Universidade de Aveiro Departamento de Biologia
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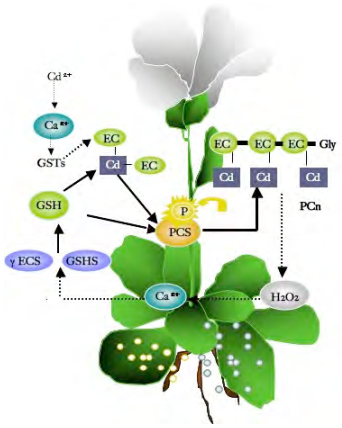
Phytochelatin signal tranduction and cadmium chelation in plants



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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor, realizada sob a orientação científica do Doutora Etelvina Maria Almeida Figueira, Professor a auxiliar do Departamento de Biologia da Universidade de Aveiro e do Professor Edgar Figueiredo da Cruz e Silva, Professor assistente do Departamento de Biologia da Universidade de Aveiro

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Dedico esta Tese a todas as pessoas que me ajudaram ao longo destes anos a concluir este trabalho.

Nunca te ofereceram um desejo sem te darem ao mesmo tempo o poder de o tornares realidade. Contudo é possível que tenhas de lutar por ele.
Ilusões Richard Bach

o júri

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palavras-chave

Sinalização de stresse, plantas, cádmio, fitoquelatinas, transdução de sinais, fosfatases proteicas, cálcio, proteínas sinalizadoras de cálcio, ROS, stresse oxidativo

resumo

As plantas utilizam diversas estratégias de sinalização para reconhecer e responder aos stresses ambientais. A maioria das vias de transdução de sinais partilham um sinal genérico, normalmente a modulação dos níveis intracelulares de Ca^{2+}. Esta por sua vez pode iniciar uma cascata de fosforilação proteica que finalmente afecta as proteínas directamente envolvidas na protecção celular ou culmina em factores de transcrição que vão determinar a resposta fisiológica ao stresse. A percepção destes sinais e a compreensão de como estes podem activar as respostas adaptativas são factores-chave para a tolerância das plantas a stresses abióticos.
Um dos principais stresses abóticos que restrigem o crescimento das plantas é a presença de metais pesados. A produção de fitoquelatinas e a subsequente quelação dos metais é o mecanismo mais conhecido de tolerância ao stresse metálico em plantas. Fitoquelatinas (PCs) são péptidos com grupos tiol que são sintetizados através da transpeptidação da glutationa (GSH), pela acção da enzima fitoquelatina sintase (PCS). No entanto, até ao momento, as vias de sinalização que levam à síntese de fitoquelatinas e à percepção do stresse metálico são pouco compreendidas. Dentro deste contexto, o presente trabalho foi elaborado com o intuito de elucidar a via de sinalização através da qual o cádmio é detectado pelas células vegetais e induz a síntese de PCs.

Quase todos, os estudos de stresses abióticos em plantas apontam para o facto de a sua sinalização se basear nos mesmos tipos de sinais moleculares, nomeadamente a sinalização por cálcio, a fosforilação proteica e a indução de espécies reactivas de oxigénio (ROS). Trabalhos recentes sugerem que a sinalização de PCs poderá envolver todos estes parâmetros. Assim, uma primeira abordagem foi efectuada para compreender a síntese de PCs na espécie *Arabidopsis thaliana*, através da monitorizaçção da actividade de enzimas relacionadas, a γ-EC sintetase, GSH sintetase e a PC sintase (PCS), assim como o tempo necessário para o elongamento das PCs e a sua acumulação. Seguidamente, ao longo deste processo foi analisada a expressão de sinais específicos, associados com sinais de cálcio, fosforilação proteica e sinalização por ROS. A importância destes factores na síntese de PCs foi também avaliada através do uso de moduladores farmacológicos de cálcio e fosfatases proteicas e também pela indução de stresse oxidativo.
Os resultados demonstraram novos dados sobre o papel do cálcio e da fosforilação proteica na produção de PCs e na síntese de GSH, revelando que a actvidade da PCS é regulada por fosforilação e que a sinalização de cálcio pode mediar a síntese de GSH. O envolvimento da sinalização de ROS na síntese de GSH, atráves de crosstalk com a sinalização de cálcio também foi proposta. Assim, os resultados aqui apresentados descrevem uma possível via de sinalização de cádmio nas plantas e da indução de fitoquelatinas. Este trabalho poderá ser portanto muito útil na implementação de novas metodologias de agricultura sustentável e práticas de fitorremediação em solos contaminados com metais pesados.

keywords

Stress signaling, plants, cadmium stress, phytochelatins, signal transduction, protein phosphatases, calcium, calcium-binding proteins, ROS, oxidative stress

abstract

Plants use diverse signalling strategies for recognizing and responding to environmental stresses. Most stress signal transduction pathways share a generic signal perception, usually the modulation of intracellular Ca^{2+} levels, which initiates a protein phosphorylation cascade that finally targets proteins directly involved in cellular protection or transcription factors controlling specific sets of stress-regulated genes. Perception of stress clues and relay of the signals to switch on adaptative responses are the key steps leading to stress tolerance.

One of the major growth restraining stresses experienced by plants is heavy metal-induced stress. Phyhtochelatin (PC) production and metal chelation is the most well-known mechanism of metal tolerance in plants. PCs are non protein thiols that are synthesised by the transpeptidation of glutathione, through the catalytic activity of the enzyme phytochelatin synthase. Until now, the pathways by which PC synthesis is triggered in plants remains to be elucidated. Under this context this work was elaborated with the aim to clarify the pathway by which Cd is detected by the plant cells and induces PC synthesis.

Most of all, if not all signal transduction studies in plants exposed to abiotic stresses point out the fact that stress signaling in plants relies on the same signals, but with different specificities, involving mostly calcium signaling, protein phosphorylation and ROS induction. Recent works suggest that metal stress detection and PC synthesis involves all of these components. Hence, a first approach was made in order to understand the process of PC synthesis and Cd chelation in *Arabidopsis thaliana*, monitoring the activity of the enzymes related to GSH and PC synthesis, such as GSH synthethase, γ-EC synthethase and PC synthase (PCS) as well as the timing for PC elongation and accumulation. Then, throughout the time-course development of PC synthesis and Cd chelation we monitored the expression of specific signal transducers associated with calcium signatures, protein phosphorylation and ROS signaling. We also assed their importance in PC synthesis, in the presence of specific pharmacological modulators of these signaling components.

Results provided important insights on the role of calcium and protein phosphorylation in PC production and GSH synthesis, showing that PC synthase activity is modulated by protein phosphorilation and that calcium is required for GSH synthesis. The involvement of ROS signaling in GSH production, through calcium signaling was also suggested. Taken altogether, results presented here describe a possible pathway for Cd signaling and PC induction in plants and can help to introduce novel practices of sustainable crop management and phytoremediation methodologies in metal contaminated soils.

Index

ABBREVIATIONS

ANOVA	analysis of variance
BSA	bovine serum albumin
Caff	caffeine
CAN	cantharidine
CBL	calcineurin B-like
Cd	cadmium
CDNB	1-chloro-2,4-dinitrobenzene
CIPK	calcium induced protein kinases
Cys	cysteine
DTE	dithioerithritol
DTT	dihiothreitol
γEC	glutamilcystein
ECL	enhanced chemiluminescence
γECS	γEC synthethase
EDTA	ethylene diamine tetra acetic acid
EGTA	ethylene glycol –bis (2-aminoethylether)-N,N,N′,N′-tetra acetic acid
(x) *g*	gravitational acceleration (when referring to centrifugation)
Glu	glutamate
Gly	glycine
GSH	glutathione
GSHS	glutathione synthethase

GSSG	oxided glutathione
HEPES	N-2-hydroxythylpiperazine – N′-2-ethanesulphonate
Hr	hours
HRP	horseradish peroxidase
RP-HPLC	reverse phase high pressure liquid chromatography
La	lanthanum
MAPK	mitogen-activivated protein kinase
mBBr	monobromobimane
min	minutes
OA	okadaic acid
OD	optical density
PAGE	polycrylamide gel electrophoresis
PBS	phosphate-buffer saline
PC	phytochelatin
PCS	phytohelatin synthase
PMSF	phenyl methylsulfoxide
PP	protein phosphatase
PP1	protein phosphatase 1
PP1c	catalytic subunit of PP1
PP2A	protein phosphatase 2A
PP2B	protein phosphatase 2B
PTP	protein tyrosine phosphatase
ROS	reactive oxygen species

RPM	rotations per minute
Rut Red	ruthenium red
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SE	standard error of the means
Ser/Thr-PP	serine threonine special protein phosphatase
TBS-T	tris-buffered saline tween solution
TFA	trifluoracetic acid
Tris	Tris(hydroxymethyl) aminomethane

Chapter 1

Introduction

1.1 Stress signal transduction in plants

Unlike animals, plants are sessile organisms that cannot move away from adverse environmental conditions; therefore, they heavily rely on high sensitivity detection and adaptation mechanisms to environmental perturbations. In nature, plants are exposed to various environmental stimulli, which affect their physiology, morphology and development; these can be of biotic nature, such as fungal or pathogenic attack or abiotic nature such as climatic alterations or heavy metal and pesticide contamination (Clark et al., 2001). A rapid and precise perception of many of these alterations is of crucial importance for plants to adapt to changing natural environments, as it allows them to rapidly perceive environmental alterations that trigger their development or avoid the deleterious effects imposed by a specific type of stress.

Plants might perceive the environmental alterations in different ways, such as by plasma membrane located receptors, intracellular or cytoskeleton-associated proteins. Subsequently, the imposed signal is recognized and a complex cascade of events involving several interacting components recognize the imposed signal is triggered, culminating into altered gene expression programs, and metabolic alterations (Kaur and Gupta, 2005). This cascade of events is called signal transduction, and normally acts through second messengers that can trigger the molecular events leading to the physiological response. Various signal pathways can operate independently

from each other or they may positively or negatively modulate other pathways. Different signalling pathways may also share components and second messengers to achieve their objectives. As a result, many signals could interact in a cooperative fashion with each other (Knight, 2000, Kaur and Gupta, 2005).

Every year, environmental stresses are the cause of considerable losses in crop productivity. Abiotic stresses, such as heat, cold, drought, salinity, and heavy metals are important factors that can severely affect plant growth and metabolism. Therefore, during the last decade, the importance of the early detection and signalling mechanisms in plant environmental responses has brought a wide interest in this area of research. Several signal transducers have been now recognized, some of which share high degrees of similarity with animals, and few have been identified has exclusive of the plant kingdom (Kudla et al., 2001; Luan, 2004). Differences in stress tolerance between genotypes may arise from differences in signal perception and transduction mechanisms (Hare et al., 1997, Kaur and Gupta, 2005). Thus, whilst most of the biochemical factors necessary for stress tolerance acquisition are present in all species, subtle differences in signal transducers can hold the key to improve plant tolerance to distinct abiotic and biotic stresses.

1.2. Most stress signalling mechanisms share the same components

Diverse studies on molecular responses of plants to various types of stresses indicate that different types of constraints provide different information to the cells. The multiplicity of this information makes the response of plants complex and hence also the stress signalling pathways (Murata et al., 1997; Knight, 2000; Xiong et al., 2002). It is now widely accepted that plants usually use similar transduction mechanisms to cope with different stresses **(**Mithöfer et al, 2004). Such examples were found in

cases of mechanical wounding and pathogen attack (Schaller and Weiler 2002), as well as salt, cold and drought stress (Xiong et al. 2002).

