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Research area: Environmental stress and adaptation
Cellular Response of pea plants to cadmium toxicity: cross-talk between reactive oxygen species, nitric oxide and calcium

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Financial sources: This work was supported by grant BIO2005-03305 from the Ministry of Education and Science, and Junta de Andalucía (Research Group BIO-0192), Spain.

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ABSTRACT

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 yet. In this work, using pea plants we have studied the effect of Cd on antioxidants, reactive oxygen species (ROS) and nitric oxide (NO) metabolism of leaves by using different cellular, molecular and biochemical approaches.

The growth of pea plants with 50 µM CdCl₂ affected differentially the expression of superoxide dismutase isozymes at both transcriptional and post-transcriptional level, giving rise to an SOD activity reduction. The CuZn-SOD down-regulation was apparently due to the calcium (Ca) deficiency induced by the heavy-metal. In these circumstances, the overproduction of ROS (H₂O₂ and O₂⁻) could be observed in vivo by confocal laser microscopy, mainly associated with vascular tissue, epidermis, and mesophyll cells and production of superoxide radicals was prevented by exogenous Ca. On the other hand, the nitric oxide synthase (NOS)-dependent NO production was strongly depressed by Cd and the treatment with Ca prevented this effect. Under these conditions, the pathogenesis-related proteins (PRPs) PrP4A, chitinase, and the HSP 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 in a cross-talk between Ca, ROS and NO.
INTRODUCTION

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 uptaken by plant roots and then can be loaded into the xylem for its transport into leaves. Most plants are sensitive to low Cd concentrations which inhibit plant growth as consequence of alterations in the photosynthesis rate and the uptake and distribution of macro and micronutrients (Lozano-Rodríguez 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 defences producing an oxidative stress (Somashekaraiah et al., 1992; Shaw, 1995; Gallego et al., 1996; Sandalio et al., 2001; Dixit 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 plants under Cd stress has been reported (Romero-Puertas et al., 2004). ROS were detected in epidermic, transfer and mesophyll cells, being plasma membrane the main source of ROS, although mitochondria and peroxisomes were also involved (Romero-Puertas et al., 2004). Concerning the mechanism of ROS production, Cd does not participate in Fenton-type reactions (Stoch and Bagchi, 1995) but can indirectly favour the production of different ROS (H₂O₂, O₂⁻, ·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 are involved in the detoxification of O₂⁻, and H₂O₂, respectively, thereby preventing the formation of ·OH radicals. Ascorbate peroxidase (APX) and glutathione reductase (GR), as well as glutathione, are important components of the ascorbate-glutathione cycle responsible for the removal of H₂O₂ in different cellular compartments (Jiménez et al., 1997; Noctor et al., 1998). Apart
from its toxic role, ROS are also involved in signalling in different processes such as growth, development and response to biotic and abiotic stress and this signalling process is controlled by a balance between ROS production and scavenging (Bailey and Mittler, 2006).

The 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 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; Pedroso and Durzan, 2000; Clark et al., 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-arginine-dependent nitric oxide synthase (NOS) (Corpas et al., 2004). However, the gene of 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 CdCl₂ 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, and the SOD activity, was also investigated. To get deeper insights into the mechanisms of cellular response to Cd toxicity, the role of different molecules that could be involved in cell signalling under metal stress, such as jasmonic acid and salicylic acid, and ethylene, was studied, as well as the expression of the antioxidative enzymes superoxide dismutases and pathogen-related proteins of pea leaves. All these pieces of information are very important to understand the mechanisms involved in the defense of plant cells against different types of abiotic stress.
RESULTS

Effect of Cd on macro- and micro-nutrient 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 signalling component in biotic and abiotic stress and disturbances in its content has been associated with toxicity by Cd, Zn, Cu, or Al (Kinraide et al., 2004), although the mechanisms involved are not well known yet. The growth of pea plants in full-nutrient solutions containing 50 µM CdCl₂ for 15 days produced an accumulation of Cd in the leaves of about 13 µg per g of dry weight (Table S1). In these conditions, a reduction in the content of the following nutrients was observed: Ca (27%), Cu (30%), Fe (19%), Mn (47%), Mg (20%) and Zn (41%). On the contrary, Cd produced a 3-fold increase in the S content, while the Na contents were not affected by the heavy-metal treatment (Table S1).

To get more insights into the role of the Cd-induced Ca deficiency in the heavy-metal toxicity, plants were supplemented with 10 mM Ca(NO₃)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 increases and Mg that decreases slightly, in control plants (Table S1).

