plants under Cd stress has been reported (Romero-Puertas et al., 2004). ROS were detected in epidermal, transfer and mesophyll cells, with plasma membrane being the main source of ROS, although mitochondria and peroxisomes were also involved (Romero-Puertas et al., 2004). Concerning the mecha-

[1] This work was supported by the Ministry of Education and Science (grant no. BIO2005–03305 and fellowship to M.R.-S.) and the Junta de Andalucía (Research Group no. BIO–0192), Spain.

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www.plantphysiol.org/cgi/doi/10.1104/pp.108.131524

nism of ROS production, Cd does not participate in Fenton-type reactions (Stoch and Bagchi, 1995) but can indirectly favor the production of different ROS, such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), and hydroxyl radical (·OH), by unknown mechanisms, giving rise to an oxidative burst (Olmos et al., 2003; Romero-Puertas et al., 2004; Garnier et al., 2006). The enzymes superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) are involved in the detoxification of $O_2^{\cdot-}$ (SOD) and H_2O_2 (CAT, POX), thereby preventing the formation of ·OH radicals. Ascorbate peroxidase and glutathione reductase, as well as glutathione, are important components of the ascorbate-glutathione cycle responsible for the removal of H_2O_2 in different cellular compartments (Jiménez et al., 1997; Noctor et al., 1998). Apart from their toxic roles, ROS are also involved in signaling in different processes such as growth, development, and response to biotic and abiotic stresses, and this signaling process is controlled by a balance between ROS production and scavenging (Bailey-Serres and Mittler, 2006).

Nitric oxide (NO) is a widespread intracellular and intercellular messenger with a broad spectrum of regulatory functions in many physiological processes (Moncada et al., 1991; Ignarro, 2002; del Río et al., 2004; Grün et al., 2006). In plants, NO was reported to be involved in ethylene (ET) emission (Leshem and Haramaty, 1996), response to drought (Leshem, 1996), disease resistance (Durner et al., 1998; Clark et al., 2000; Delledonne et al., 2001), growth and cell proliferation (Ribeiro et al., 1999), maturation and senescence (Leshem and Haramaty, 1996; Corpas et al., 2004), apoptosis/programmed cell death (Magalhaes et al., 1999; Clark et al., 2000; Pedroso and Durzan, 2000), and stomatal closure (García-Mata and Lamattina, 2002; Neill et al., 2002). There are several enzymatic systems that have been shown to produce NO, mainly nitrate reductase (Rockel et al., 2002) and L-Arg-dependent nitric oxide synthase (NOS; Corpas et al., 2004). However, the gene for the plant NOS has not been identified yet (Zemojtel et al., 2006; Neill et al., 2008).

In this work, the effect of growing pea plants with $CdCl_2$ on the production of ROS and NO in leaves was studied in vivo by confocal laser microscopy. Taking into account that in pea plants the NOS-derived NO production is dependent on Ca (Corpas et al., 2004), the effect of this metal on NO and ROS production as well as the SOD activity were also investigated. To get deeper insights into the mechanisms of cellular response to Cd toxicity, the roles of different molecules that could be involved in cell signaling under metal stress, such as jasmonic acid (JA) and salicylic acid (SA), as well as ET, were studied. In addition, the expression of the antioxidative enzymes SOD, pathogen-related proteins (PRs), and defense-related proteins have been analyzed. All of these pieces of information are very important to understanding the mechanisms involved in the defense of plant cells against different types of abiotic stress.

RESULTS

Effects of Cd on Macronutrient and Micronutrient Content of Pea Leaves

In a previous work, it was found that Cd induced a strong reduction in the Ca content of leaves (Sandalio et al., 2001). Ca is an important signaling component in biotic and abiotic stresses, and disturbances in its content have been associated with toxicity by Cd, zinc (Zn), copper (Cu), or aluminum (Al; Kinraide et al., 2004), although the mechanisms involved are not well known. The growth of pea plants in full-nutrient solutions containing 50 μM $CdCl_2$ for 15 d produced an accumulation of Cd in the leaves of about 13 $\mu g\ g^{-1}$ dry weight (Supplemental Table S1). In these conditions, a reduction in the content of the following nutrients was observed: Ca (27%), Cu (30%), iron (Fe; 19%), manganese (Mn; 47%), magnesium (Mg; 20%), and Zn (41%). On the contrary, Cd produced a 3-fold increase in the sulfur content, while the sodium contents were not affected by the heavy metal treatment (Supplemental Table S1).

To get more insights into the role of the Cd-induced Ca deficiency in heavy metal toxicity, plants were supplemented with 10 mM $Ca(NO_3)_2$. The addition of Ca to the nutrient solution produced an increase in the content of this element in both control and Cd-treated plants, and a 30% reduction in the Cd accumulation in the leaves of Cd-treated plants, without affecting the content of the remaining elements, except the Mn, which increased, and Mg, which decreased slightly, in control plants (Supplemental Table S1).

Response of SODs to Cd

The growth of pea plants for a long period of time with 50 μM $CdCl_2$ produced reductions in the activities of Mn-SOD, Fe-SOD, and CuZn-SOD of 60%, 80%, and 90%, respectively (Sandalio et al., 2001). The analysis by semiquantitative reverse transcription (RT)-PCR of pea plants treated with Cd showed a differential effect of this heavy metal on the expression of these antioxidative enzymes (Fig. 1). Cd up-regulated the expression of Fe-SOD but down-regulated the CuZn-SODs and Mn-SOD (Fig. 1). These results suggest that, under Cd stress conditions, Mn-SOD is regulated at the transcriptional level while CuZn-SOD and Fe-SOD are regulated at both the transcriptional and post-transcriptional levels. To investigate the involvement of Ca in the Cd-dependent regulation of SOD expression, pea plants were supplemented with $Ca(NO_3)_2$ during the Cd treatment and the CuZn-SOD transcript levels were analyzed. The exogenous Ca supply reversed the effect of Cd on CuZn-SOD expression, reaching the same levels as in control plants (Fig. 1). The enzymatic analysis of CuZn-SOD showed that this activity was also recovered by Ca (data not shown).

ROS and NO in the Plant Response to Cd

Confocal laser scanning microscopy (CLSM) has been widely used to study fluorescent probe distribution in

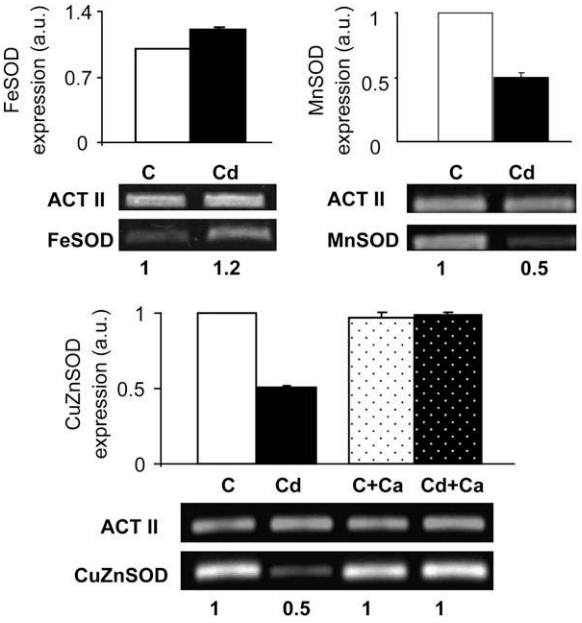

Figure 1. Cd effect on SOD expression in pea leaves. Analysis of mRNA expression of SODs was carried out by semiquantitative RT-PCR. Representative agarose electrophoresis gels show the amplification products visualized by ethidium bromide staining under UV light. Numbers express the change in the SOD band intensity relative to the untreated control (C; as detailed in "Materials and Methods"). Columns represent means of mRNA expression of SODs ± SE of three independent experiments with three replicates each in arbitrary units (a.u.).