Most signal transduction pathways share a generic signal perception, usually the modulation of intracellular Ca^{2+} levels, which initiates a protein phosphorylation cascade that finally targets proteins directly involved in cellular protection or transcription factors controlling specific sets of stress-regulated genes (Kaur and Gupta, 2005). It is becoming increasingly clear that in plants, these basic elements show a great similarity with animals and can include Ca^{2+}, inositol phospholipids, G-proteins, cyclic nucleotides, protein kinases and protein phosphatases, among others (Fig. 1.2) (Clark et al., 2001).

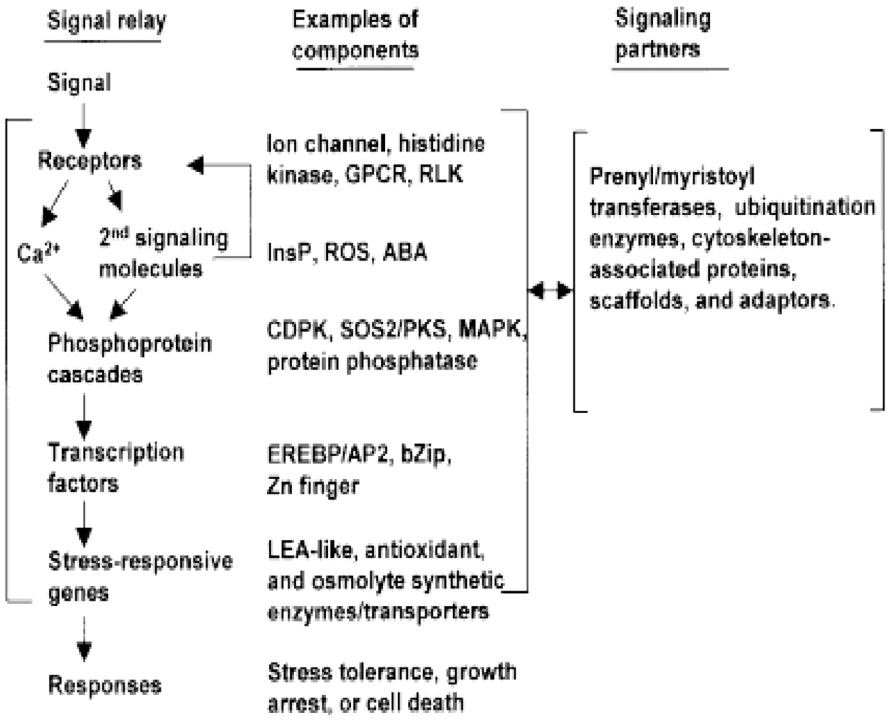


Fig. 1.2: A generic pathway for abiotic stress transduction in plants (adapted from Clark et al., 2001).

Recent advances have also brought to light novel specific signal transducers that are exclusive to the plant kingdom. Most of them function as Ca^{2+} sensors, namely the recently discovered family of novel calcium sensors CBLs from *Arabidopsis* and their target proteins, the calcium-induced protein kinases, (CIPKs), involved in various abiotic stresses, such as salt, drought,

cold and heat (Kudla et al., 2001; Luan, 2004). Reactive oxygen species ROS have also emerged as important signalling molecules that control various processes including pathogen defense, programmed cell death, and stomatal behavior (Mithöfer et al., 2004; Maksymiec, 2007)(Fig 1.2). Overall, these components can act together in a multiplicity of ways, according to the final metabolic adjustment. Since stress tolerance mechanisms can involve several physiological responses, a complex crosstalk between different pathways, all acting towards the same tolerance response can occur. One example of this complexity is illustrated in Fig 1.2, adapted from Kaur and Gupta (2005)

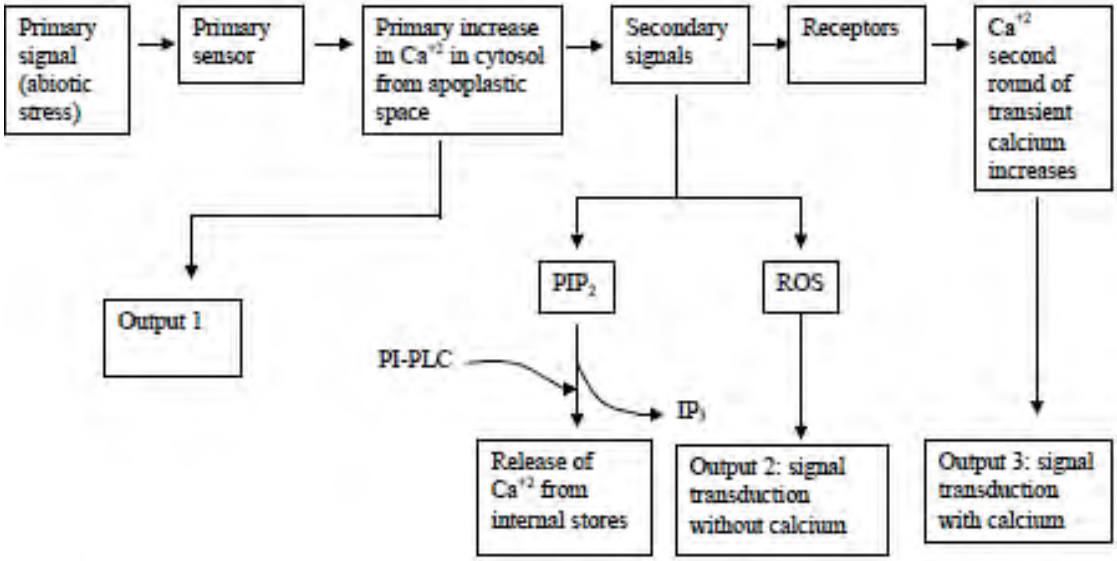


Fig. 1.3. Role of secondary signal molecules in signal transduction pathways induced by abiotic stresses. Abiotic stresses usually result in transient increases in cytosolic Ca^{+2} either through influx from the apoplastic space or release from the internal stores. The primary increase in cytosolic calcium leads to output 1 and generates a secondary signal (hormone and second messengers) which initiates another cascade of signalling events and stimulates a second round of transient calcium increases, resulting in output 3. Secondary signalling molecules such as ROS can also regulate signal transduction without calcium (output 2). Internal Ca^{+2} release is regulated by ligand-sensitive Ca^{+2} channels. Inositol polyphosphates, cyclic ADP ribose, nicotinic acid adenine dinucleotide phosphate could act as second messengers and were described to induce Ca^{+2} release in plant cells. Adapted from Kaur and Gupta (2005).

1.3 Heavy metals stress in plants

Heavy metals have become one of the main abiotic stress agents for living organisms because of their increasing use in the developing fields of

industry and agrotechnics their high bioaccumulation rates and high toxicity levels. Over the last several decades there has been an increasing awareness of how heavy metals act as environmental pollutants (Baker and Walker, 1989) and their effects on plants (Sanitá di Toppi and Gabrielly, 1999; Cobbet, 2000; Rauser, 2000; Cobbet and Goldsbrought, 2002; Hall, 2002; Clemmens, 2006). Cadmium is one of the most important environmental pollutants, particularly in areas where there is high anthropogenic pressure. Its presence in the atmosphere, soil, and water (even in trace concentrations) can cause serious toxicity to all organisms, and its bioaccumulation in the food chain can be highly dangerous (Wagner, 1994; Sanitá di Toppi and Gabbrielli, 1999). In plants, Cd is known to inhibit seed germination and root growth, induce chromosomal aberrations and micronucleus formation (Fojtová and Kovarík, 2000). Cd can also cause membrane depolarization and cytoplasmic acidification, leading to the disruption of cellular homeostasis (Pinto et al., 2003). The toxicity symptoms seen in the presence of Cd mostly result from metal binding to the sulphydryl groups in proteins, forming a metal–protein complex, which can affect the enzymatic systems, growth, photosynthesis, respiration, reproduction, nutrient assimilation, and molecular synthesis (Morin, 2003). In addition, excess Cd may stimulate the formation of free radicals and reactive oxygen species (ROS), which in turn may cause lipid peroxidation, enzyme inactivation, DNA damage and loss of membrane selectivity (Hall, 2002; Boominathan and Doran, 2003).

1.3.2. The importance of PCs

Plants can respond to heavy metal toxicity in a variety of different ways. Such responses include immobilization, exclusion, chelation and compartmentalization of the metal ions, as well as the expression of more general stress response mechanisms such as ethylene and stress proteins (Cobbet, 2000; Cobbet and Goldsbrought, 2002; Hall, 2002; Clemmens, 2006). One recurrent general mechanism for heavy metal detoxification in plants and other organisms is the chelation of the metal by a specific ligand (Rauser, 2000). A number of metal-binding ligands have now been recognized in

plants, but by far, the most important are phytochelatins (Zenk, 1996; Rauser, 1999; Cobbet and Goldsbrought, 2002; Hall, 2002; Clemmens, 2006).

Phytochelatins (PCs) are a family of Cys-rich, small non-protein thiol peptides with the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ and are synthesised in a wide variety of plant species, algae, yeast and nematodes (Zenk, 1996; Rauser, 1999, 2000; Cobbett and Goldsbrough, 2002). The phytochelatin-mediated tolerance an interesting phenomena of stress defence known in plants. These peptides are exclusively formed in the presence of heavy metals, by the transpeptidation of the tripeptide glutathione (GSH), through the action of a constitutive enzyme, known as PC synthase (PCS, EC 2.3.2.15) (Zenk, 1996; Rauser, 1999; Cobbett and Goldsbrough, 2002). When in the presence of toxic metal concentrations, particularly cadmium, PCs form complexes with the metal ions, hence preventing them from interfering with the cellular metabolism (Grill et al., 1985; Vögelli–Lange and Wagner, 1990; Ortiz et al., 1995, Zenk, 1996). These complexes are then stored in the vacuole, where they are rendered harmless to the cell (Grill et al., 1985; Vögelli–Lange and Wagner, 1990; Ortiz et al., 1995, Zenk, 1996).

The knowledge of how plants perceive the metal presence and switch on or off the PC synthesis pathway can be of crucial importance to better understand the metal tolerance mechanisms in plants, to improve crop tolerance in metal polluted soils and also to enhance metal phytoremediation techniques. Nonetheless, the signal transductions pathways that underlie Cd signalling and the subsequent PC production pathway are still very far from being understood.

1.4 Metal signal transduction in plants – possible pathways

The analysis of the mechanisms behind metal tolerance has become an important focus of research, especially in the case of cadmium. Several factors suggest that Cd tolerance can be achieved through the same signal transduction pathways that plants use for other abiotic stresses (Mithöfer et al., 2005). Previous studies showed that a pre-exposure to metals also induces enhanced tolerance to biotic factors, suggesting a type of chemical memory,

obtained by enhanced signaling pathways, that can be triggered upon the first stress (Trewavas, 1999). For example, Ghoshroy et al. (1998) and Mittra et al. (2004) showed that a mild dose of Cd pre-exposure increased plant resistance to viral and fungus infections. Also, other works point out that pre-exposure to a metal can enhance tolerance to other metals. For example, Avilés et al. (2003) have shown that a pre-exposure to Hg enhanced Cd accumulation in *Euglena gracilis*. Metal stress can also induce alterations in ROS accumulation and in glutathione pools, two important signaling mediators in many abiotic stresses, such as salt, osmotic and temperature stress (May et al., 1998; Clarck et al., 2001; Mithöfer et al., 2005; Maksymiec, 2007). Toghether, these data indicate the existence of a signal transduction pathway underlying metal tolerance that shares at least some signalling components with other biotic and abiotic stresses. But how they can be related to phytochelatin induction remains to be elucidated.