Response of superoxide dismutases to Cd

The growth of pea plants for a long period of time with 50 µM CdCl₂ produced reductions in the activities of Mn-SOD, Fe-SOD and Cu,Zn-SOD of 60, 80 and 90%, respectively (Sandalio et al., 2001). The analysis by semiquantitative 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 transcriptional level, while Cu,Zn-SOD and Fe-SOD are regulated at both transcriptional and posttranscriptional level. To investigate the involvement of Ca in the Cd-dependent regulation of SOD expression, pea plants were supplemented with Ca(NO₃)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 probes distribution in 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. DCF-DA was used to detect $\text{H}_2\text{O}_2$/peroxides, DHE for $\text{O}_2^-$, and DAF-2 DA for NO, and samples were observed by confocal laser microscopy (CLSM) (Sandalio et al., 2008). Three dimensional re-construction of the pea leaf sections showing chlorophyll autofluorescence (blue colour) and fluorescence due to DHE and DCF-DA are presented in the Figure 2, Figure S1 and Figure S2. Red $\text{O}_2^-$-dependent fluorescence of DHE was not visible in leaves from control plants (Fig 2, panel A), but in Cd-treated plants red fluorescence was observed mainly in xylem vessels, and adaxial sclerenchyma (Fig 2, panel B and C; Fig S2, panel B), epidermis, stomata and mesophyll cells (Fig 2, panel B and D; Fig S2, panel D). The preincubation of leaf sections with TMP, a $\text{O}_2^-$ scavenger, abolished completely the fluorescence (Fig 2, panel E), thus showing the specificity of the fluorescent probe DHE for the $\text{O}_2^-$. To investigate if the Cd-dependent $\text{O}_2^-$ production was related to the Ca deficiency previously observed, $\text{O}_2^-$ 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 2, panel G). In these tissues $\text{O}_2^-$-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 $\text{H}_2\text{O}_2$, in xylem vessels from vascular tissues and epidermis (Fig 2, panel H and I; Fig S2, panel F). However, in Cd treated leaves the fluorescence increased considerably in palisade mesophyll cells and, in less extent, in sclerenchyma cells (Fig 2, panel I; Fig S2, panel F and H). When pea leaf sections were incubated with 1 mM ascorbate, a peroxide scavenger, the fluorescence was considerably reduced (Fig 2, panel J). Some of the fluorescence due to DCF-DA, and DHE in mesophyll cells overlapped with the tissue autofluorescence which indicates that part of ROS production observed in leaf sections could be associated with chloroplasts (Fig 2, panel D and L), although some fluorescence punctates, different from chloroplasts, can be also observed in the cytoplasm. A higher magnification of the pictures shows fluorescence in chloroplasts and
spheric organelles which could be peroxisomes or mitochondria (Fig 2, panels D and L; Fig S2, panel H). In the sclerenchyma, the production of both ROS was mainly located in the apoplast, especially in the intercellular space connecting cells (Fig 2, panels C and K; Fig S2, panels B and F). The analysis of H$_2$O$_2$ by cytochemistry with CeCl$_3$ and electron microscopy showed that in xylem vessels from control plants the H$_2$O$_2$-dependent precipitates took place in the inner side of cell wall (Fig S3).

The NO-derived DAF-2DA green fluorescence was found in xylem vessels, sclerenchyma, and epidermic cells of control plants (Fig 3, panel A) but, in contrast with ROS generation, Cd treatment produced a significant reduction of NO-dependent fluorescence observed in control leaves (Fig 3, panel B). The incubation of control leaves with aminoguanidine or L-NAME (not shown), two well known inhibitors of animal NOS, also produced a strong reduction of DAF-2DA fluorescence (Fig 3, panel C), 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 3, panel D), showing the specificity of DAF-2DA for NO.

The constitutive L-arginine-dependent NOS activity previously described in pea plants is dependent of Ca and calmoduline (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, panels E and F; Fig S4). This suggests that the NO decrease by Cd could be in part due to an inactivation of the NOS activity as consequence of the Cd-induced of Ca deficiency in leaves. A higher magnification of images from Ca-Cd-treated plants shows the production of NO associated to the apoplast in xylem vessels, and also in sclerenchyma cells (Fig 3, panel G; Fig S4, panel B). A punctate pattern of fluorescence was also observed in mesophyll cells (Fig 3, panel H; Fig S4, panel D). Images of chlorophyll autofluorescence are shown in Figure S5.

Jasmonic acid, salicylic acid and ethylene under Cd stress

Jasmonic acid is a component of the signalling processes under biotic and abiotic stress (Devoto and Turner, 2005). To determine whether JA was involved in the cell response to Cd toxicity, this molecule was analyzed by GC-MS in leaves from control and Cd-treated pea
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 is 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 content of conjugated (MeSA) and free SA were slightly reduced in Cd-treated plants (Fig 4). Analysis of ethylene by gas chromatography showed an increase of two times in leaves from pea plants grown with 50 μM CdCl₂ (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).