fixed and living plant tissues (Fricker and Meyer, 2001; Sandalio et al., 2008). To image ROS and NO accumulation in leaves from pea plants treated with Cd, specific fluorescent probes were used. 2′,7′-Dichlorofluorescein diacetate (DCF-DA) was used to detect H_2O_2/peroxides, dihydroethidium (DHE) was used for $O_2^{\cdot-}$, and 4,5-diaminofluorescein diacetate (DAF-2DA) was used for NO, and samples were observed by CLSM (Sandalio et al., 2008). Three-dimensional reconstruction of the pea leaf sections showing chlorophyll autofluorescence (blue) and fluorescence due to DHE and DCF-DA are presented in Figure 2 and Supplemental Figures S1 and S2. Red $O_2^{\cdot-}$-dependent fluorescence of DHE was not visible in leaves from control plants (Fig. 2A), but in Cd-treated plants, red fluorescence was observed mainly in xylem vessels and adaxial sclerenchyma (Fig. 2, B and C; Supplemental Fig. S2B), epidermis, stomata, and mesophyll cells (Fig. 2, B and D; Supplemental Fig. S2D). The preincubation of leaf sections with 2,2,6,6-tetramethylpiperidinooxy (TMP), an $O_2^{\cdot-}$ scavenger, abolished completely the fluorescence (Fig. 2E), thus showing the specificity of the fluorescent probe DHE for the $O_2^{\cdot-}$. To investigate if the Cd-dependent $O_2^{\cdot-}$ production was related to the Ca deficiency previously observed, $O_2^{\cdot-}$ was also imaged in Ca-supplemented plants. Ca prevented the accumulation of superoxide radicals induced by Cd in mesophyll cells but not in epidermis and vascular tissues (Fig. 2G). In these tissues, $O_2^{\cdot-}$-dependent fluorescence was even higher in control plants, which suggests a possible involvement of Ca in ROS production, especially in the xylem vessels.

Cross sections of control and Cd-treated leaves incubated with DCF-DA showed a very bright green fluorescence due to peroxides, mainly to H_2O_2, in xylem vessels from vascular tissues and epidermis (Fig. 2, H and I; Supplemental Fig. S2F). However, in Cd-treated leaves, the fluorescence increased considerably in palisade mesophyll cells and, to a lesser extent, in sclerenchyma cells (Fig. 2I; Supplemental Fig. S2, F and H). When pea leaf sections were incubated with 1 mM ascorbate, a peroxide scavenger, the fluorescence was considerably reduced (Fig. 2J). Some of the fluorescence due to DCF-DA and DHE in mesophyll cells overlapped with the tissue autofluorescence, which indicates that part of the ROS production observed in leaf sections could be associated with chloroplasts (Fig. 2, D and L), although some fluorescence punctates, different from chloroplasts, can also be observed in the cytoplasm. A higher magnification of the images shows fluorescence in chloroplasts and spheric organelles, which could be peroxisomes or mitochondria (Fig. 2, D and L; Supplemental Fig. S2H). In the sclerenchyma, the production of both ROS was mainly located in the apoplast, especially in the intercellular space connecting cells (Fig. 2, C and K; Supplemental Fig. S2, B and F). The analysis of H_2O_2 by cytochemistry with $CeCl_3$ and electron microscopy showed that in xylem vessels from control plants, the H_2O_2-dependent precipitates were found on the inner side of cell wall (Supplemental Fig. S3).

The NO-derived DAF-2DA green fluorescence was found in xylem vessels, sclerenchyma, and epidermal cells of control plants (Fig. 3A); however, in contrast with ROS generation, Cd treatment produced a significant reduction of NO-dependent fluorescence observed in control leaves (Fig. 3B). The incubation of control leaves with aminoguanidine or L-NAME (data not shown), two well-known inhibitors of animal NOS, also produced a strong reduction of DAF-2DA fluorescence (Fig. 3C), which is indicative of the involvement of a NOS-like activity in the production of the NO detected. As a positive control, Cd-treated pea plants were incubated with 10 μM sodium nitroprusside (SNP), a NO donor, and the NO-dependent fluorescence was observed by confocal laser microscopy. In these conditions, a NO-dependent increase in DAF-2DA fluorescence in the leaf tissue was observed (Fig. 3D), showing the specificity of DAF-2DA for NO.

The constitutive L-Arg-dependent NOS activity previously described in pea plants is dependent on Ca and CaM (Corpas et al., 2004). To investigate if the Cd-dependent reduction of NO observed was due to a Ca deficiency, pea plants were supplemented with Ca $(NO_3)_2$ during the Cd treatment. The reduction of constitutive NO production by Cd was reversed by supplying Ca to the nutrient solution, reaching similar levels of NO to those observed in control leaves (Fig. 3,

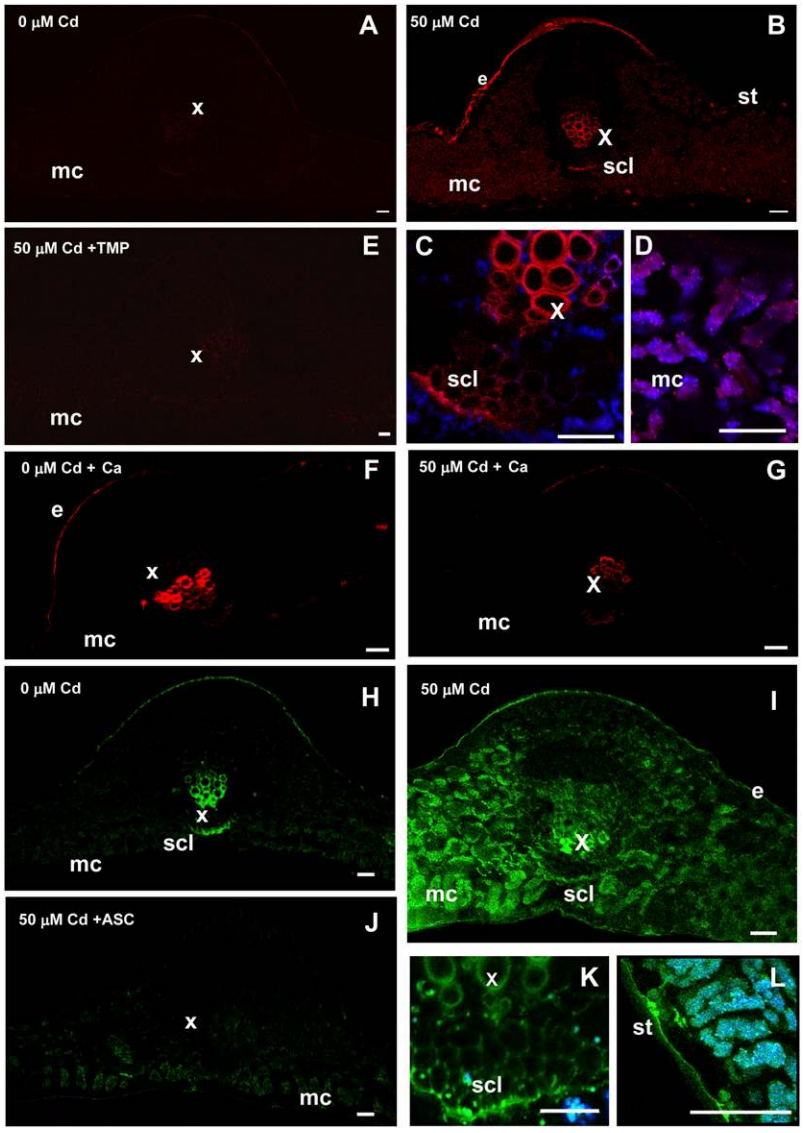

Figure 2. Imaging of ROS production in pea leaves by CLSM. Images are projections of several optical sections collected by confocal microscopy showing the autofluorescence (blue; excitation at 633 nm, emission at 680 nm) and fluorescence due to DHE and DCF-DA. A to G, $O_2^{\cdot-}$-dependent DHE fluorescence (red; excitation at 450–490, emission at 520) in leaf cross sections from control (A) and Cd-treated (B–D) pea leaves. C and D correspond to magnifications of vascular cylinder and mesophyll cells, respectively. As a negative control, leaves were incubated with 1 mM TMP, an $O_2^{\cdot-}$ scavenger (E). F and G represent the effects of exogenous Ca supply [10 mM $Ca(NO_3)_2$] on $O_2^{\cdot-}$ in control and Cd-treated plants, respectively. H to L, H_2O_2-dependent DCF-DA fluorescence (green; excitation at 485, emission at 530) in leaf cross sections from control (H) and Cd-treated (I–L) pea plants. As a negative control, leaves were incubated with 1 mM ascorbate (ASC), which acts as a H_2O_2 scavenger (J). K and L correspond to magnifications of vascular cylinder and mesophyll cells, respectively. These results are representative of at least six independent experiments. e, Epidermis; mc, mesophyll cells; scl, sclerenchyma; st, stomata; X, xylem vessels. Bars = 50 μm.