1.4.1 Possible points of PC signalling regulation

Since phytochelatins are not gene products, their signalling pathways would have to encompass the enzymes involved in their production. Indeed, some reports have shown that increasing the activity of the enzymes involved in the GSH pathway enhanced Cd tolerance and PC synthesis. In fact, increasing both γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GSHS) activity enhances Cd tolerance and PC synthesis (Chen and Goldsbrough, 1994; May et al., 1998; Noctor and Foyer, 1998**;** Schafer et al., 1998; Xiang and Oliver, 1998). On the other hand, specific signalling components have already been pointed out as regulators of Cd stress. He et al. (2005) showed that calcium can be important for Cd tolerance both reducing the toxic effects exerted by this metal but also directly affecting PC synthase. Protein phosphorylation, mostly through MAPK kinases is also emerging as an important mediator in Cd signaling (Nakagami et al., 2004; Rios-Barrera et al., 2009). Finally, several reports have revealed that ROS production is involved in GSH synthesis (Griffith and Meister, 1979) and calcium signalling (Lamb and Dixon, 1997; Xing et al., 1997; Grant et al., 2000;

Yang and Poovaiah, 2002), which suggests that ROS may be related with PC synthesis. These findings have opened new possibilities to the unravel of the signalling pathway involved in Cd tolerance and PC synthesis and evoke the need to pursue further studies to identify possible Cd-induced signalling pathways.

1.5 Objectives

Understanding the mechanisms by which plants perceive environmental signals and transmit them to the cellular machinery activating adaptive responses is of fundamental importance to biology. The knowledge about stress signal transduction can be applied in the development of rational breeding programs and transgenic strategies to improve stress tolerance in crops.

Heavy metal stress is one of the most important factors limiting the growth and distribution of plants in the world and has become a widespread problem. However, the signalling mediators that trigger metal tolerance mechanisms such as phytochelatins are still largely unknown. The understanding of these signalling networks can open new possibilities to design crops with increased abilities to adapt to excess metal conditions that can be agronomicaly and environmentally significant.

Since stress sensors are not known and most of the signalling intermediates have not been identified in plants, one important approach for understanding the stress-induced pathways would be to analyse the signal transduction components that are highly conserved and present in all signal pathways. Most signal transduction studies in plants exposed to abiotic stresses show that stress signalling relies mostly on Ca signals, protein phosphorylation and ROS induction. Taking into consideration the few reports on Cd signalling, as well as the studies on plant signal transducers we came to hypothesise that metal stress signalling and PC synthesis can involve all of these components. Under this context this work was elaborated with the following aims:

1) Elucidate the pathway by which Cd is detected by the plant cells and induces PC synthesis, by analyzing the role of calcium signals, protein phosphatases and ROS induction during the metal detection and response process in *Arabidopsis thaliana*.

2) Understand how this information could be used for improving the current practices involving plant tolerance abilities and metal uptake, which can be used in both the fields of agronomy (where a higher tolerance with reduced metal uptake in the edible plant organs is desired) and phytoremediation (where more tolerant plants with increased metal uptake are preferred).

Therefore, a first approach was made in order to analyze the process of PC synthesis and Cd chelation in *Arabidopsis thaliana* (chapter 3) by timing the major events that culminated in thiol alterations and enzymatic modulation. Subsequently, the importance of specific signal transducers associated with calcium, protein phosphorilation and ROS signals was monitored during PC synthesis. Hence, in chapter 4, the role of calcium signalling and calcium-binding proteins in Cd responses was adressed, using different pharmacological modulators of calcium levels and analyzing the expression of the calcium sensors (particularly CBLs) involved in this process. In chapter 5, the role of protein phosphatases was investigated, namely PP1 and PP2A, in Cd signalling and PC induction. In Chapter 6 the involvement of ROS signals in PC, GSH and calcium modulation, as well as on GST activity was adressed. Finally, in Chapter 7, a possible pathway for Cd signalling and PC induction in plants was presented. Possible strategies for regulating Cd uptake and tolerance in plants were also suggested.

Chapter 2

Materials and Methods

2.1. Plant material

Arabidopsis thaliana (L.) Heynh (cell line DMZ PC-0011) suspensions were grown in a rotary shaker at 100 rpm at 25°C, in a modified MS basal medium (Murashige and Skoog, 1962), composed of 1.650 mg.l^{-1} NH_4NO_3, 6.2 mg.l^{-1} H_3BO_3, 440 mg.l^{-1} $CaCl_2$, 0.025 mg.$l^{-1}$$CoCl_2$, 370 mg.$l^{-1}$ $MgSO_4$, 0.025 mg.l^{-1} $CuSO_4$, 170 mg.$l^{-1}$$KH_2PO_4$, 27.8 mg.$l^{-1}$ $FeSO_4$, 1.9 mg.$l^{-1}$$KNO_3$, 22.3 mg.$l^{-1}$$MnSO_4$, 0.83 mg.$l^{-1}$KI, 0.25 mg.$l^{-1}$ Na_2MoO_4, 8.6 mg.$l^{-1}$$ZnSO_4$, 37.2 mg.$l^{-1}$ Na_2EDTA, 100 mg.l^{-1} i-Inositol, 0.5 mg.l^{-1}Nicotinic acid, 0.5 mg.l^{-1}Pyridoxine, 0.1 mg.l^{-1} Thiamine, 30 mg.l^{-1}IAA, supplemented with 2% sucrose, 0.2 mg. l^{-1} kinetin, 2 mg. l^{-1} 2,4-Dichlorophenoxyacetic acid and 2 g. l^{-1} casein. The cells were subcultured every 7 days, during the exponential growth phase, by making a 1:10 dilution in fresh medium. Cell cultures with 4 days after subculturing were used for all the experiments.

2.2. Cd exposures

Different volumes of a Cd stock solution (100 mM $CdCl_2$) were added to the nutrient solution, to yield concentrations of 1, 10, 100, 300 or 500 μM Cd and cells were collected after each period of exposure (5 minutes, 15 minutes, 30 minutes, 1 hour, 3 hours, 12 hours, 1 day or 2 days). At each collection, cells were centrifuged at 9000 *g*, at 4°C, for 10 minutes; the supernatant was discarded and the cells were weighed, frozen in liquid N_2 and stored at -80°C for each biochemical analysis. All procedures were repeated in 3 to 5 independent experiments.

2.3. Plant cell viability determinations

In all experiments, the viability of the cell cultures (in the indicated time of exposure) was assayed by addition of Evans Blue to a final concentration of 0.05% (w/v). As Evans Blue can only permeate ruptured plasma membranes, it specifically invades dead cells. At 15 min after addition of Evans Blue, the percentage of living cells was determined by scoring at least 300 cells (Horemans et al., 2007). All procedures were repeated in 3 to 5 independent experiments.

2.4. Treatments with calcium modulators, protein phosphatases inhibitor and H_2O_2

Four days after changing the media, cells were exposed to the different signalling modulators by adding different concentrations of each reagent to the nutrient solution, as described in Rentel and Knight (2004). After 1 hour, $CdCl_2$, was added, in order to achieve a final concentration of 100 µM Cd and after another hour cells were collected. Cell viabilities were analysed after 0, 30, 60 and 120 minutes, in control and exposure conditions according to the procedure described above. Cells were weighed and individual samples were frozen in liquid N_2 and stored at -80ºC for each biochemical analysis. All procedures were repeated in 3 to 5 independent experiments.

2.4.1. Calcium modulation assays

Concomitantly to Cd exposure, cells were exposed to several calcium blockers, as described by Rentel and Knight (2004). Cells were incubated with the different pharmacological calcium modulators, $CaCl_2$, caffeine, EGTA, $LaCl_3$ (La) or Ruthenium red (Rut Red) solutions to reach a final concentration of 100 mM of calcium, 1 mM or 10 mM of caffeine, 0.1, 0.5 or 1 mM EGTA; 0.5 or 1 mM La or 0.1 or 0.5 mM Rut Red. After 1 hour of incubation samples were exposed to 100 µM Cd for 1 hour and were harvested and frozen as described. The effect of each reagent on cell viability was monitored as

described above, in order to assure that the concentration was not inhibiting normal cell metabolism.

2.4.2. Phosphatase inhibition assays

As described for other modulators, cells were exposed to 1 nM, 10 nM, 100 nM or 1 μM of cantharidin (Can), a PP2A and PP1 inhibitor. The effect the phosphatase inhibitor on cell viability was monitored during 2 hours, in order to assure that the concentrations were not inhibiting normal cell metabolism, as described for Ca treatments. After 1 hour of incubation with Can, samples were exposed to 100 μM Cd, harvested and frozen at the different times of exposure, also as described before.

2.4.3. H_2O_2 modulation assay

Cells were treated as described previously for the other modulators, but with the addition of 1, 10 and 100 mM H_2O_2 for 1 hour previous to 100 μM Cd exposure. Cells were also collected as described previously.

2.5. PC Analysis

2.5.1 Extraction procedure for thiol analysis

Peptide extraction was performed as described by Lima et al. (2006). Frozen samples were homogenised in a mortar and pestle on ice with 0.1 N HCl at a ratio of 1g of plant cells to 4 ml of acid, while under a stream of N_2. The homogenates were centrifuged at 40.000 *g*, for 10 min, at 4 ^{o}C and the resulting supernatant was immediately used or stored at -80^{o}C.

2.5.2. Derivatization

The method for derivatization of thiol compounds was performed as described earlier (Lima et al., 2006). Extracts (100 μl) were neutralised with 0.1 M NaOH, after the addition of 200 μl of 0.1 M Tris-HCl buffer (pH 8.0), 1 mM EDTA and 25 μl of 2mM DTE. After incubation for 1 h at room temperature, 50 μl of 20 mM mBBr (Calbiochem) were added. Derivatization was performed in the dark, for 40 min at a temperature of 35 ^{o}C. The reaction was

stopped by the addition of 5 % (v/v) acetic acid, up to a total volume of 1.5 ml. Samples were stored at 4°C before HPLC-RP analysis.

2.5.3. HPLC analysis of thiol compounds

The highly fluorescent bimane derivatives were separated by RP-HPLC (Gilson liquid chromatograph, model 306), as described earlier (Lima et al, 2006). Samples were centrifuged at 40.000 *g*, for 5 min, at 4 °C and filtered. Twenty µl aliquots were injected on a RP C_{18} column (250x4.6 mm i.d., 5µ, Gilson). The column was equilibrated with previously degassed eluant A [0.01% aqueous TFA (v/v)] and developed by a linear gradient of 0-20 % eluant B (90% acetonitrile, in 0.01% aqueous TFA) during the first 20 min, followed by an isocratic elution of 20 % eluant B for the next 30 min and finally a linear gradient of 20-90% of eluant B during the last 10 min. The complete analysis was performed in 60 min. After each run the column was washed by raising the concentration of eluant B to 100% and re-equilibrated with eluant A. All thiols were resolved and eluted at a flow rate of 1 ml. min^{-1} and detected by fluorescence (Jasco 821-FP Intelligent Spectrofluometer) with excitation at 380 nm and emission at 480 nm (Klapheck, 1988, Sneller et al., 2000). In order to overcome the problem of the decrease in derivatisation with chain length increase, adverted by other authors (Sneller et al. 2000), thiol identification was based on Cys and GSH standards (Sigma) and synthesised PC standards (kindly offered by Dr. M.H. Zenk), with *n* ranging from 2-5.