**Effect of Cd on pathogen-related proteins**

Studies on gene regulation under different stress conditions are important to get deeper insights into the regulation of defenses involved in each particular condition and the cross-talk processes occurring in different stress conditions. Some pathogenesis-related proteins have been described to be upregulated under abiotic stress (Tateishi et al., 2001; Streessman et al., 2004) which evidence the existence of common effectors with biotic stress. The analysis of the expression of pathogenesis-related proteins (PRPs) and the HSP 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 the HSP 71.2, while the expression of PAL transcripts did not change significantly by the treatment (Fig 6). The induction of PrP4A and HSP 71.2 was reverted by the supply of ASC, a H₂O₂ 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-diamidine-2-phenylindol (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 (DIC). FISH results showed an intense hybridization signal in leaves of Cd-treated plants (Fig 8, panel C), in contrast with control plants where a very faint signal was observed (Fig 8, panel B). No hybridization signal was observed in control experiments with the sense probe (Fig 8, panel A). PrP4A
expression was observed in mesophyll cells (Fig 8, panel C), especially in pallisade cells from Cd-treated pea plants (Fig 8, panel E); the hybridization signal was localized in the cytoplasms, being the nuclei (visualized by DAPI with blue fluorescence) free of signal (Fig 8, panel C magnification). However, the signal was absent in epidermis (Fig 8, panel C and E) and xylem tissue (Fig 8, panel G), although a weak/low FISH signal was observed in stomata (Fig 8, panel C and E), and in parenchymatic cells surrounding xylematic vessels (Fig 8, panel G).
DISCUSSION

**Cd produces disturbances in cations accumulation**

Cd is well known to produce disturbances in both 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 Cl₂Cd produced a decrease in the content 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, S was accumulated 3-fold in Cd treated plants respect to the control plants. The induction of sulfur metabolism by Cd has been previously described and involves a coordinate transcriptional regulation of genes for sulfate uptake and its assimilation, as well as GSH and phytochelatins (PCs) biosynthesis (Howarth et al., 2003; Nocito et al., 2006). Induction of PCs 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 in the cells by the same uptake systems used by cations such as Fe, Cu, Ca and Zn. Excess of 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. By the contray, the addition of Ca did not alter considerably the accumulation of the rest of elements, except the Mn and Mg in control plants. It has been observed that Cd not only competes with Ca for the transporters but also for intracellular Ca binding proteins (Rivet et al., 1997) and plasma membrane (Kinraide, 1998). Recently Tsyganov et al. (2007) have observed a relationship between Cd tolerance and homeostasis of Ca in both root and shoots in a Cd tolerant pea mutant (SGECdᵀ). Ca has been also reported to alleviate Cd toxicity in radish (Rivet et al., 1997), rice roots (Kim and Yang, 2002) and Arabidopsis seedlings (Suzuki, 2005) by reducing the Cd uptake. Ca also prevents aluminium-dependent growth inhibition in wheat (Kinraide and Parker, 1987).