E and F; Supplemental Fig. S4). This suggests that the NO decrease by Cd could be due in part to an inactivation of the NOS activity as a consequence of the Cd-induced Ca deficiency in leaves. A higher magnification of images from Ca-Cd-treated plants shows the production of NO associated with the apoplast in xylem vessels and also in sclerenchyma cells (Fig. 3G; Supplemental Fig. S4B). A punctate pattern of fluorescence was also observed in mesophyll cells (Fig. 3H; Supplemental Fig. S4D). Images of chlorophyll autofluorescence are shown in Supplemental Figure S5.

JA, SA, and ET under Cd Stress

JA is a component of the signaling processes under biotic and abiotic stresses (Devoto and Turner, 2005). To determine whether JA was involved in the cell response to Cd toxicity, this molecule was analyzed by gas chromatography-mass spectrometry (GC-MS) in leaves from control and Cd-treated pea plants. Under Cd stress, an increase of two times in methyl jasmonate (MeJA) took place in pea leaves (Fig. 4), and free JA was detected neither in control nor in Cd-treated plants. The analysis of SA content shows that free SA was the main form present in pea leaves. On the contrary, Cd treatment did not produce any statistically significant effect on the SA levels, although the contents of conjugated (methyl salicylate [MeSA]) and free SA were slightly reduced in Cd-treated plants (Fig. 4). Analysis of ET by GC showed an increase of two times in leaves from pea plants grown with 50 μM $CdCl_2$ (Fig. 5), and this increase was reversed by supplying Ca to the nutrient solution, although a slight increase of ET emission was also observed in control plants (Fig. 5).

Effects of Cd on PRs and Defense Proteins

Studies of gene regulation under different stress conditions are important to get deeper insights into the regulation of defenses involved in each particular

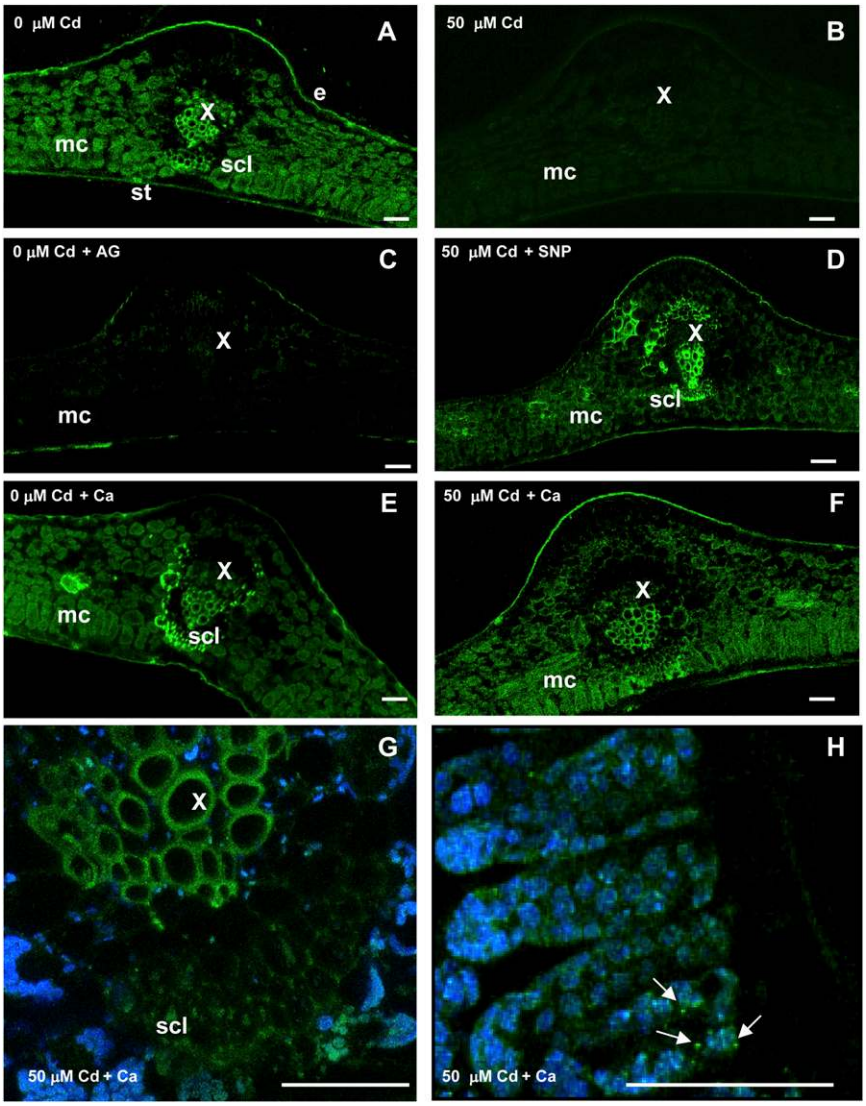

Figure 3. Imaging of NO production in pea leaves by CLSM. Images are projections of several optical sections collected by confocal microscopy showing the autofluorescence (blue; excitation at 633 nm, emission at 680 nm) and NO-dependent DAF-2DA fluorescence (green; excitation at 495 nm, emission at 515 nm) from control (A, C, and E) and Cd-treated (B, D, and F–H) pea plants. As a negative control, leaves were incubated with 1 mM aminoguanidine (AG), an inhibitor of mammalian NOS (C), and as a positive control, Cd-treated plants were grown with 100 μM SNP (D). E and F show the effect of exogenous Ca supply [10 mM $Ca(NO_3)_2$] on NO production in control and Cd-treated plants, respectively. G and H correspond to magnifications of vascular cylinder and mesophyll cells, respectively. Arrows represent small organelles showing NO-dependent fluorescence. These results are representative of at least six independent experiments. e, Epidermis; mc, mesophyll cells; scl, sclerenchyma; st, stomata; X, xylem vessels. Bars = 50 μm.

condition and the cross talk processes occurring in different stress conditions. Some PRs have been described to be up-regulated under abiotic stress (Tateishi et al., 2001; Stressmann et al., 2004), which suggests the existence of common effectors with biotic stress. The analysis of the expression of PRs and *HSP71.2* (for heat shock protein 71.2) in pea plants treated with Cd was carried out by semiquantitative RT-PCR and is shown in Fig. 6. Cd treatment up-regulated chitinase, *PrP4A*, and *HSP71.2*, while the expression of *PAL* (for Phe ammonia-lyase) transcripts did not change significantly with the treatment (Fig. 6). The induction of *PrP4A* and *HSP71.2* was reverted by the supply of ascorbate, a H_2O_2 scavenger, which suggests that both genes are at least partially regulated by ROS (Fig. 7). However, Ca did not change the expression level of *PrP4A* in both control and Cd-treated plants (data not shown). To study if there was a differential response of different cell types to Cd, the expression of the *PrP4A* gene was observed in situ on cross sections of pea leaves by fluorescence in situ hybridization (FISH; Fig. 8). FISH assays were analyzed by confocal microscopy, and various images were collected after excitation with different laser channels: the green fluorescence emission of the hybridization signal corresponding to PrP4A mRNAs, the blue fluorescence emission of the 4,6-diamidino-2-phenylindole (DAPI) staining for visualization of the nuclei, and the merged images of both, DAPI/FISH, fluorescence signals. Additionally, the structure of the leaf section was visualized and captured by differential interference contrast. FISH results showed an intense hybridization signal in leaves of Cd-treated plants (Fig. 8C), in contrast to control plants, where a very faint signal was observed (Fig. 8B). No hybridization signal was observed in control experiments with the sense probe (Fig. 8A). *PrP4A* expression was observed in mesophyll cells (Fig. 8C), especially in palisade cells from Cd-treated pea plants (Fig. 8E); the hybridization signal was localized in the cytoplasm,