2.6. Determinations of γ-ECS, GSHS and PCS activity

Different enzymatic activity analysis was performed according to Tsuji et al. (2003). Different cell extracts with different treatments were harvested by centrifugation (3.500 *g* for 10 min at 4°C) and resuspended in 100 mM Tris–HCl buffer (pH 8.0) containing 10 mM $MgCl_2$ and 5 mM EDTA. The cells were pulverized with a mortar and a pestle under liquid nitrogen, and ultracentrifuged at 80.000 *g* for 45 min at 4°C. Supernatants were used for enzyme analysis.

2.6.1. PC synthase

PC synthase activity was determined in all sample in a reaction mixture (final volume of 200 μl) containing 100 mM Tris–HCl buffer (pH 8.6), 5 mM mercaptoethanol, 10 mM glutathione, 0.5 mM $CdCl_2$ and the extracted sample, in a volume that yielded a final concentration of 1 mg protein in the final volumes. The incubation was performed at 37°C for 120 min and terminated by addition of 40 μl of 3.6 N HCl. The amount of PCs synthesized was determined by HPLC analysis as described earlier. For phosphatase inhibitory assays, the addition of cantharidine or EGTA to the reaction mixture, using a control sample, was also tested, in order to evaluate the influence of Can on PCS activity, in the presence or absence of Cd. Final concentrations in the reaction volume for Can and EGTA were 100 nM and 100 mM, respectively.

2.6.2. GSH synthethase

GSHS activity was determined in a reaction mixture (final volume of 200 μl) containing 50 mM Tris–HCl buffer (pH 8.0), 10 mM $MgCl_2$, 30 mM glycine, 5 mM γ-EC, 10 mM ATP, and the extracted sample, in a volume that yielded a final concentration of 1 mg protein in the final volumes of the assay. The incubation was performed at 37°C for 60 min and terminated by addition of 40 μl of 3.6 N HCl. The amount of GSH synthesized was determined by HPLC analysis as described above. PC synthesis was not observed in this reaction.

2.6.3. γ-ECS synthethase

γ-ECS activity was determined in a reaction mixture (final volume of 200 μl) containing 100 mM HEPES buffer (pH 8.0), 40 mM MgCl2, 30 mM glutamate, 1 mM cysteine, 5 mM ATP, 0.5 mM phosphoenolpyruvate, 0.5 unit pyruvate kinase, 0.5 mM dithioerythritol, and the extracted sample, in a volume that yielded a final concentration of 1 mg protein in the final volumes of the assay. The incubation was performed at 37°C for 45 min and terminated

by addition of 40 μl of 3.6 N HCl. The amount of γ-ECS synthesized was determined by HPLC analysis as described above.

2.7. CaBPs isolation throught affinity chromatography

Plant cells exposed to the different exposure periods to Cd were collected as described above and homogeneized in buffer A containing 50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 10% glycerol, with a proteinase mix of 2mM PMSF, 10 μg. l^{-1} aprotinin, 10 μg. l^{-1} leupeptine and 1 μg. l^{-1} of pepstatin. Extracts were centrifuged at 12000 RPM at 4ºC for 15 minutes. The protein content of the supernantants was analyzed and normalized amounts of protein of each sample were loaded into a Phenyl Sepharose collumn (GE Healthcare). Elution was performed with a buffer A containing 1 mM $CaCl_2$. Calcium-binding proteins, which contain a hydrophobic surface were bound to the column. All other proteins were collected in the flow through. Calcium-binding protein elution was performed by removing the bound calcium from the protein. This was accomplished by an elution with buffer A containing 1 mM EGTA. Eluted fractions were concentrated with Vivaspin 10.000 concentrators, at 4000 RPM and 4ºC. Alternatively proteins were concentrated by precipitation with cold acetone 1:4 (v/v) Samples were incubated for several hours or overnight at -20ºC and precipitated proteins were collected by centrifugation at 5000 RPM at 4ºC. The amount of eluted proteins was quantified and samples were immediately frozen at -80ºC.

2.8 Protein extractions and protein determination

Plant cells were extracted with 50 mM Tris-HCL, pH 7.5 and 2mM PMSF, in a ratio of 1g of plant material to 5 ml of extraction buffer and centrifuged for 12000 RPM, at 4ºC, for 15 minutes. Soluble protein concentration was measured following the method described by Bradford (1976) using bovine serum albumin (BSA) as standard. The assay is based on the stable dye–protein complex, which can be quantified spectrophotometrically at 595 nm

2.9. SDS-PAGE Electrophoresis

Cells were suspended in 200 µl SDS sample buffer (Hames, 1981), followed by a 5-min heat treatment at 90 ^{o}C. Lysates were centrifuged at 10000 *g* for 10 min to remove cell debris. The supernatant was collected and used for protein separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), carried out in 15% acrylamide gels, following the system of Laemmli (1970). Molecular weight standards used were purchased from Bio-Rad. Gels were stained with Coomassie brilliant blue R-250 (Bio-Rad). Densitometric readings were performed by a Bio-Rad densitometer (model GS 710). The molecular weight and relative amount of proteins corresponding to each band were calculated using Quantity-One Software (Bio-Rad) (Figueira et al., 2005).

2.10. Immunoblot detection of PP1 and CBL expression

Samples were separated on 15% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in TBS-T buffer for 1h, followed by incubation with antirabbit antibodies for PP1 or CBL proteins, for 2 hours at room temperature with shaking. After washing, the blots were incubated with antirabbit secondary antibody conjugated with horseradish peroxidase (diluted 1:1000) for 1h at room temperature. Blots were washed and developed with an ECL chemiluminescence kit (Amersham). The intensity of blot bands was determined by densitometry using a GS-710 calibrated imaging densitometer and Quantaty One software (BioRad).

2.11. Hydrogen peroxide (H_2O_2) quantification

Cell extracts (1 g) frozen in liquid nitrogen were crushed in a mortar and homogenized in 2 ml of 50 mM phosphate buffer (pH 7.0) containing 1% Triton X-100 (v/v) and 1% PVP (w/v) and was centrifuged at 10000 *g* for 10 minutes at 4 ^{o}C. H_2O_2 content was determined according to Pick and Mizel (1981) with some modifications. The assay was based on horseradish

peroxidase-dependent oxidation of phenol red by H_2O_2.The reaction mixture in a final volume of 1110 µl consisted of 100 µl of sample extract and 1000 µl of 0.28 mM of phenol red solution in 10 mM potassium buffer (pH 7.0) with 8.5 U/ml of horseradish peroxidase. The mixture was shaken for 10 minutes at 25ºC and then 10 µl of 1 M NaOH was added. The absorbance was measured at 610 nm. The H_2O_2 level was calculated according to a standard curve of H_2O_2.

2.12. GST activity assays

GST activity was determined spectrophotometrically with CDNB (1-chloro-2,4-dinitrobenzene) by the method of Habig and Jakobi (1981). CDNB (1 mM final concentration in ethanol), and GSH (1mM final concentration in 0.1M sodium phosphate buffer, pH 6.5) were prepared as substrates (at 25 ºC). GST activity was obtained based on the extinction coefficients of 9.6 (mM. cm^{-1}) for CDNB. Changes in absorbance at 340 nm, were monitored for product formation using a UV/VIS spectrophotometer (Beckman Model DU-68) and activity was calculated using protein concentrations determined via the Bradford assay, with BSA used as standard.

2.13. Analysis of intracellular cadmium absorption and Cd binding

Cd determination was performed as described in Lima (2004). After the several exposures, cells were collected as described earlier and washed with a solution of 5 mM $CaCl_2$ in order to remove extracellular Cd. Cells were pulverized as described earlier, and intracellular Cd was extracted in 0.1N HNO_3, at a ratio of 1:3 (w/v). Extracts were centrifuged at 50.000 *g* for 15 min (Meuwly and Rauser, 1995) and filtered. Cd concentration was measured by ICP-AES (Inductively Coupled Plasma-Atomic Emission Spectroscopy), in a Jobin Yvon Jy Plus model. Cd binding to SH groups was monitored by its absorvance at 254 nm (Casterline and Barnett, 1982, Rauser (2000). Shortly, cells were extracted with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, as described earlier, at a ratio of 1:4 (w/v). After centrifugation at 15.000 *g*, the absorbance of the supernatant was measured at 254 nm. Control samples (without the addition of Cd) and standards with known GSH+Cd concentrations, as well

as Cd and GSH alone were used.

2.14. Statistical analysis

All data values were submitted to statistical analysis with SIGMASTAT v 2.03 software, by one-way or two-way ANOVA and with Tukey's Multiple Range Test, with a significance of $P<0.05$ or $P<0.001$.

Chapter 3

PC formation and Cd chelation in *A. thaliana*

3.1 Introduction

PCs are considered to be the principal mechanism responsible for cadmium detoxification in the majority of plant species, as supported by numerous inhibitory (Grill et al., 1987; Reese and Wagner, 1987; Gussarsson et al., 1996), biochemical (Kneer and Zenk, 1992) and mutant analysis studies (Mutoh and Hayashi, 1988; Howden et al., 1995; Ha et al., 1999). In order to chelate heavy metals such as Cd, PC molecules with different chain lengths are successively produced. In the structural model of a PC-Cd complex, the Cd ion can coordinately bind one to four sulfur atoms from either single or multiple PC molecules, hence forming amorphous complexes as shown in Fig. 3.1.1. The ratio of Cd to cysteine in the complexes is about 2 (Grill et al., 1985).

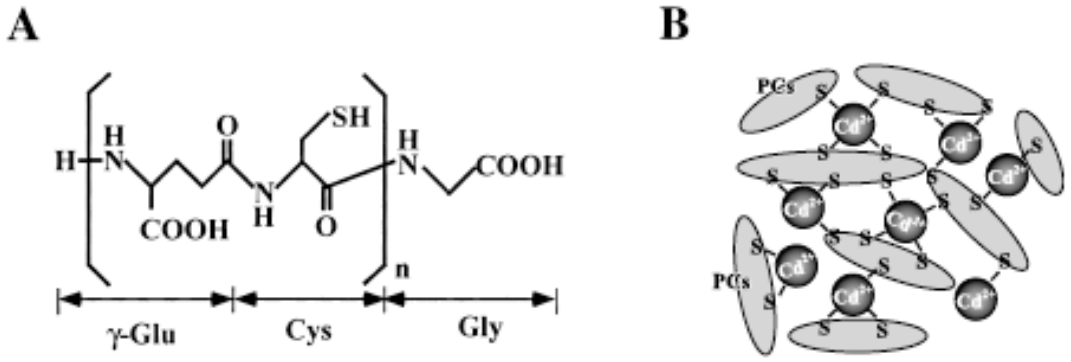


Fig. 3.1.1. The primary structures of PCs (A) and PC-Cd complexes (B). Dications such as Cd coordinately bind one, two, three or, at maximum capacity, four sulfur atoms from either single or multiple PC molecules, resulting in amorphous complexes. In the optimized structure of the $Cd(PC_2)_2$ complex, only the S atoms enter the coordination sphere of Cd (adapted from Hirata et al., 2005).