**Differential expression of superoxide dismutases by Cd**

Cd-dependent reduction of SOD activity has been reported in wheat (Milone et al., 2003), peas (Sandalio et al., 2001) and beans (Cardinaels et al., 1984), although the opposite effect was observed in Alyssum plants (Schickler and Caspi, 1999) sunflower (Laspina et al.,
2005), coffee 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 plants, SOD increased at short time 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 previously observed (Sandalio et al., 2001; Romero-Puertas et al., 2007). The plastidic Fe-SOD, in its turn, was up-regulated although its activity was previously observed to be reduced by the metal (Sandalio et al., 2001). This suggests a possible post-translational regulation of Fe-SOD by oxidation (Fe-SODs are sensitive to H₂O₂) 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 Cu,Zn-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 transcriptional level, although the mechanism involved is unknown. Cu,Zn-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 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 catalase, previously observed (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 been also observed in Medicago sativa roots (Ortega-Villasante et al., 2005) and pea roots (Rodríguez-Serrano et al., 2006) by using DCF-DA and CLSM, and overproduction of O₂⁻ was also observed by using DHE in Lupinus luteus roots (Kopyra and Gwóźdz, 2003). The analysis of H₂O₂ in pea leaf sections by DCF-DA fluorescence showed an induction of this ROS production by Cd mainly in mesophyll cells probably associated to chloroplasts, mitochondria and peroxisomes, and in plasma membrane from epidermal cells. In a previous work, using CeCl₃ cytochemistry the Cd-dependent accumulation of H₂O₂ in
peroxisomes, mitochondria and plasma membrane was demonstrated, being NADPH oxidase the main source of ROS in plasma membrane (Romero-Puertas et al., 2004). An oxidative burst has also been associated with Cd toxicity in *Nicotiana tabacum* cells suspensions, being an NADPH oxidase 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 to 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, such as it 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$_2^-$ production. Thus, exogenous Ca supply reversed the Cd-treated phenotype getting similar results to those obtained in control plants grown in presence of Ca, reducing considerably the Cd-dependent O$_2^-$ production in mesophyll cells. However, in control plants Ca produced an slight increase of O$_2^-$ 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), electron transport chain in mitochondria (Romero-Puertas et al., 2004; Garnier et al, 2006), glycolate oxidase in peroxisomes (Romero-Puertas et al., 1999; 2004) or 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 DAF2-DA 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 previously observed in pea roots and leaves under the same experimental conditions (Rodríguez-Serrano et al., 2006; Barroso et al., 2006). Aluminium 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 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 a 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 which can react with $\text{O}_2^-$, and thus, regulate its accumulation in the tissue (Romero-Puertas and Delledonne, 2003). The reduction of NO under Cd treatment could favor $\text{O}_2^-$ accumulation promoting oxidative damages. This fact is supported by the reduction of $\text{O}_2^-$ 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 *Lupinus luteus* roots the supply of NO as SNP reduced the negative effects of Cd, NaCl, ethylene and paraquat and reduced the $\text{O}_2^-$ production induced by Cd and Pb (Kopyra and Gwózdz, 2003). A protective role of NO has been also observed in sunflower leaves under Cd toxicity (Laspinas 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 $\text{O}_2^-$/NO ratio could favour oxidative conditions but could also interfere in the signal transduction pathways of the defence 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 to 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 jasmonic acid and ethylene**

To get deeper insights into the mechanisms involved in the cell response to Cd toxicity, the analysis of jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) contents was carried out. Cd induced an increase of JA and ethylene which suggest that these molecules are involved in the cellular response to Cd toxicity. JA is an oxylipin which act as signalling compound in different defense situations such as response to pathogens and herbivore attack (Wastermack and Partnier, 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 damages. In a previous work we have demonstrated that growth of pea plants with Cd induced lipid peroxidation in leaves (Sandalia 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 GSH and phytochelatins synthesis under Cd treatment (Xiang and Olivier, 1998). The increase of JA could also contribute to metal toxicity through the activation of lipoxygenase activity, H$_2$O$_2$ production and lipid peroxidation (Wang and Wu, 2005; Maksymiec et al., 2007).

Ethylene plays a pleiotropic role in plant growth and development and is involved in a number of processes, including germination, senescence or fruit ripening, but also participates in a variety of defense responses (Guo and Ecker, 2004). The stimulation of ethylene biosynthesis by Cd has been reported in different plant species (Sanita di Toppi and Gabbrielli, 1999), although the molecular relathionship between ethylene biosynthesis and Cd stress has not been well established (Sanita di Toppi and Gabbrielli, 1999). In this work, we have 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 Figs 2 and 4. Ethylene and NO are antagonistic (Leshem 2000) which would explain the reduction of NO and the rise of ethylene emission observed in this work. This fat is supported by the effect of Ca on the accumulation of NO and ET. Leshem (2000) has proposed that NO could inhibit ACC synthase or ACC oxidase and so preventing ethylene formation. Recently, Lindermayr et al (2006) have demonstrated the reversible inhibition of methionine adenosyltransferases (MAT-1) by S-nitrosylation which can originate a reduction of S-adenosylmethionine pool and, therefore, a decrease of ethylene 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 ethylene 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 roots but the mechanisms involved are not well known (Metwally et al., 2003).
Pathogenesis-related proteins (PRPs) could protect against Cd toxicity

The analysis of PRPs expression in pea plants under Cd stress showed the up-regulation of chitinases, PrP4A and the HSP 71.2, while PAL did not change. Chitinase catalyse the hydrolytic cleavage of the β-1,4-glycoside bond of N-acetylglucosamine 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 (Békésiova et al., 2007), and also by osmotic stress (Tateiishi 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 (Danna et al., 2006), and chitinase isoforms are differentially modified by different metals (Békésiova et al., 2007). 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 which binds chitin, can inhibit 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 of ET in chinese cabbage plants (Chung et al., 2005) and was sensitive to ET and JA in Arabidopsis (Tomma et al., 2001), and in both cases 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 contrast with 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 PRPs in biotic and abiotic stress conditions.