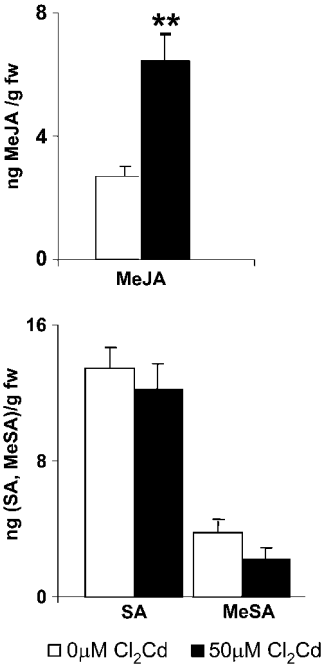

Figure 4. MeJA and MeSA production in leaves from control and Cd-treated pea plants. The content of JA and SA was determined by GC-MS as described in "Materials and Methods." Each column represents the mean ± SE of three independent experiments with six replicates each. **, Differences were significant at $P < 0.01$, according to Duncan's multiple range test. fw, Fresh weight.

with the nuclei (visualized by DAPI with blue fluorescence) being free of signal (Fig. 8C, magnification). However, the signal was absent in epidermis (Fig. 8, C and E) and xylem tissue (Fig. 8G), although a weak/low FISH signal was observed in stomata (Fig. 8, C and E) and in parenchyma cells surrounding xylem vessels (Fig. 8G).

DISCUSSION

Cd Produces Disturbances in Cation Accumulation

Cd is well known to produce disturbances in both the uptake and distribution of elements in pea plants (Hernández et al., 1998; Sandalio et al., 2001; Tsyganov et al., 2007) and other plant species (Gussarson et al., 1996; Rogers et al., 2000). In this work, long-term growth with 50 μM $CdCl_2$ produced a decrease in the contents of Ca, Cu, Fe, Mn, and Zn in pea leaves. Similar results have been observed previously in the same species (Sandalio et al., 2001) as well as in other plant species (Salt et al., 1995; Gussarson et al., 1996; Shukla et al., 2003; Azevedo et al., 2005). On the contrary, sulfur was accumulated 3-fold in Cd-treated plants with respect to the control plants. The induction of sulfur metabolism by Cd has been described previously and involves a coordinated transcriptional regulation of genes for sulfate uptake and its assimilation, as well as glutathione and phytochelatin biosynthesis (Howarth et al., 2003; Nocito et al., 2006). Induction of phytochelatins is one of the main detoxification strategies against Cd, by chelating Cd ions and preventing its toxicity (Howarth et al., 2003; Nocito et al., 2006).

Several studies have demonstrated that Cd can enter the cells by the same uptake systems used by cations such as Fe, Cu, Ca, and Zn. Excess Cd could compete with those elements for the transporters promoting a reduction in both uptake and accumulation of those cations (Clemens, 2006). The exogenous supply of Ca to the nutrient solution reduced the accumulation of Cd in the tissue, which demonstrates the competition between both elements for the same transporters. In contrast, the addition of Ca did not alter considerably the accumulation of the rest of the elements, except for Mn and Mg in control plants. It has been observed that Cd competes with Ca not only for the transporters but also for intracellular Ca-binding proteins (Rivetta et al., 1997) and plasma membrane (Kinraide, 1998). Recently, Tsyganov et al. (2007) observed a relationship between Cd tolerance and homeostasis of Ca in both roots and shoots in a Cd-tolerant pea mutant ($SGECd^t$). Ca has also been reported to alleviate Cd toxicity in radish (*Raphanus sativus*; Rivetta et al., 1997), rice (*Oryza sativa*) roots (Kim et al., 2002), and Arabidopsis (*Arabidopsis thaliana*) seedlings (Suzuki, 2005) by reducing Cd uptake. Ca also prevents Al-dependent growth inhibition in wheat (*Triticum aestivum*; Kinraide and Parker, 1987).

Differential Expression of SODs by Cd

Cd-dependent reduction of SOD activity has been reported in wheat (Milone et al., 2003), pea (Sandalio et al., 2001), and bean (*Phaseolus vulgaris*; Cardinaels et al., 1984), although the opposite effect was observed

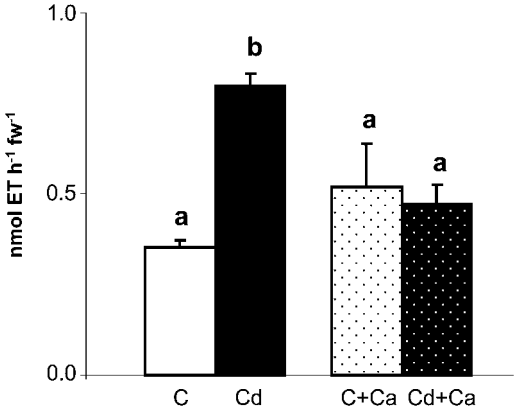

Figure 5. Effects of Cd and Ca on ET emission in pea leaves. The content of ET was assayed by GC, as described in "Materials and Methods." Each column represents the mean ± SE of two independent experiments with six replicates each. Values indicated by the same letter are not significantly different ($P < 0.05$), according to Duncan's multiple range test. fw, Fresh weight.

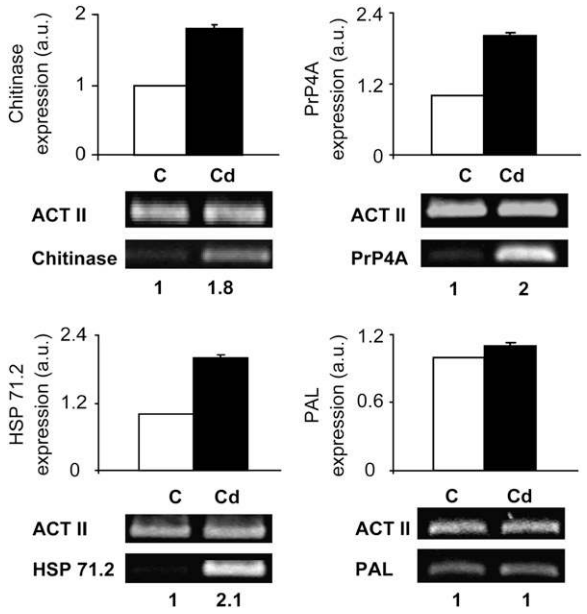

Figure 6. Cd effects on the expression of *PrP4A, PAL,* chitinase, and *HSP71.2* in pea leaves. Analysis of mRNA expression was carried out as described for Figure 1. Columns represent means of mRNA expression of SODs ± SE of three independent experiments with three replicates each in arbitrary units (a.u.).

in *Alyssum* plants (Schickler and Caspi, 1999) sunflower (*Helianthus annuus*; Laspina et al., 2005), coffee (*Coffea arabica*) cells (Gomes-Junior et al., 2006), and radish roots (Vitória et al., 2001). These discrepancies are due to differences in the metal concentration and also in the period of treatment used in each case, in addition to the plant tissue studied. Thus, in garlic (*Allium sativum*) plants, SOD increased under short Cd treatment but decreased after long-term exposure (Zang et al., 2005). In this work, long-term exposure to high Cd concentrations produced in pea leaf the down-regulation of Mn-SOD and CuZn-SOD transcripts, which is correlated with the reduction of their activities observed previously (Sandalio et al., 2001; Romero-Puertas et al., 2007). The plastidic Fe-SOD, in turn, was up-regulated, although its activity was previously observed to be reduced by the metal (Sandalio et al., 2001). This suggests a possible posttranslational regulation of Fe-SOD by oxidation (Fe-SODs are sensitive to H_2O_2) or by reduction of Fe availability (del Río et al., 1991). A similar effect was observed in pea roots under the same experimental conditions, except for the Mn-SOD, which was up-regulated by Cd at both transcript and activity levels (Rodríguez-Serrano et al., 2006). Down-regulation of CuZn-SOD by Cd was reverted by Ca supply, and the same results were observed in Arabidopsis plants (data not shown), which suggests a role of this element in the regulation of CuZn-SOD at the transcriptional level, although the mechanism involved is unknown. CuZn-SOD activity was also recovered by Ca (data not shown). Ca deficiency has also been associated with a reduction of SOD activity and oxidative stress in tomato (*Solanum lycopersicum*) plants (Schmitz-Eiberger et al., 2002). Concerning the CuZn-SOD regulation, Sunkar et al. (2006) have demonstrated that microRNA (miR398) regulates this protein in Arabidopsis under oxidative stress.