According to some authors, the longer the chain (more repeated units of γ-GluCys), the stronger the pH stability and the binding capacity towards heavy metals per molecule (Mehra et al., 1988, 1995). Therefore, it has been hypothesized that Cd concentrations and time of exposure are *grosso modo* correlated with the amount of PCs produced and with the ability to produce PCs with longer chain lengths (Grill et al. 1987; 1985; Mehra et al., 1995; Sanitá di Toppi and Gabrielli, 1999).

3.1.1 The process of vacuolar metal sequestration

At the light of previous reports (Reese and Winge, 1988; De Knecht et al., 1992; Reese et al., 1995; Rauser, 2000; Sousa and Rauser, 2004; Lima et al, 2006), it has been proposed that Cd sequestration is based on two steps, where Cd is firstly complexed by PCs in the cytosol, forming a low molecular weight complex (LMW), which is subsequently transferred to the vacuole where a higher molecular weighted complex (HMW) is formed (Ortiz et al., 1995).

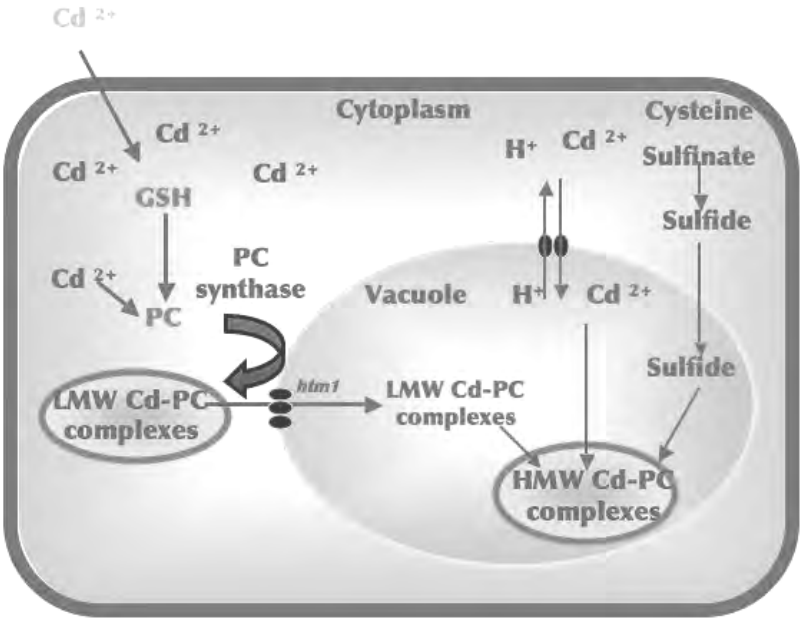


Fig. 3.1.2: Vacuolar sequestration of PC-Cd complexes. LMW (low molecular weight) complexes are formed in the cytosol and are transported to the vacuole where a higher molecular weighted complex (HMW) is accumulated. Vacuolar Cd sequestration is mediated by a 90.5 kDa vacuolar ATP-binding cassette transporter, HMT1 (heavy metal tolerance factor 1) or a similar one, that catalyzes the MgATP-energized uptake of Cd-PCs and apo-PCs into the vacuoles (Ortiz et al., 1995; Salt and Rauser, 1995; Vatamaniuk et al., 2000).

The HMW complex is found to be more stable in the vacuole's acidic environment and has a higher ability to bound metal ions when compared to LMW complexes (Ortiz et al., 1995; Zenk, 1996). In some plants and yeasts, HMW PC-Cd complexes also contain both Cd and acid-labile sulfide (Dameron et al., 1989; Reese et al., 1992). The incorporation of sulfide into the HMW complexes increases both the amount of Cd per molecule and the stability of the complex (Speiser et al., 1992).

3.1.2 Enzymatic pathways of PC formation: possible regulation points for Cd signalling

Regulation of γ-EC synthethase and GSH synthetase

Since phytochelatins are not gene products, the signaling pathways should involve the enzymes that catalize their production. PC synthesis begins with the formation of the tripeptide GSH, from the amino acids L-cysteine, L-glutamate acid and glycine. It is the sulfhydryl (thiol) group (SH) of cysteine that serves as a proton donor and is responsible for the biological activity of glutathione. (May et al., 1998; Noctor et al., 1998) (Fig.3.1.3)

Glutathione is synthesized in two ATP-dependent steps. Firstly, the formation of γ-glutamylcysteine (γ-EC) from glutamate and cysteine, is catalyzed by γ-glutamylcysteine synthetase (γ-ECS, EC 6.3.2.2), as descrived in Fig. 3.1.3. This is generally accepted as the rate-limiting step in the biosynthetic pathway (May et al., 1998; Noctor et al., 1998). γ-ECS is a redox sensitive homodimeric enzyme, conserved in the plant kingdom (Horthon et al., 2006). In addition to the redox dependent control, the plant γ-ECS enzyme is also feedback inhibited by GSH (Hicks et al., 2007). In the second step, glycine is added to the C-terminal of gamma-glutamylcysteine via the enzyme glutathione synthetase (GSHS, EC 6.3.2.3) (see Fig. 3.2.3), which is also involved in the regulation of GSH levels (Wolf et al., 2005).

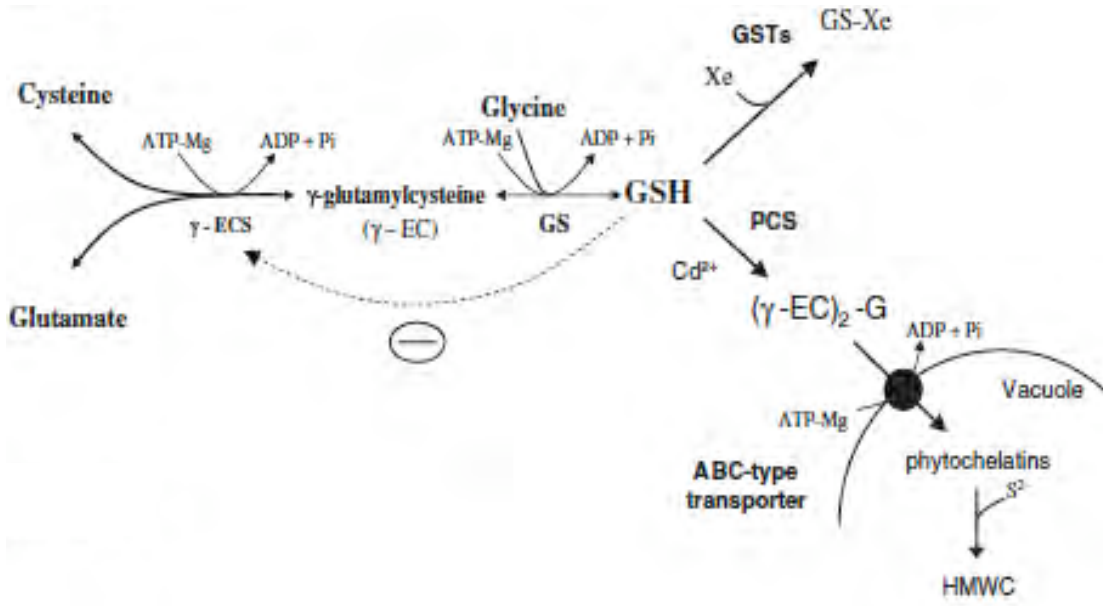


Fig. 3.1.3. The enzymatic pathways leading to PC synthesis in Arabidopsis. First, the biosynthesis of GSH occurs in two steps. The first catalysed by γ-ECS forms the dipeptide γ-glutamylcysteine from glutamate and cystein. The second is catalysed by GSHS, which adds a terminal glycine, hence forming the tripeptide GSH. GSH suffers a cleavage of the Gly bond and further trasnspeptidation by the enzyme PCS, forming PCs with different chain lengths. These are transported through the vacuole by an ABC-type transporter where HMW complexes are formed. GSH levels are also modulated by the action of GSTs that catalyse the formation of glutathione complexes. The figure is a composite of different functions described in different organisms. Enzyme abbreviations are: γECS, glutamylcystein synthethase; GSHS, glutathione synthetase; PCS, Phytochelatin synthase, GST: glutathione-s-transpherases. Adapted from Mendoza and Sánchez (2005).

The regulation of both enzymes of the GSH biosynthetic pathways have been found to be regulated by Cd. Studies of transgenic Indian mustard (*Brassica juncea*) plants, with increased expression of the enzymes of the GSH biosynthetic pathway had increased PC biosynthesis and Cd tolerance (Yong et al., 1999; Zhu et al., 1999). Tsuji et al (2003) also reported that γ-ECS and GSHS were activated by Zn and Cd. Another work shows that a Cd-tolerant tomato cell line has increased γ-ECS activity (Chen and Goldsbrough, 1994) and wild-type Indian mustard plants also responded to Cd exposure to Cd with increased levels of a γ-ECS transcript (Schafer et al., 1998). There is also circumstantial evidence supporting post-transcriptional regulation of GSSH expression in addition to its well-recognized regulation through GSH feedback inhibition (May et al., 1998; Noctor and Foyer, 1998), which is highly observed during PC synthesis. All of these informations support the idea that

the regulation of GSH biosynthesis is an endogenous mechanism by which PC expression might be modulated and influenced by Cd signals.

Regulation of Phytochelatin synthase

Once GSH is formed in the cell, the biosynthesis of PCs is catalyzed by PCS (EC.2.3.2) (see also Fig 3.1.2). PCS is a γ-glutamyl-cysteine transpeptidase with a papain-like catalytic triad (Rea, 2006). The regulation of PC synthase activity is obviously expected to be the primary point of PC synthesis regulation. PCS is a constitutively expressed, ubiquitous enzyme in *Arabidopsis* (Vatamaniuk, 1999; 2000). Previous studies indicated that PC biosynthesis occurs within minutes of exposure to Cd and is independent of *de novo* protein synthesis, thus suggesting an absence of regulation at the transcription level (Ha et al., 1999; Vatamaniuk et al., 1999; Cobbett, 2000). Also, the enzyme appears to be ubiquitously expressed, independently of heavy metal exposure, but in an inactive state. (Grill et al., 1989). This suggests a mechanism of autoregulation that balances between the cellular Cd concentration and PCS activity, however, the factors that trigger this activity remain to be fully elucidated (Wang et al. 2009).

In 1999, three separate groups achieved the genetic characterization of PCS. Isolated from *Arabidopsis*, S*chizosaccharomyces pombe* and *Triticum aestivum*, these genes – designated *AtPCS1*, *SpPCS* and *TaPCS1*, respectively, encoded similar 50-55 kDa polypeptides active in the synthesis of PCs from GSH (Ha et al., 1999; Clemens et al., 1999; Vatamaniuk et al., 1999)**.** The two terminal domains of PCS (Fig. 3.1.4) were shown to present different roles in PC synthesis. Several works suggested that the N-terminal is the most conserved and the catalytic domain, which catalyzes the deglycylation of GSH to -Glu-Cys (Ha et al., 1999), whereas the C-terminal domain is more variable, but can be involved in PC elongation (Rea et al., 2004; Ruotolo, et al., 2004; Tsuji et al., 2005; Harada et al., 2004)

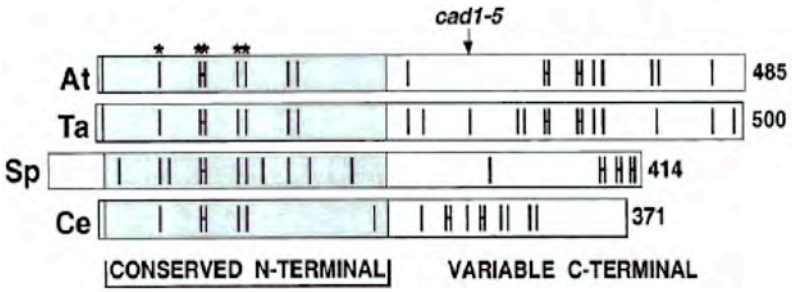


Figure 3.1.4. Schematic comparison of PC synthase polypeptides from different organisms. At, *Arabidopsis* (CAD1/AtPCS1); Ta, *Triticum aestivum* (TaPCS1); Sp, S*chizosaccharomyces pombe* (SpPCS;); Ce, Caenorhabditis elegans (CePCS1). The total number of amino acids in each polypeptide is shown on the right. Approximate positions of all Cys residues are indicated by vertical bars; adjacent Cys residues are connected by a horizontal bar; and Cys residues conserved across the three sequences are highlighted with an asterisk. The conserved N-terminal domains exhibit at least 40% identical amino acids in pair-wise comparisons of the four sequences. Adapted from Cobbett (2000).