The third gene induced by Cd corresponds to the citosolic heat shock protein, HSP 71.2 which is known to be up-regulated under heat stress and its function is to act as
molecular chaperone facilitating protein transport into organelles (DeRocher and Vierling, 1995) or preventing protein aggregation (Ma et al., 2006). The induction of HSPs in pea plants under Cd treatment is apparently regulated by H₂O₂ overproduction since ASC reversed the up-regulation induced by Cd, in addition to that, transcription factors involved in the HSPs regulation can act as H₂O₂ sensors (Miller and Mitller, 2006). In Arabidopsis plants, the regulation of HSP71.2 by JA has been also 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 PRPs, PAL expresion was not affected by the metal. The regulation of PAL is dependent on NO (Wang and Wu, 2005; de Pinto et al., 2002) 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 upregulation of PRPs, and the NO reduction are common features of senescence (Obregón et al., 2001; Corpas et al., 2004; Rodríguez-Serrano et al., 2006), which suggest that Cd accelerates senescence processes in plants. This fat 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 PRPs genes under biotic and abiotic conditions suggest 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 a ROS overproduction, NO reduction and PRP upregulation. However, unlike ozone or pathogen attack, Cd did not produce any visible symptom 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 previously reported, a cross-talk between ROS, NO and Ca in the regulation of cellular response to long-term Cd exposure is proposed (Fig. 9). Cd produces nutrient disturbances, being Ca 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 plants cells some of the proteins modulated by CaM include NAD kinases, glutamate decarboxylase, HSPs (Lu and Harrington, 1994) and catalase (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 what 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, direct or indirectly, promote the accumulation of superoxide radicals (O$_2^-$) and induce oxidative stress. In its turn, ROS accumulation can cause membrane damages which are involved in the JA and ET production. Finally, the overproduction of JA, ET and ROS could activate the cell response with the induction of pathogenesis-related proteins (PRPs) in order to protect proteins from damages associated to Cd toxicity.
MATERIALS AND METHODS

Plant material and growth conditions
Pea (*Pisum sativum* L., cv Lincoln) plants were obtained from Royal Sluis (Enkhuizen, Holland). Plants were grown in the greenhouse in aerated full-nutrient media under optimum conditions during 14 days (Sandalio et al., 2001). Then, the media either remained unsupplemented (control plants) or were supplemented with 50 μM CdCl₂ (Cd-treated plants), and plants were grown for 14 days. To determine the effect of Ca, control and Cd-treated plants were supplemented with 10 mM Ca(NO₃)₂ one day before the addition of Cd and were grown for 14 days. The effect of ASC (a H₂O₂ scavenger) was studied by infiltrating the leaves with 10 mM ASC.

ROS and NO detection by confocal laser scanning fluorescence microscopy (CLSM)
For NO detection, pea leaf segments of approximately 10 mm² were incubated for 1 h at 25°C, in darkness, with 10 μM 4,5-diaminoflorescein diacetate (DAF-2 DA, Calbiochem; excitation 495 and emission 515) 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 dihydroethidium (DHE, Fluka; excitation at 488 and emission at 520) for 30 min, as indicated by Sandalio et al (2008). H₂O₂ was detected by incubation with 25 μM 2′,7′-dichlorofluorescin diacetate (DCF-DA, Calbiochem; excitation at 485, emission at 530) (Rodríguez-Serrano et al., 2006). As negative controls, leaf sections were incubated with two ROS scavengers, 1 mM 2,2,6,6-tetramethylpiperidinoxy (TMP, O₂⁻ scavenger), and 1 mM ascorbate (H₂O₂ 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 by vibratome and mounted for examination with a confocal laser scanning microscope (Leica TCS SL; Leica Microsystems, Wetzlar, Germany). Autofluorescence due to the chlorophyll was detected by excitation at 633 nm and emission at 680nm.

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 μg of total RNA from leaves was used as template for the
reverse transcriptase (RT) reaction. It was added to a mixture containing 0.5 μg oligo dT23 anchored (Sigma, St. Louis, MO, USA), 1X RT-buffer, 20 U Rnasin ribonuclease inhibitor, 15 U AMV reverse transcriptase (FYNZYMES). The reaction was carried out at 42°C for 40 minutes, 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 Table 1. cDNAs were amplified by polymerase chain reaction (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 MgCl₂, 1X PCR buffer, 1.25 U of Hot Mastertaq polymerase (Eppendorf, Hamburg, Germany) and 0.4 μmol of each primers (see Table 2S) 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, 20-40 s at annealing temperature and 30 s at 65°C with a final extension of 10 min at 65°C. Amplified PCR products were detected after electrophoresis in 0.8 % agarose gels (Serva) stained with ethidium bromide. Quantification of the bands was performed using a Chemidoc system (Bio-Rad Laboratories, Hercules, CA) coupled with a high sensitive 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 *Pisum sativum* was obtained by the protocol described before, and used for PCR amplification with the specific primers for the PrP4A included in the Table 2. The amplified fragment 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 a DIG RNA labelling kit according to the manufacturer protocols (Roche).