Cd Induces ROS Accumulation and Reduction of NO

The reduction observed in SOD activity and other antioxidants such as CAT observed previously (Sandalio et al., 2001; Romero-Puertas et al., 2007) could be responsible for the overproduction of ROS detected by CLSM, which would produce oxidative damages at macromolecules, being responsible for the Cd toxicity. A Cd-dependent accumulation of peroxides has also been observed in alfalfa (*Medicago sativa*) roots (Ortega-Villasante et al., 2005) and pea roots (Rodríguez-Serrano et al., 2006) using DCF-DA and CLSM, and overproduction of $O_2^{\cdot -}$ was also observed using DHE in *Lupinus luteus* roots (Kopyra and Gwózdz, 2003). The analysis of H_2O_2 in pea leaf sections by DCF-DA fluorescence showed an induction of this ROS production by Cd mainly in mesophyll cells, probably associated with chloroplasts, mitochondria, and peroxisomes, and in plasma membrane from epidermal cells. In a previous work, using $CeCl_3$ cytochemistry,

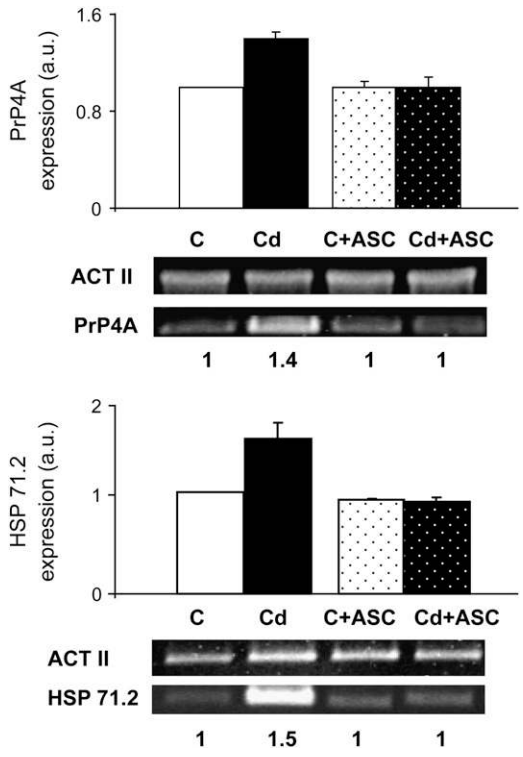

Figure 7. Effects of ascorbate on the expression of *PrP4A* and *HSP71.2* in control (C) and Cd-treated pea leaves. Analysis of mRNA expression was carried out as described for Figure 1. Columns represent means of mRNA expression of SODs ± SE of three independent experiments with three replicates each in arbitrary units (a.u.).

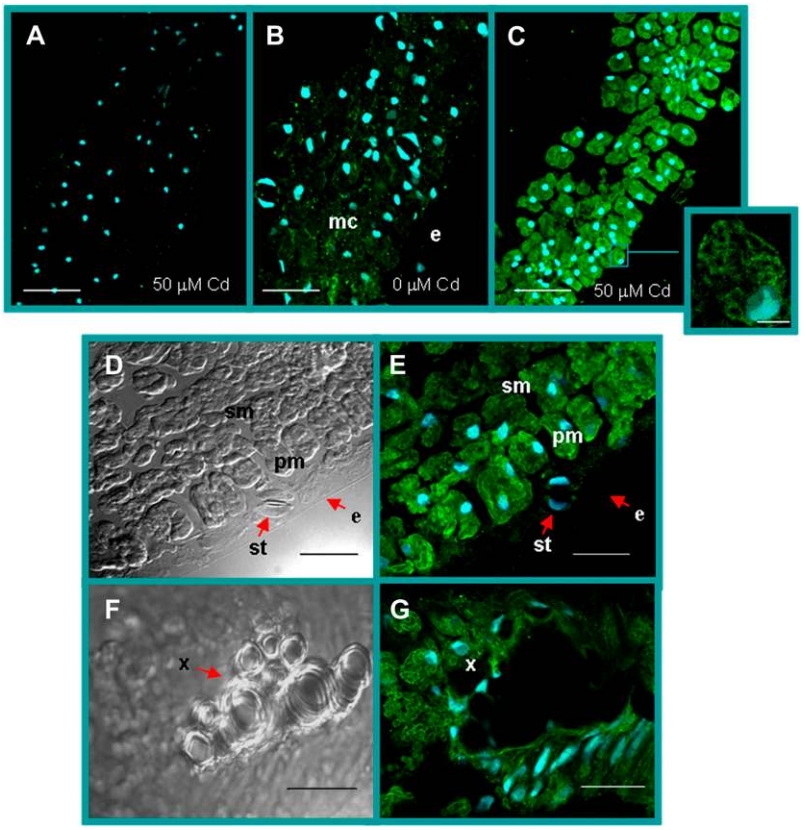

Figure 8. Localization of *PrP4A* expression by FISH on pea leaf sections under Cd toxicity. Images are projections of several optical sections collected by confocal microscopy showing the merged fluorescence provided by the DAPI staining of nuclei in blue and the FISH signal in green. A, Cross section of Cd-treated pea leaf hybridized with the sense probe showing the absence of hybridizing signal. B, Cross section of control pea leaf hybridized with the antisense probe (green). C, Cross section of Cd-treated pea leaf hybridized with the antisense probe showing intense green hybridization signal in the mesophyll cells. The small inset box shows a higher magnification (bar = 10 μm) of a single mesophyll cell showing fluorescence of the cytoplasm. E and G, Details of mesophyll and xylem vessels in Cd-treated plants hybridized with the antisense probe. D and F, Same as E and G, showing Nomarski images. These results are representative of three independent experiments. e, Epidermis; mc, mesophyll cells; pm, palisade mesophyll; sm, spongy mesophyll; st, stomata; x, xylem. Arrows indicate stomata (st), epidermis (e), and xylem vessels (x). Bars = 50 μm.

the Cd-dependent accumulation of H_2O_2 in peroxisomes, mitochondria, and plasma membrane was demonstrated, with NADPH oxidase being the main source of ROS in the plasma membrane (Romero-Puertas et al., 2004). An oxidative burst has also been associated with Cd toxicity in tobacco (*Nicotiana tabacum*) cell suspensions, with a NADPH oxidase being involved (Olmos et al., 2003; Garnier et al., 2006). The highest fluorescence detected was localized in the cell wall of the xylem vessels (Fig. 2). Similar results have been observed in pea roots, where the highest ROS production was associated with the vascular tissue (Rodríguez-Serrano et al., 2006). In different plant species, ROS production has been associated with cell wall lignification in the xylem (Ogawa et al., 1997; Ros-Barceló, 1999). Apart from lignification, the production of ROS in vascular tissues could serve as a signal under stress conditions, as has been proposed in wounding damage (Orozco-Cardenas and Ryan, 1999). ROS overproduction was partially due to the Ca deficiency induced by Cd treatment, to judge by the negative effect of Ca supply on $O_2^{\cdot-}$ production. Thus, exogenous Ca supply reversed the Cd-treated phenotype, getting similar results to those obtained in control plants grown in the presence of Ca, reducing considerably the Cd-dependent $O_2^{\cdot-}$ production in mesophyll cells. However, in control plants, Ca produced a slight increase of $O_2^{\cdot-}$ accumulation in xylem vessels and epidermis, which can be explained by the NADPH oxidase activation by Ca. These results suggest that other sources different from NADPH oxidase can be involved in the Cd-dependent ROS production in mesophyll cells, such as peroxidases in the plasma membrane (Choi et al., 2007), the electron transport chain in mitochondria (Romero-Puertas et al., 2004; Garnier et al., 2006), glycolate oxidase in peroxisomes (Romero-Puertas et al., 1999, 2004), and chloroplasts (Azpilicueta et al., 2007). The protecting role of Ca can be also explained by the up-regulation of antioxidants such as CuZn-SOD.