Over the last 20 years, the activation of the catalytic mechanism of PC synthase has been a focus of research for several groups. Several conflicting reports have been arising and came to show that PCS activation is much more complex than initially thought. Since the work reported by Vatamaniuk et al. (2000, 2004) that suggested that PCS does not bind directly Cd ions and that blocked thiols are sufficient for its activation, most lines of evidence favor the notion that the transfer of metal ions from thiol ligands such as GSH to PCS proteins occurs (see Fig. 3.1.5).

In summary, in the absence of heavy metals, PC synthase generally adopts an inactive conformation. By exposure to heavy metals, such as Cd, most of the intracellular ions form complexes with GSH such as Cd-GS_2. The complexes have stronger affinity to PC synthase than free heavy metal ions and function as enzyme activators by causing the folding of the enzyme protein. Then, the heavy metal may bind with PC synthase and facilitate a conformation change for its activation (Vatamaniuk et al., 2002). The first step (step 1) of PC synthesis is the formation of a γ-EC acyl-enzyme intermediate concomitant with the cleavage of Gly from the first substrate (Fig. 3.1.5). The second step (step 2) is the reaction for the transfer of the γ-EC unit from the substituted enzyme intermediate to the second substrate, GSH or PCn, to generate a product containing an additional γ-EC moiety, PC_2 or PC_{n+1}

(Vatamaniuk et al., 2004). These two reactions are performed in the conserved N-terminal domain (Fig. 3.1.5).

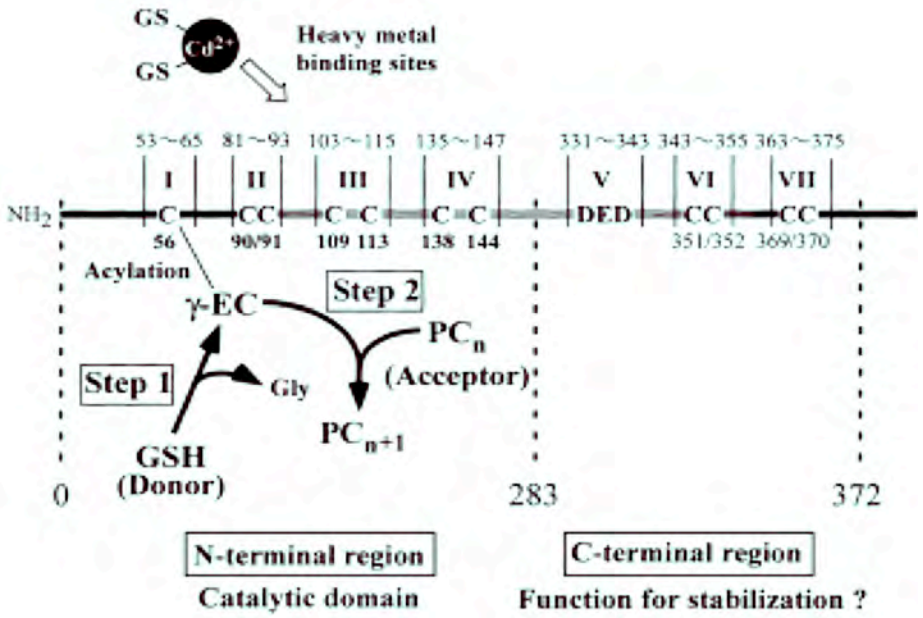


Fig. 3.1.5. Model for the hypothetical catalytic mechanism of phytochelatin synthase in eukaryotes. (Adapted from Hirata et al., 2005).

Nonethless, Oven et al. (2002) found only very minor activity of AtPCS1 with S-alkylated GSH in the absence of Cd, observed activation of PC synthases with metal thiolates that clearly were no substrates (e.g. dithiothreitol, cysteine). Furthermore, there is strong evidence for Cd binding to AtPCS1 *in vivo* because the C-terminal part of AtPCS1 was able to enhance Cd tolerance of a sensitive *S. cerevisiae* mutant *in vivo,* in the absence of any PCS activity (Ruotolo et al., 2004), suggesting that Cd is sequestered efficiently by the protein even in the presence of a vast excess of thiol ligands in the cell. Thus, the understanding of the metal-dependent transpeptidation step is still far from being understood. According to Clemens (2010), two principal scenarios can be distinguished. In the first one, a GS-metal complex or a PC-metal complex binds to a PCS protein, or more precisely, to the second as yet unknown acylation site and serves as a substrate for dipeptide transfer (Vatamaniuk et al, 2004). In the second hypothesis, a metal ion is transferred from a GS-metal complex to the PCS protein, thereby activating it.

3.1.3 Objectives

The process of PC synthesis is a complex phenomenon, involving several key steps with fundamental importance on its synthesis and most importantly on metal chelation. These events include Cd absorption, GSH synthesis and depletion, metal-binding to GSH, PCS activation and the synthesis of the olygomeric chains of PCs. All of these key steps should be regulated or involved in specifical signalling networks. In order to study these pathways, their timeline development needs to be well understood. Nonetheless, their kinetics and time-course activation are still poorly investigated. This knowledge will constitute an important basis for the study of the signaling events that culminate in the formation of PCs and metal tolerance. Under this context, this chapter focuses on the analysis of the time-course synthesis of PCs during a Cd exposure, as an attempt to understand its kinetics in the model plant *Arabidopsis thaliana*. Several important parameters were analyzed, aiming to:

1. Understand the metal tolerance abilities of *A. thaliana* to Cd stress in order to choose a concentration and to define a timeline period for the subsequent studies.

2. Determine the timing of PCS catalytic activation and the cellular events that are involved with its activity.

3. Determine the timing of GSH modulation induced by Cd and PC formation, as well as the kinetics of its catalytic synthesis.

4. Define a timeline for PC formation, chain elongation and cadmium chelation.

Results obtained in this chapter will provide important information on the chronology and sequence of events involved in PC synthesis and Cd sequestration in this species, which will be important for the subsequent chapters.

3.2 Results

3.2.1 Cd tolerance in *A. thaliana*

In order to understand the metal tolerance abilities of *A. thaliana*, and to choose a Cd concentration for the subsequent studies, plant cells were exposed to a range of Cd concentrations, during a three-hour period. Fig. 3.2.1 shows the effects of exposure to 1, 10, 100, 300 and 500 μM Cd on cell viability, throughout the time of exposure, expressed as percentage of viable cells relatively to controls.

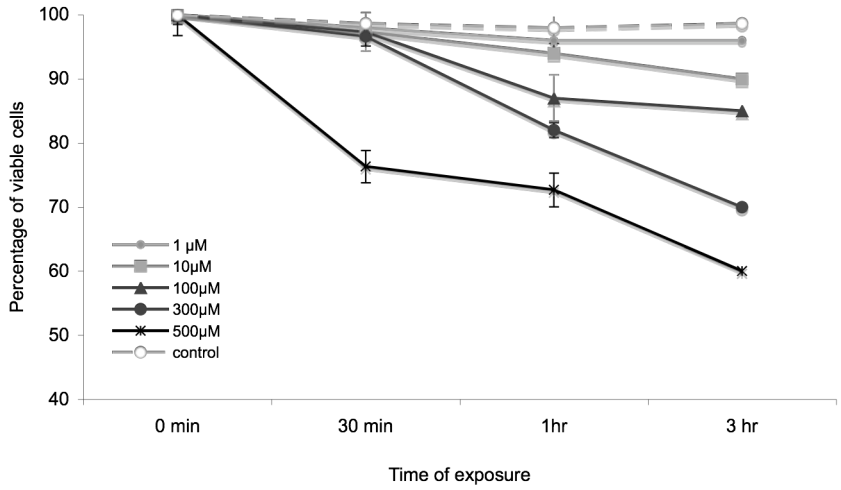


Fig.3.2.1. The effect of different Cd concentrations on *Arabidopsis thaliana* cells, expressed as percentage of cell viability, during 3 hours of exposure to 1, 10, 100, 300 and 500 μM Cd. Values are the means of at least three replicate observations ±SE.

Increasing metal concentrations induced a significant inhibition of cell viability ($P<0.001$) and three hours of exposure to the higher metal concentrations (300 and 500 μM Cd) were enough to significantly decrease the cell viability to almost 50% of controls ($P<0.05$). At 30 min of exposure only 500 μM Cd concentration induced a significant cell inhibition, whereas the other concentrations were not significantly different from controls ($P>0.05$). Nonetheless at 1 hour of exposure significant reductions in the cell viability of exposures were already observed for 100, 300 and 500 μM Cd ($P<0.05$), these alterations were maintained after 3 hours of exposure, but with higher reductions.