Fluorescence *in situ* hybridization (FISH)

Pea leaves pieces were fixed in 4% paraformaldehyde in PBS (pH 7.0) for 16 hours, at 4°C, cryoprotected with 2.3 M sucrose, embedded in tissue freezing medium, frozen on dry ice and
sectioned in a cryostat (Leica CM 1800, Vienna, Austria) at -30°C. Cryostat sections (40-60 μm thickness) were thawed and pretreated to facilitate penetration of the labelling reagents: sections were dehydrated in a series of 30, 50, 70 and 100% methanol/water, then rehydrated in a series of 70, 50, 30% methanol/water, and finally in PBS, for 5 min 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 previously described (Massoneau et al. 2005). Sections were incubated with hybridization solution at 50°C overnight. The hybridization solution consisted of digoxigenin-labelled 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, 2xSSC, at room temperature, and in 0.1xSCC at 50°C (1x SSC: 150 mM sodium chloride, 15 mM sodium citrate, pH 7.2). After washing in PBS; sections were incubated in 5% BSA for 5 min. Then, the hybridization signal was detected by incubation with mouse anti-digoxigenin antibodies (Sigma), diluted 1/5000 in 1% BSA in PBS for 90 min at room temperature, followed by a fluorescence anti-mouse-ALEXA 488 (Molecular Probes Inc., Eugene, OR, USA; excitation at 495nm and emission at 519nm) antibody, applied 1/25 in PBS for 45 min at room temperature. After washing in PBS, the sections were counterstained with DAPI (excitation at 345nm and emission at 455nm), mounted in Mowiol and observed. Confocal optical section stacks were collected using a confocal laser scanning microscope Leica TCS-SP. Controls were performed substituting the antisense RNA probe for the sense one at the same concentration in the hybridization solution.

**Jasmonic and salicylic acid 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 homogenised in 10 ml acetone. Extracts were dried by speed vacuum at 40°C and resuspended in 5 % ethyl ether. The ethyl ether fraction was purified by gas chromatography 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 SiO2 column eluted with 50 ml of hexane:ether (90:10). This fraction contained free SA and JA. To obtain the SA and JA derived fraction (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 gas chromatography-mass spectrometry (GC-MS) by select ion monitoring (SIM) at m/z 120 and 152 for the MeSA and at m/z 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 x 0.25 x 0.25 m film DB-MS column. The temperature was as follows: from 40ºC (1 min) to 20ºC/min until 150ºC (3 min), and from 5ºC/min to 230ºC. Helium was used as carrier gas (1 ml/min) and split less injection (1μl).

**Ethylene determination**

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

**Macronutrients and Micronutrients determination**

Leaves were dried and mineralized with H₂O₂/nitric acid and microwave and the content of the elements were assayed by inductively coupled plasma emission spectrometry (ICP-EOS) analysis.

**FIGURE LEGENDS**

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

**Figure 2.** Imaging of ROS production in pea leaves by confocal laser scanning microscopy. Images are projections of several optical sections collected by confocal microscopy showing the autofluorescence (blue colour, excitation at 633 nm and emission at 680nm) and fluorescence due to DHE and DCF-DA. A-G, O₂⁻-dependent DHE fluorescence (excitation at 450-490 and emission at 520, red colour) in leaf cross-sections from control (A) and Cd-treated pea leaves (B,C,D). Panel C and D correspond to magnifications of vascular cylinder and mesophyl cells, respectively. As negative control, leaves were incubated with 1 mM
TMP, an O$_2^-$ scavenger (E). Panels F and G represent the effect of exogenous Ca supply (10 mM Ca(NO$_3$)$_2$) on O$_2^-$ in control and Cd-treated plants, respectively. H-L, H$_2$O$_2$-dependent DCFDA fluorescence (excitation at 485 and emission at 530, green colour) in leaf cross sections from control (H) and Cd-treated pea plants (I-L). As negative control, leaves were incubated with 1 mM ascorbate (ASC) which acts as H$_2$O$_2$ scavenger (J). Panels K and L correspond to magnifications of vascular cylinder and mesophyl cells, respectively. Figure representative of at least six independent experiments. scl, sclerenchyma; st, stomata; x, xylem vessels; mc, mesophyll cells. Bar = 50 µm.