The analysis of NO production by DAF-2DA fluorescence microscopy showed that fluorescence of control leaves was mainly due to a NOS-like activity, to judge by its inhibition by aminoguanidine (Corpas et al., 2004). But, in contrast with ROS, the production of NO was strongly reduced by Cd (Fig. 3). The reduction of NO levels by Cd was also observed previously in pea roots and leaves under the same experimental conditions (Barroso et al., 2006; Rodríguez-Serrano et al., 2006). Al treatment also led to a reduction of NO production in roots from *Hibiscus* (Tian et al., 2007) and Arabidopsis (Illéš et al., 2006) plants. However, Bartha et al. (2005) and Kopyra et al. (2006) observed a Cd-dependent increase of NO accumulation in roots from pea seedlings and soybean (*Glycine max*) cell suspensions, respectively, after short-term treatment with Cd. This discrepancy could be attributed to differences in the cell response to short and long periods of metal treatment. The constitutive NOS activity described in pea plants is dependent on Ca

and CaM (Corpas et al., 2004, 2006). The Ca-dependent restoration of NO accumulation in Cd-treated plants shows that Ca could be a key point in Cd toxicity by reducing NOS activity and modulating NO production and, therefore, those proteins regulated by *S*-nitrosylation. This result suggests the existence of cross talk between NO, ROS, and Ca under Cd toxicity. In rat brain, Cd also produced an inhibition of a constitutive Ca-CaM-dependent NOS activity, which was prevented by exogenous Ca supply (Demontis et al., 1998).

NO is a free radical that can react with $O_2^{\cdot-}$ and, thus, regulate its accumulation in the tissue (Romero-Puertas and Delledonne, 2003). The reduction of NO under Cd treatment could favor $O_2^{\cdot-}$ accumulation, promoting oxidative damages. This fact is supported by the reduction of $O_2^{\cdot-}$ accumulation after the restoration of NO production induced by Ca treatment. The involvement of NO in different biotic and abiotic stresses has been demonstrated (Gould et al., 2003). In *L. luteus* roots, the supply of NO as SNP reduced the negative effects of Cd, NaCl, ET, and paraquat and reduced the $O_2^{\cdot-}$ production induced by Cd and lead (Kopyra and Gwózdz, 2003). A protective role of NO has also been observed in sunflower leaves under Cd toxicity (Laspina et al., 2005). NO is also a signal molecule involved in triggering the defense response of cells against different stress conditions (Romero-Puertas and Delledonne, 2003; Neill et al., 2008). The imbalance of the $O_2^{\cdot-}$/NO ratio could favor oxidative conditions but could also interfere in the signal transduction pathways of the defense mechanism against stress (Delledonne et al., 2001).

In this work, the main production of ROS and NO took place in the xylem, sclerenchyma, and epidermis. These results are consistent with reports from other authors showing that in cell wall lignification of xylem elements, an oxidative burst is involved and a NO burst also participates in the programmed cell death associated with the differentiating vessels (Gabaldón et al., 2005). Moreover, ROS and NO production could be involved in signal transduction pathways to activate the response to stress in other tissues.

The Cellular Response to Cd Is Mediated by JA and ET

To get deeper insights into the mechanisms involved in the cell response to Cd toxicity, an analysis of JA, SA, and ET contents was carried out. Cd induced an increase of JA and ET, which suggests that these molecules are involved in the cellular response to Cd toxicity. JA is an oxylipin that acts as a signaling compound in different defense situations such as response to pathogens and herbivore attack (Wasternack and Parthier, 1997). However, responses mediated by JA can be also triggered by diverse abiotic stresses (Devoto and Turner, 2005). JA is obtained from linolenic acid, and its production is associated with lipid peroxidation and membrane damage. In a previous work, we demonstrated that growth of pea plants with Cd induced lipid peroxidation in leaves (Sandalio et al., 2001), which would explain the increase observed in this work in JA production. The activation of the pathogen-dependent JA receptor is linked to the ion channel stimulation and ROS production (McDowell and Dangl, 2000; Garrido et al., 2003), and these conditions come together under Cd stress. In Arabidopsis plants, JA regulates genes involved in glutathione and phytochelatin synthesis under Cd treatment (Xiang and Oliver, 1998). The increase of JA could also contribute to metal toxicity through the activation of lipoxygenase activity, H_2O_2 production, and lipid peroxidation (Wang and Wu, 2005; Maksymiec et al., 2007).

ET plays a pleiotropic role in plant growth and development and is involved in a number of processes, including germination, senescence, and fruit ripening, but it also participates in a variety of defense responses (Guo and Ecker, 2004). The stimulation of ET biosynthesis by Cd has been reported in different plant species (Sanitá di Toppi and Gabbrielli, 1999), although the molecular relationship between ET biosynthesis and Cd stress has not been well established (Sanitá di Toppi and Gabbrielli, 1999). In this work, we observed that Ca supply reversed the induction of ET by Cd, and this fact could be due to an indirect effect of Ca on ROS and NO production, as shown in Figures 2 and 4. ET and NO are antagonistic (Leshem, 2000), which would explain the reduction of NO and the rise of ET emission observed in this work. This fact is supported by the effect of Ca on the accumulation of NO and ET. Leshem (2000) has proposed that NO could inhibit 1-aminocyclopropane-1-carboxylic acid synthase or 1-aminocyclopropane-1-carboxylic acid oxidase and so prevent ET formation. Recently, Lindermayr et al. (2006) demonstrated the reversible inhibition of Met adenosyltransferases (MAT-1) by *S*-nitrosylation, which can originate a reduction of the *S*-adenosylmethionine pool and, therefore, a decrease of ET biosynthesis. Thus, the Cd-dependent reduction of the NO level in leaves, observed in this work, could alter MAT-1 regulation by NO and, therefore, increase ET biosynthesis.

SA plays an important role in signal transduction pathways, being involved in the induction of the hypersensitive response (Alvarez, 2000). However, the results obtained in this work suggest that, under Cd toxicity, SA is not involved in the cellular response in leaves, although in pea roots, different results were obtained (Rodríguez-Serrano et al., 2006). SA alleviates Cd toxicity in barley (*Hordeum vulgare*) roots, but the mechanisms involved are not well known (Metwally et al., 2003).

PRs and Defense-Related Proteins Could Protect against Cd Toxicity

The analysis of PR expression in pea plants under Cd stress showed the up-regulation of chitinases, *PrP4A*, and *HSP71.2*, while *PAL* did not change. Chitinases

catalyze the hydrolytic cleavage of the β-1,4-glycoside bond of GlcNAc and is considered a defense mechanism against pathogens (Kasprzewska, 2003). An induction of chitinase activity has been observed in pea plants by Cd (Metwally et al., 2003) and other heavy metals like lead and arsenic (Békésiová et al., 2008) and also by osmotic stress (Tateishi et al., 2001), low temperature (Stressmann et al., 2004), and wounding (Wu and Bradford, 2003). Chitinases are probably components of the general defense response program of cells, although they can also play unknown specific roles in heavy metal stress. Thus, transgenic plants expressing fungal chitinases showed enhanced tolerance to metals (Dana et al., 2006), and chitinase isoforms are differentially modified by different metals (Békésiová et al., 2008). Concerning the regulation of chitinases, Wu and Bradford (2003) demonstrated that they are regulated by ET and JA in tomato leaves, and Rakwal et al. (2004) reported a regulation by ET, JA, and ROS in rice plants.