3.2.2 PC production is dependent on Cd concentrations

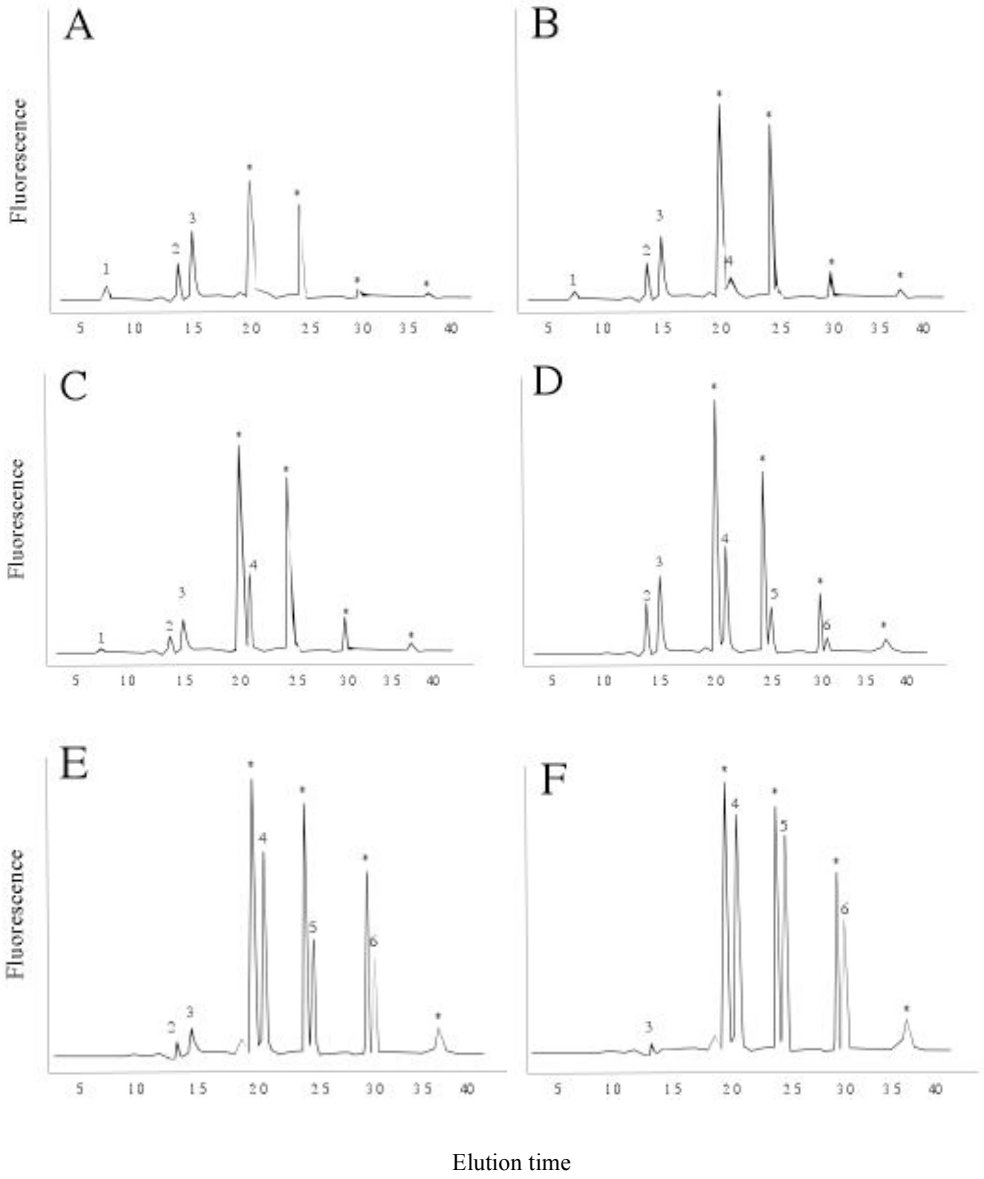


Fig.3.2.2. HPLC profiles of the time-course monitorizations of PC induction in *A. thaliana* cells after 1 hour exposure to different Cd concentrations: A: control; B: 1 μM, C: 10 μM, D: 100 μM, E: 300 μM and F: 500 μM Cd. The retention times of PC peaks in the sample correlated perfectly with standard peaks. Residence times and peak identification were as follows: 1: Cys (11 min); 2: γ-EC (12 min); 3: GSH (15 min); 4: PC_2 (21 min); 5: PC_3 (31 min); 6: PC_4 (39 min). Derivatization peaks are marked with *.

The induction of PCs was monitored after 1 hour of exposure to the different Cd concentrations, the minimum time required for significant growth reductions in most of the Cd concentrations (Fig. 3.2.1). Fig. 3.2.2 shows examples of chromatographic profiles of PC induction after 1 hour of exposure to 1 μM, 10 μM, 100 μM Cd, 300 μM or 500 μM Cd. PCs were detected in all concentrations with *n* ranging from 2 to 4, as well as the

constitutive thiols GSH, Cys and also γEC. Results show that increasing Cd concentrations induced consistently and significantly higher PC production as well as an enhanced amount of olygomeric repeats ($P<0.05$). Fig 3.2.3 demonstrates the thiol production with different Cd concentrations and the increase in the different γ-EC repeats.

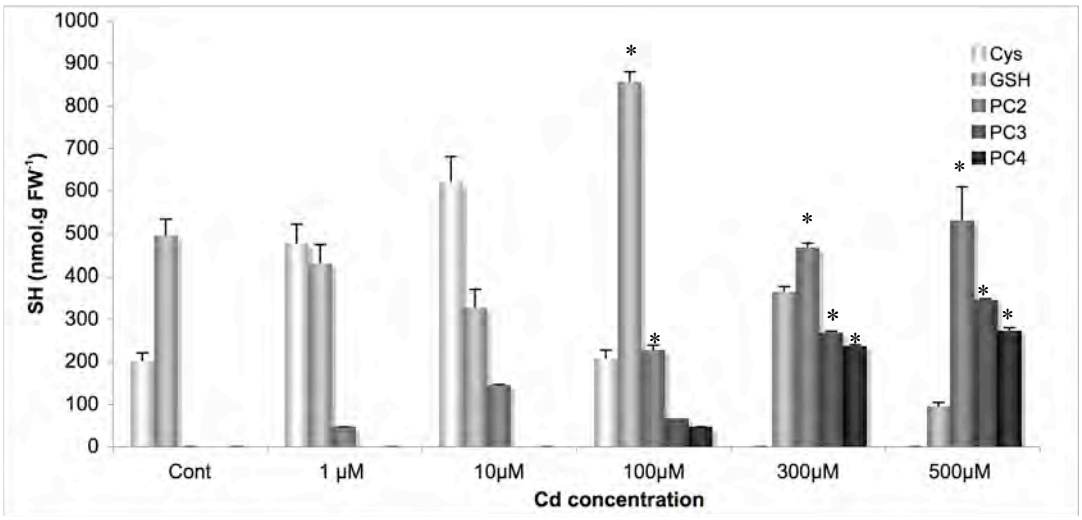


Fig.3.2.3. Thiol production (Cys, GSH, PC_2, PC_3 and PC_4) in *Arabidopsis thaliana* cells exposed to 1 µM, 10 µM, 100 µM, 300 µM and 500 µM Cd. Values are an average of three replicate observations ±SD. Values significantly different from controls are marked with * ($P<0.05$).

No PCs were detected in controls, but after 1 hour of exposure to 1 µM Cd, a small amount of the dimeric form (PC_2) was already synthesized. With 10 µM Cd, PC_2 was also detected and in all concentrations from 100 µM Cd, PCs ranging from two to four olygomeric repeats were detected. Interestingly, in 100 µM Cd, GSH was higher than in all the other concentrations, even comparing to controls. This was also the lower Cd concentration that induced all the olygomeric repeats after 1 hour exposure. When analyzing the relation between the chain length and the Cd exposures, although PC_2 was always present in higher amounts than the other PCs, there was a consistent increase in PC_2, PC_3 and PC_4 with higher Cd concentrations. Reversely, both Cys and GSH were highly reduced at 300 and 500 µM Cd concentrations.

3.2.3 **Timeline of PC synthesis**

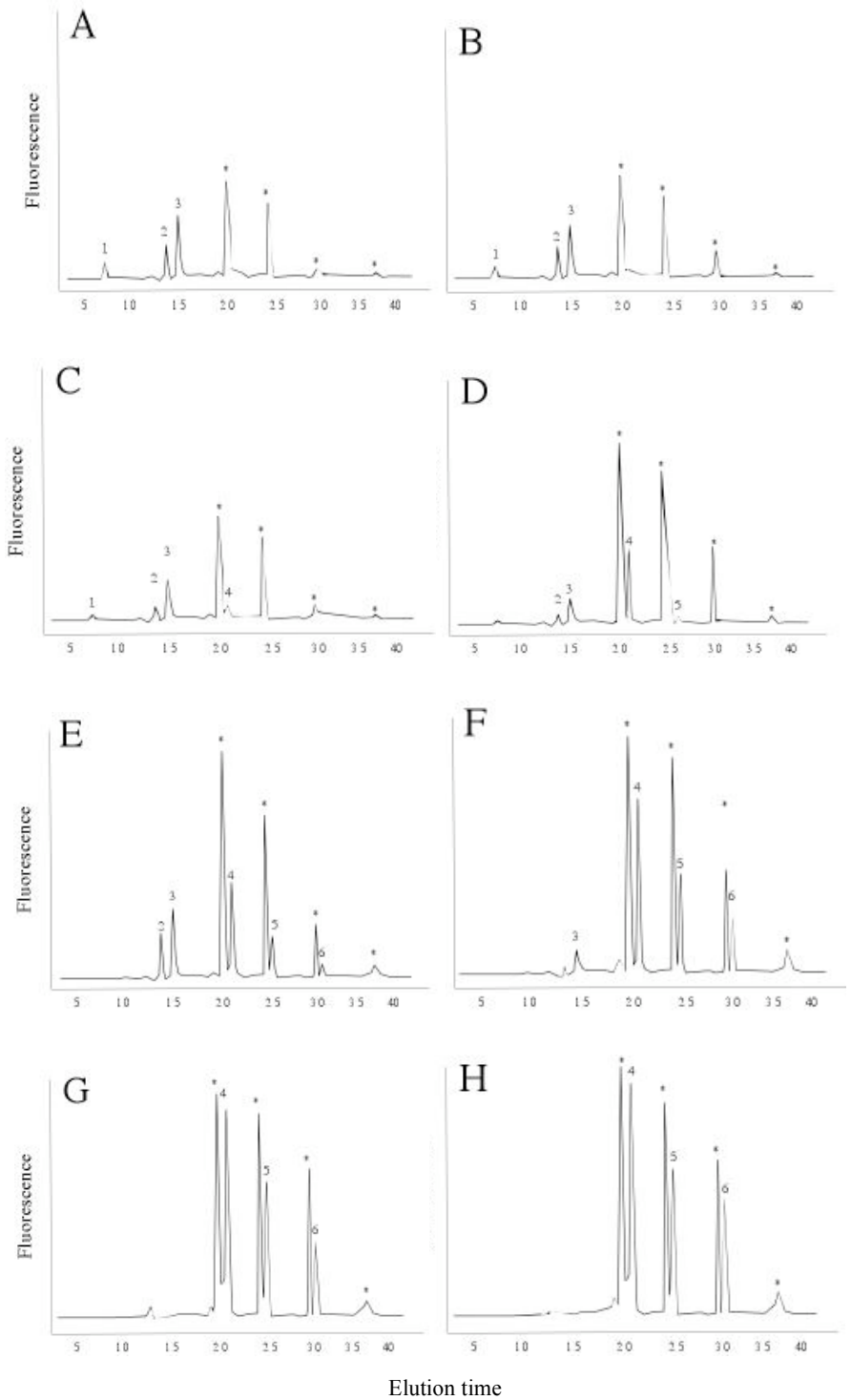


Fig 3.2.4. HPLC profiles of the time-course monitorizations of PC induction in *A. thaliana* cells after exposure to 100μM Cd. A: 0min; B: 5min; C:15min; D: 30 min; E: 1hr; F: 12hr, G: 1d and H: 2 d. Residence times and peak identification were as follows: 1: Cys (11 min); 2: γ-EC (12 min); 3: GSH (15 min); 4: PC_2 (21 min); 5: PC_3 (31 min); 6: PC_4 (39 min). Derivatization peaks are marked with *.

At the light of the previous results, the exposure to 100 μM Cd was chosen for the rest of the experiments. Fig. 3.2.4 shows the HPLC profiles with

the time alterations experienced in the different thiols (Cys, GSH, PC_2, PC_3 and PC_4) through a time-course exposure of 2 days. PCs were already detected after 15 min of exposure to Cd. With increasing time of exposure, the concentration of constitutive monothiols GSH and Cys declined, with the concomitant increase in PC accumulation. After 1 hour of exposure, all four types of oligomeric repeats were found (n=2 to 4), which were strongly enhanced until 2 days of exposure.