**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 colour; excitation at 633 nm and emission at 680 nm) and NO-dependent DAF-2 DA fluorescence (excitation at 495 nm and emission at 515 nm, green colour) from control (A, B, E) and Cd-treated pea plants (B, D, F-H). As 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). Panels 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. Panels G and H corresponds to magnifications of vascular cylinder and mesophyl cells, respectively. Arrows represent small organelles showing NO-dependent fluorescence. Figure representative of at least six independent experiments. scl, sclerenchyma; st, stomata; x, xylem vessels; mc, mesophyll cells. Bar = 50 µm.

**Figure 4.** Methyl-jasmonic acid (MeJA) and salicylic acid (SA) production in leaves from control and Cd-treated pea plants. The content of JA and SA was determined by gas chromatography-mass spectrometry as described in Materials and Methods. MeJA, methyl-jasmonate. MeSA, methyl-salicylate. Each column represents the mean±SEM of three independent experiments with six replicates each one. Differences were significant at p<0.01 (**), according to the Duncan´s Multiple Range Test.

**Figure 5.** Effect of Cd and Ca on ethylene (ET) emission in pea leaves. The content of ET was assayed by gas chromatography, as described in Materials and Methods. Each column represents the mean±SEM of two independent experiments with six replicates each one. Values indicated by the same letter are not significantly different (p< 0.05) according to the Duncan´s Multiple Range Test.
Figure 6. Cadmium effect on the expression of the pathogenesis related proteins PrP4A, PAL, chitinase and the HSP 71.2 in pea leaves. Analysis of mRNA expression was carried out as described in Fig 1. Columns represent the mean of mRNA expression of SODs ±SEM of three independent experiments with three replicates each one in arbitrary units (a.u.).

Figure 7. Effect of ascorbate on the expression of PrP4A, and HSP 71.2 in control and Cd-treated pea leaves. Analysis of mRNA expression was carried out as described in Fig 1. Columns represent the mean of mRNA expression of SODs ±SEM of three independent experiments with three replicates each one in arbitrary units (a.u.).

Figure 8. Localization of PrP4A expression by fluorescence in situ hybridization (FISH) on pea leaf sections under cadmium 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 anti-sense probe (green colour). C, cross-section of Cd-treated pea leaf hybridized with the anti-sense probe showing intense and green hybridization signal in the mesophyll cells. Small box shows a higher magnification (bar represents 10 μm) of a single mesophyll cell showing fluorescence of the cytoplasm. E and G show details of mesophyll and xylem vessels in Cd-treated plants hybridized with the antisense probe. D and F, the same as E and G, showing Nomarsky images. Figure is representative of three independent experiments. e, epidermis; mc, mesophyll cells; pm, palisade mesophyll; sm, spongy mesophyll; st, stomata; x, xylem. Arrows represent stomata (st), epidermis (e), and xylem vessels (x). Bar = 50 μm.

Figure 9. Model proposed for cross-talk between calcium, ROS and NO and its role in the regulation of the plant response to Cd toxicity. +, upregulation; - , downregulation; ↓, reduction; ↑, increase. PRs, pathogenesis related proteins; MAT-1, methionine adenosyltransferase 1.
SUPPLEMENTARY MATERIAL

Table S1. Effect of Cd and Ca treatment on nutrient contents of leaves of pea plants.

Table S2. Oligonucleotides used in this work for the semi-quantitative PCR analysis.

Figure S1. Imaging of chlorophyl autofluorescence in pea leaves by confocal laser scanning microscopy corresponding to the figure 2.

Figure S2. Imaging of chlorophyl autofluorescence and ROS production in pea leaves treated with Cd by confocal laser scanning microscopy.

Figure S3. Localization of H₂O₂ in control pea leaves by cytochemistry with CeCl₃ and electron microscopy.

Figure S4. Imaging of chlorophyl autofluorescence and NO production in pea leaves treated with Cd+Ca by confocal laser scanning microscopy.

Figure S5. Imaging of chlorophyl autofluorescence in pea leaves corresponding to the figure 3 by confocal laser scanning microscopy.
ACKNOWLEDGEMENTS

M. Rodríguez-Serrano acknowledges fellowships from Ministry of Education and Science, Spain. Special thanks are given to Miss N. de la Casa for her skilful technical assistance. The laser confocal microscopy analyses were carried out at the Technical Services of the University of Jaén. The ICP analysis was carried out at the Ionomic Service of CEBAS (CSIC), Murcia, and JA and SA analysis were carried out at the Technical Services of the Instituto de la Grasa (CSIC), Sevilla.