PrP4A is a hevein-related protein that binds chitin, can inhibit the growth of fungus, and belongs to chitinase I and II classes (Broekaert et al., 1990). The Cd-dependent up-regulation of *PrP4A* is in accordance with data reported by Sävestrand et al. (2000), who showed an induction of this gene under ozone, UV-B radiation, and pathogen attack. The regulation of this gene was dependent on ET in Chinese cabbage (*Brassica campestris* ssp. *pekinensis*) plants (Chung et al., 2005) and was sensitive to ET and JA in Arabidopsis (Thomma et al., 2001), and in both cases it was not dependent on SA, which could explain its up-regulation by Cd. In this work, we have demonstrated that *PrP4A* is also regulated by ROS, although exogenous Ca supply does not affect the up-regulation under Cd toxicity. In situ localization of *PrP4A* transcripts in pea leaves revealed an accumulation mainly in the cytoplasm of palisade mesophyll cells, suggesting that *PrP4A* gene products have specific functions in these cells. The absence of transcripts in epidermis is in contrast to the data obtained for chitinase, β-1-glucanase, teonin, and *SAR8.2*, which were accumulated in phloem and epidermal cells of different plant species under pathogen infection (Wubben et al., 1996; Lee and Hwang, 2003). These discrepancies could be explained by the differential distribution of PRs in biotic and abiotic stress conditions.

The third gene induced by Cd corresponds to the cytosolic heat shock protein, *HSP71.2*, which is known to be up-regulated under heat stress; its function is to act as a molecular chaperone facilitating protein transport into organelles (DeRocher and Vierling, 1995) or preventing protein agregation (Ma et al., 2006). The induction of HSPs in pea plants under Cd treatment is apparently regulated by H_2O_2 overproduction, since ascorbate reversed the up-regulation induced by Cd; in addition to that, transcription factors involved in *HSP* regulation can act as H_2O_2 sensors (Miller and Mittler, 2006). In Arabidopsis plants, the regulation of HSP71.2 by JA has also been described (Cheong et al., 2002). HSPs are also up-regulated in response to wounding, osmotic stress, light (Wang et al., 2004), and oxidative stress (Ma et al., 2006). The up-regulation of genes involved in protein folding in response to Cd treatment has been observed in Arabidopsis plants (Suzuki et al., 2001), which demonstrates that Cd toxicity is in part due to the induction of protein denaturation, probably by oxidative modifications (Romero-Puertas et al., 2002). In contrast with the former PRs, *PAL* expression was not affected by the metal. The regulation of *PAL* is dependent on NO (de Pinto et al., 2002; Wang and Wu,

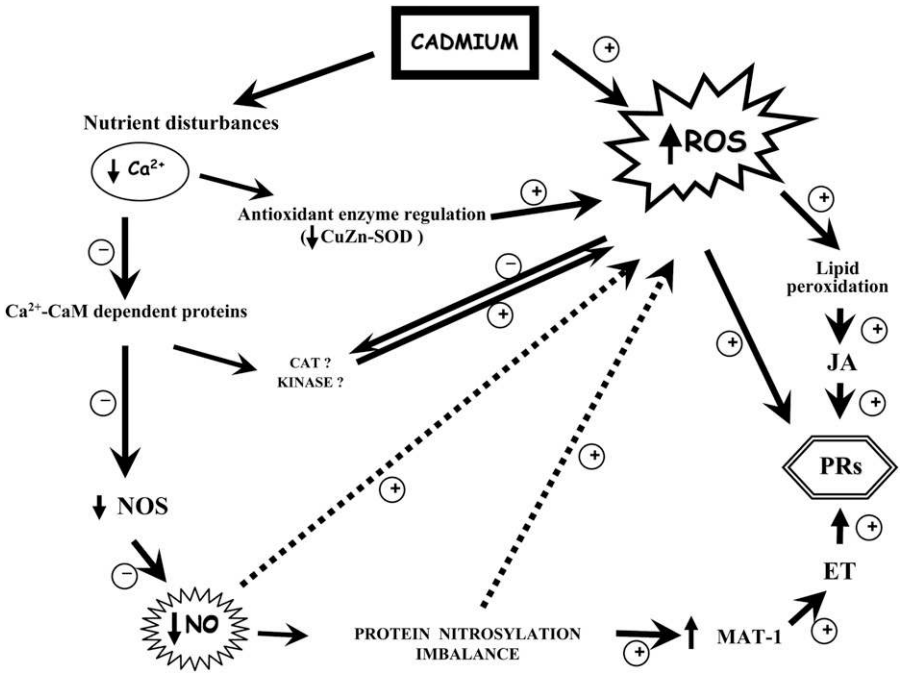

Figure 9. Model proposed for cross talk between Ca, ROS, and NO and its role in the regulation of the plant response to Cd toxicity. +, Up-regulation; −, down-regulation; ↓, reduction; ↑, increase; MAT-1, Met adenosyltransferase 1.

2005), and the reduction of NO induced by the Cd treatment could explain the absence of changes in the *PAL* transcript levels.

The increase of JA, ET, and ROS production, the up-regulation of PRs, and the NO reduction are common features of senescence (Obregón et al., 2001; Corpas et al., 2004; Rodríguez-Serrano et al., 2006), which suggests that Cd accelerates senescence processes in plants. This fact is supported by the results obtained by McCarthy et al. (2001), who demonstrated that Cd induces senescence symptoms in peroxisomes from pea leaves. The induction of PR genes under biotic and abiotic conditions suggests that different stresses induce the same set of genes by sharing effectors of gene regulation.

To judge by the results obtained in this work, in pea plants, Cd could generate a similar response induced by pathogen attack, which is characterized by ROS overproduction, NO reduction, and PR up-regulation. However, unlike ozone or pathogen attack, Cd did not produce any visible symptoms of local necrosis (Sandalio et al., 2001), although the formation of microlesions not visually detectable cannot be excluded. On the basis of the results obtained in this work and others reported previously, cross talk between ROS, NO, and Ca in the regulation of the cellular response to long-term Cd exposure is proposed (Fig. 9). Cd produces nutrient disturbances, with Ca being one of the most negatively affected elements. Ca and CaM are involved in the control of many physiological and biochemical processes, mainly through different signal transduction pathways. In plant cells, some of the proteins modulated by CaM include NAD kinases, Glu decarboxylase, HSPs (Lu and Harrington, 1994), and CAT (Yang and Poovaiah, 2002), among others. A reduction of Ca content could interfere with the expression of antioxidant enzymes like CuZn-SOD or could inactivate Ca-CaM-dependent proteins (Rivetta et al., 1997). One of these proteins could be the NOS, which would explain the reduction in NO accumulation observed in leaves from pea plants treated with Cd. The reduction of NO could affect the activity of proteins regulated by *S*-nitrosylation, such as MAT-1 involved in ET biosynthesis, and this could be the reason for the induction of ET emission in Cd-treated plants. A decrease in the level of NO could, directly or indirectly, promote the accumulation of $O_2^{\cdot-}$ and induce oxidative stress. In turn, ROS accumulation can cause membrane damage that is involved in JA and ET production. Finally, the overproduction of JA, ET, and ROS could activate the cell response with the induction of PRs in order to protect proteins from damage associated with Cd toxicity.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Pea (*Pisum sativum* 'Lincoln') plants were obtained from Royal Sluis. Plants were grown in the greenhouse in aerated full-nutrient medium under optimum conditions during 14 d (Sandalio et al., 2001). Then, the medium either remained unsupplemented (control plants) or was supplemented with 50 μM $CdCl_2$ (Cd-treated plants), and plants were grown for 14 d. To determine the effect of Ca, control and Cd-treated plants were supplemented with 10 mM Ca $(NO_3)_2$ 1 d before the addition of Cd and were grown for 14 d. The effect of ascorbate (a H_2O_2 scavenger) was studied by infiltrating the leaves with 10 mM ascorbate.

ROS and NO Detection by CLSM

For NO detection, pea leaf segments of approximately 10 mm^2 were incubated for 1 h at 25°C, in darkness, with 10 μM DAF-2DA (Calbiochem; excitation at 495 nm, emission at 515 nm) prepared in 10 mM Tris-HCl (pH 7.4), as described by Sandalio et al. (2008). Superoxide radicals were detected by incubating leaf sections with 10 μM DHE (Fluka; excitation at 488 nm, emission at 520 nm) for 30 min, as indicated by Sandalio et al. (2008). H_2O_2 was detected by incubation with 25 μM DCF-DA (Calbiochem; excitation at 485 nm, emission at 530 nm; Rodríguez-Serrano et al., 2006). As negative controls, leaf sections were incubated with two ROS scavengers, 1 mM TMP ($O_2^{\cdot-}$ scavenger), and 1 mM ascorbate (H_2O_2 scavenger) and with two NOS inhibitors, 1 mM L-NAME and 1 mM aminoguanidine. Then, they were washed twice in the same buffer for 15 min each and were embedded in 30% (w/v) polyacrylamide blocks. Leaf sections were cut with a Vibratome and mounted for examination with a confocal laser scanning microscope (Leica TCS SL; Leica Microsystems). Autofluorescence due to the chlorophyll was detected by excitation at 633 nm and emission at 680 nm.