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Figure 1. Cadmium effect on superoxide dismutase expression in pea leaves. Analysis of mRNA expression of SODs was carried out by semiquantitative RT-PCR. Representative agarose electrophoresis gels of 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 (as detailed in Materials and Methods). Columns represent the mean of mRNA expression of SODs ±SEM of three independent experiments with three replicates each one in arbitrary units (a.u.)
Figure 2. Imaging of ROS production in pea leaves by confocal laser scanning microscopy. Images are projections of several optical sections collected by confocal microscopy showing the autofluorescence (blue colour, excitation at 633 nm and emission at 680 nm) and fluorescence due to DHE and DCF-DA. A-G, \( \text{O}_2^- \) dependent DHE fluorescence (excitation at 450-490 and emission at 520, red colour) in leaf cross-sections from control (A) and Cd-treated pea leaves (B, C, D). Panel C and D correspond to magnifications of vascular cylinder and mesophyl cells, respectively. As negative control, leaves were incubated with 1 mM TMP, an \( \text{O}_2^- \) scavenger (E). Panels F and G represent the effect of exogenous Ca supply (10 mM Ca(NO\(_3\))\(_2\)) on \( \text{O}_2^- \) in control and Cd-treated plants, respectively. H-L, \( \text{H}_2\text{O}_2 \) dependent DCF-DA fluorescence (excitation at 485 and emission at 530, green colour) in leaf cross-sections from control (H) and Cd-treated pea plants (I-L). As negative control, leaves were incubated with 1 mM ascorbate (ASC) which acts as \( \text{H}_2\text{O}_2 \) scavenger (J). Panels K and L correspond to magnifications of vascular cylinder and mesophyll cells, respectively. Figure representative of at least six independent experiments. scl, sclerenchyma; st, stomata; x, xylem vessels; mc, mesophyll cells. Bar = 50 µm.
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 colour; excitation at 633 nm and emission at 680 nm) and NO-dependent DAF-2 DA fluorescence (excitation at 495 nm and emission at 515 nm, green colour) from control (A, B, E) and Cd-treated pea plants (B, D, F-H). As 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). Panels 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. Panels G and H corresponds to magnifications of vascular cylinder and mesophyll cells, respectively. Arrows represent small organelles showing NO-dependent fluorescence. Figure representative of at least six independent experiments. scl, sclerenchyma; st, stomata; x, xylem vessels; mc, mesophyll cells. Bar = 50 µm
Figure 4. Methyl-jasmonic acid (MeJA) and salicylic acid (SA) production in leaves from control and Cd-treated pea plants. The content of JA and SA was determined by gas chromatography-mass spectrometry as described in Materials and Methods. MeJA, methyl-jasmonate. MeSA, methyl-salicylate. Each column represents the mean±SEM of three independent experiments with six replicates each one. Differences were significant at p<0.01 (**), according to the Duncan’s Multiple Range Test.
Figure 5. Effect of Cd and Ca on ethylene (ET) emission in pea leaves. The content of ET was assayed by gas chromatography, as described in Materials and Methods. Each column represents the mean±SEM of two independent experiments with six replicates each one. Values indicated by the same letter are not significantly different (p<0.05) according to the Duncan’s Multiple Range Test.
Figure 6. Cadmium effect on the expression of the pathogenesis related proteins PrP4A, PAL, chitinase and the HSP 71.2 in pea leaves. Analysis of mRNA expression was carried out as described in Fig 1. Columns represent the mean of mRNA expression of SODs ±SEM of three independent experiments with three replicates each one in arbitrary units (a.u.)
Figure 7. Effect of ascorbate on the expression of PrP4A, and HSP 71.2 in control and Cd-treated pea leaves. Analysis of mRNA expression was carried out as described in Fig 1. Columns represent the mean of mRNA expression of SODs ±SEM of three independent experiments with three replicates each one in arbitrary units (a.u.)
**Figure 8.** Localization of PrP4A expression by fluorescence *in situ* hybridization (FISH) on pea leaf sections under cadmium 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 anti-sense probe (green colour). **C**, cross-section of Cd-treated pea leaf hybridized with the anti-sense probe showing intense and green hybridization signal in the mesophyll cells. Small box shows a higher magnification (bar represents 10 μm) of a single mesophyll cell showing fluorescence of the cytoplasm. **E** and **G** show details of mesophyll and xylem vessels in Cd-treated plants hybridized with the antisense probe. **D** and **F**, the same as **E** and **G**, showing Nomarsky images. Figure is representative of three independent experiments. e, epidermis; mc, mesophyll cells; pm, palisade mesophyll; sm, spongy mesophyll; st, stomata; x, xylem. Arrows represent stomata (st), epidermis (e), and xylem vessels (x). Bar = 50 μm
Figure 9. Model proposed for cross-talk between calcium, ROS and NO and its role in the regulation of the plant response to Cd toxicity. +, upregulation; -, downregulation; , reduction; , increase. PRs, pathogenesis related proteins; MAT-1, methionine adenosyltransferase 1.