RT-PCR Analysis of Gene Expression

Total RNA was isolated from leaves by the acid guanidine thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (1987) using the Trizol reagent kit according to the manufacturer's instructions. Two micrograms of total RNA from leaves was used as template for the RT reaction. It was added to a mixture containing 0.5 μg of oligo$(dT)_{23}$ anchored (Sigma), 1× RT buffer, 20 units of RNasin ribonuclease inhibitor, and 15 units of AMV reverse transcriptase (Fynzymes). The reaction was carried out at 42°C for 40 min, followed by a 5-min step at 98°C and then by cooling to 4°C.

Amplification of actin II cDNA from pea was chosen as a control. The oligonucleotides used in this work are shown in Supplemental Table S2. cDNAs were amplified by PCR as follows: 1 μL of the produced cDNA (diluted 1:10 or 1:20) was added to 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1× PCR buffer, 1.25 unit of Hot Master Taq polymerase (Eppendorf), and 0.4 μmol of each primer (Supplemental Table S2) in a final volume of 20 μL. Reactions were carried out in a Master Cycler (Eppendorf). A first step of 2 min at 94°C was followed by 30 cycles of 20 s at 94°C, 20 to 40 s at annealing temperature (55°C–65°C, depending on the gene), and 30 s at 65°C, with a final extension of 10 min at 65°C. Amplified PCR products were detected after electrophoresis on 0.8% agarose gels (Serva) stained with ethidium bromide. Quantification of the bands was performed using a Chemidoc system (Bio-Rad Laboratories) coupled with a high-sensitivity CCD camera. Band intensity was expressed as relative absorbance units. Each cDNA band density was first normalized by dividing it by the density of the actin II band in the same lane (to compensate for the variations in the cDNA loading onto the gel). Then, the relative increase or decrease in gene expression in the Cd-treated leaves was calculated by dividing the normalized band density of the gene from the Cd-treated leaves by that of the same gene from the untreated (control) leaves (Marone et al., 2001). Consequently, the relative density of the control gene band is presented as 1.

Preparation of Probes

The cDNA from pea was obtained by the protocol described before and used for PCR amplification with the specific primers for the PrP4A included in Supplemental Table S2. The amplified fragments were isolated from an agarose gel and cloned using a pGEMT-Easy cloning system (Promega). Specific probes of single-stranded RNA were generated by in vitro transcription using the DIG RNA labeling kit according to the manufacturer's protocol (Roche).

FISH

Pea leaf pieces were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.0) for 16 h at 4°C, cryoprotected with 2.3 M Suc, embedded in

tissue-freezing medium, frozen on dry ice, and sectioned in a cryostat (Leica CM 1800) at −30°C. Cryostat sections (40–60 μm thickness) were thawed and pretreated to facilitate penetration of the labeling reagents: sections were dehydrated in a series of 30%, 50%, 70%, and 100% methanol/water, then rehydrated in a series of 70%, 50%, and 30% methanol/water, and finally in PBS, for 5 min at each step. Then, the sections were treated with 2% (w/v) cellulase (Onozuka R-10) in PBS for 1 h at room temperature, washed in PBS and water, and dried. The hybridization was performed essentially as described previously (Massonneau et al., 2005). Sections were incubated with hybridization solution at 50°C overnight. The hybridization solution consisted of digoxigenin-labeled RNA probe diluted 1:40 in the hybridization buffer (50% formamide, 10% dextran sulfate, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 300 mM NaCl, and 2× SSC) at room temperature and in 0.1× SSC at 50°C (1× SSC = 150 mM sodium chloride and 15 mM sodium citrate, pH 7.2). After washing in PBS, sections were incubated in 5% bovine serum albumin for 5 min. Then, the hybridization signal was detected by incubation with mouse anti-digoxigenin antibodies (Sigma), diluted 1:5,000 in 1% bovine serum albumin in PBS for 90 min at room temperature, followed by a fluorescence anti-mouse Alexa 488 (Molecular Probes; excitation at 495 nm, emission at 519 nm) antibody, applied 1:25 in PBS for 45 min at room temperature. After washing in PBS, the sections were counterstained with DAPI (excitation at 345 nm, emission at 455 nm), mounted in Mowiol, and observed. Confocal optical section stacks were collected using a confocal laser scanning microscope (Leica TCS-SP). Controls were performed by substituting the antisense RNA probe for the sense one at the same concentration in the hybridization solution.

JA and SA Determination

JA and SA were quantitated as described by Vigliocco et al. (2002) with some modifications (Rodríguez-Serrano et al., 2006). Pea leaves (5 g fresh weight) were homogenized in 10 mL of acetone. Extracts were dried by speed vacuum at 40°C and resuspended in 5% ethyl ether. The ethyl ether fraction was purified by GC on a SPE C18 column and eluted with 25 mL of ether. The fraction was dried at 25°C and redissolved in 5 mL of hexane:ether (90:10). This fraction was purified on a SPE SiO_2 column eluted with 50 mL of hexane: ether (90:10). This fraction contained free SA and JA. To obtain the SA and JA derived fractions (MeSA and MeJA, respectively), the C18 column was eluted with 50 mL of ether and derivatized with diazomethane. The eluted fractions were quantitated and identified by GC-MS by selected ion monitoring at mass-to-charge ratio 120 and 152 for the MeSA and at 224 and 151 for the MeJA. Standards of MeSA and MeJA were used in the range 10 to 500 ng. The GC-MS system was equipped with a 30- × 0.25- × 0.25-m film DB-MS column. The temperature was as follows: from 40°C (1 min) at 20°C min^{-1} to 150°C (3 min), and at 5°C min^{-1} to 230°C. Helium was used as the carrier gas (1 mL min^{-1}) with splitless injection (1 μL).

ET Determination

For the determination of endogenous ET production, fresh leaves (45 g) were placed in 100-mL hermetic vials, flushed with ET-free air, and incubated for 3 h at room temperature. The ET concentration was determined on a Perkin-Elmer 8600 gas chromatograph fitted with a flame-ionization detector and a Poropak-R column. Nitrogen was used as the carrier gas, and a commercial standard mixture of ET was used for calibration of the gas chromatograph.

Macronutrient and Micronutrient Determination

Leaves were dried and mineralized with H_2O_2/nitric acid and microwaved, and the contents of the elements were assayed by inductively coupled plasma emission spectrometry analysis.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Imaging of chlorophyll autofluorescence in pea leaves by CLSM (corresponding to Fig. 2).

Supplemental Figure S2. Imaging of chlorophyll autofluorescence and ROS production in pea leaves treated with Cd by CLSM.

Supplemental Figure S3. Localization of H_2O_2 in control pea leaves by cytochemistry with $CeCl_3$ and electron microscopy.

Supplemental Figure S4. Imaging of chlorophyll autofluorescence and NO production in pea leaves treated with Cd and Ca by CLSM.

Supplemental Figure S5. Imaging of chlorophyll autofluorescence in pea leaves by CLSM (corresponding to Fig. 3).

Supplemental Table S1. Effects of Cd and Ca treatments on nutrient contents of leaves of pea plants.

Supplemental Table S2. Oligonucleotides used in this work for the semiquantitative PCR analysis.

ACKNOWLEDGMENTS

Special thanks are given to Miss N. de la Casa for her skillful technical assistance. The laser confocal microscopy analyses were carried out at the Technical Services of the University of Jaén. The inductively coupled plasma analysis was carried out at the Ionomic Service of Centro de Edafología y Biología Aplicada del Segura (Consejo Superior de Investigaciones Científicas) in Murcia, and JA and SA analyses were carried out at the Technical Services of the Instituto de la Grasa (Consejo Superior de Investigaciones Científicas) in Seville.

Received October 21, 2008; accepted March 8, 2009; published March 11, 2009.

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