Fig. 3.2.5 shows the dynamics of GSH and Cys concentrations as well as the increase in PC concentration, quantified through the different times of exposure. After 15 min a small amount of PC_2 was already detected and with time, increasingly higher PC amounts were accumulated in cells, first through an increment in PC_2 and later on in PC_3 and PC_4. Interestingly, PC_3 and PC_4 appeared at the same time and only after 1hour of exposure. In fact, if we monitor the PC increment and the GSH and Cys alterations, it is easily observed that most of the major alterations occurred during the first hour of exposure.

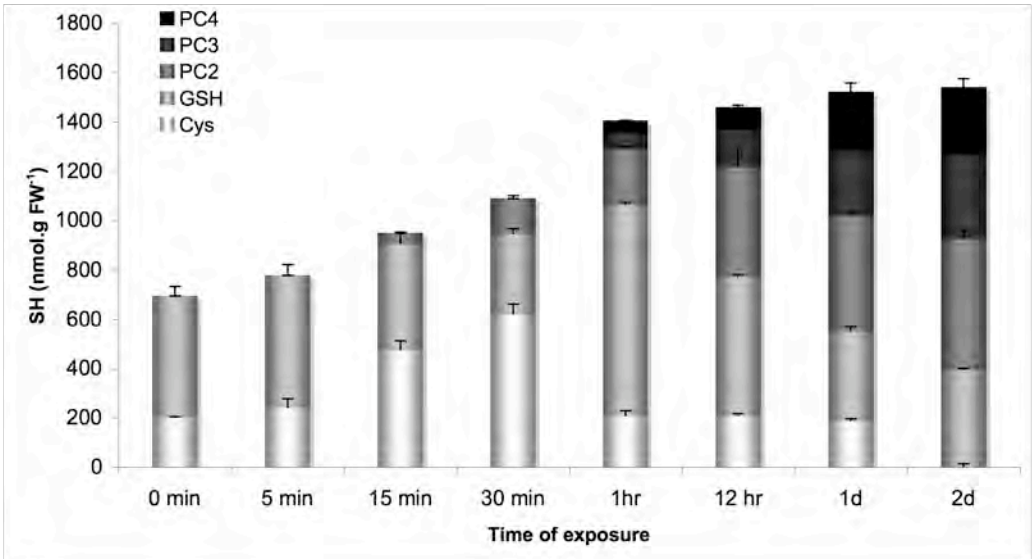


Fig. 3.2.5. Thiol (Cys, GSH and PC_2, PC_3 and PC_4) amounts produced during a two-day time-course exposure of *A. thaliana* cells to 100 μM Cd. Values are means of 3 to 5 replicates ±SE.

Fig.3.2.6 shows the time-dependence and relation between the constitutive thiols (Cys and GSH) and the synthesized polythiols (total PCs) throughout the time of exposure. Results show that until 30 min of Cd

exposure, important changes occurred, namely a Cys increase at the same time that GSH became significantly depleted (P<0.001).

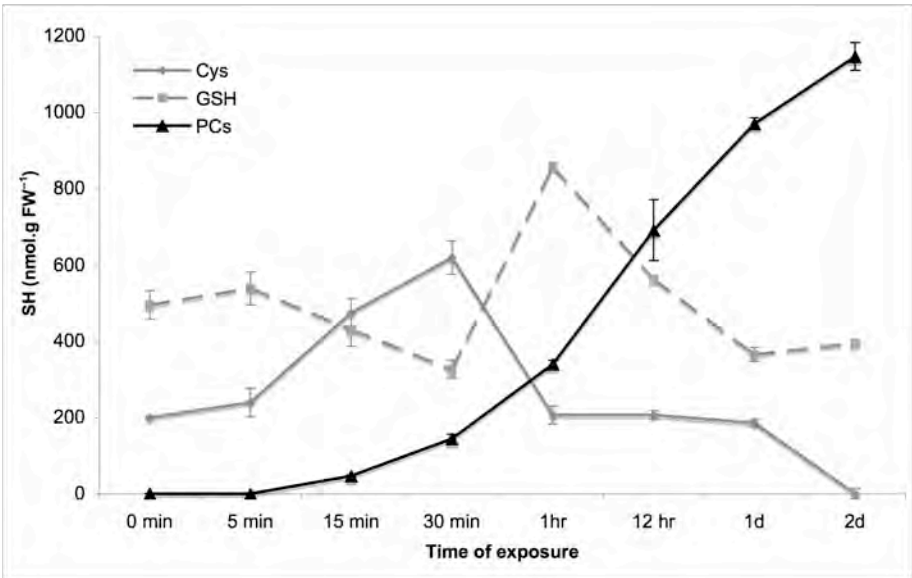


Fig. 3.2.6. Thiol (Cys, GSH and total PC) amounts produced during the time-course exposure of *A. thaliana* cells to 100 μM Cd. Values are means of 3 to 5 replicates ±SD.

This GSH reduction matched the first appearance of PCs in the cell (15 minutes). However, after 30 minutes of exposure, Cys levels began to decrease while an increase in GSH was observed, reaching higher levels than those obtained at 0 min of Cd treatments. This trend was reversed after 1 hour, as the PCs begin to be accumulated and GSH was reduced and stabilized from 1 day to the end of the exposure.

3.2.4 Timeline of Cd absorption and Cd chelation

The rate of intracellular Cd accumulation was analyzed during 1-day exposure to 100 μM Cd, in order to understand its relation to the timing of PC synthesis and is demonstrated in Fig. 3.2.7. During the time of exposure, Cd content was significantly increased (P<0.05), reaching consistently higher values throughout time. However, the rate of Cd absorption per minute was not linear, being higher until 1 hour of Cd exposure and decreasing steadily until 12 hours, after which stabilized until the end of exposure (1 day).

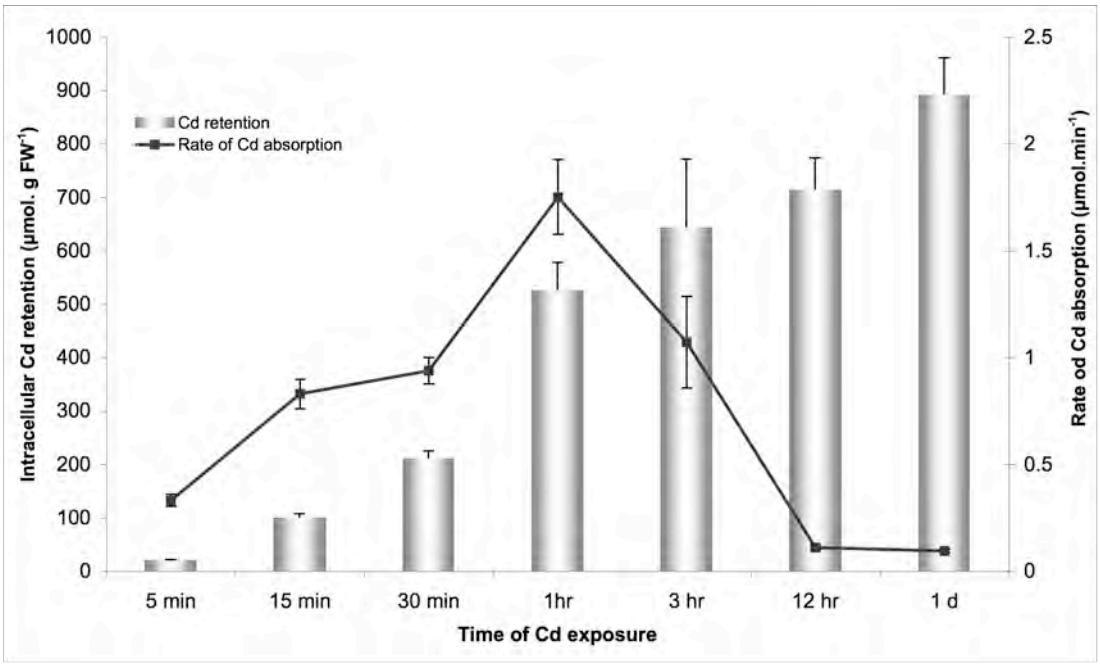


Fig. 3.2.7. Time-course intracellular Cd accumulation and rate of metal absorption throughout time in *A. thaliana* cells during 1-day exposure to 100 µM Cd. Values are the means of 3 to 5 replicates ±SE.

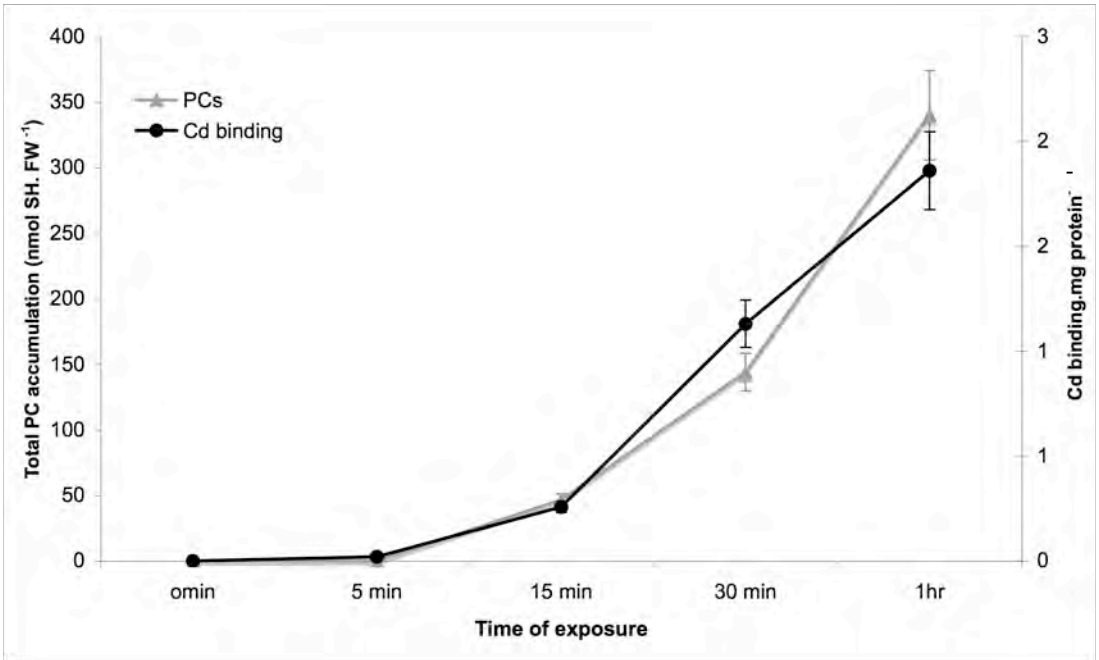


Fig. 3.2.8. Alterations in Cd binding and PC and GSH accumulation in *A. thaliana* cells during 1hour of exposure to 100 µM Cd. Values are means of 3 to 5 replicates ±SE.

The Cd-peptide binding was also monitored throughout the time of exposure, in order to understand its relation with the timing of PC synthesis. Fig. 3.2.8 shows the increase in Cd chelation throughout the first hour of metal exposure, as well as the dynamics of PC accumulation. Results show

that the Cd-peptide bond is significantly increased after 15 min, ($P<0.05$) increasing linearly until 1 hour of exposure, which is consistent with the rate of Cd absorption (Fig.3.2.7) and similar to PC synthesis (Fig.2.3.8).

3.2.5.Kinetics of GSH and PC synthesis

Time-course influence of Cd on γ-ECS and GSHS activities

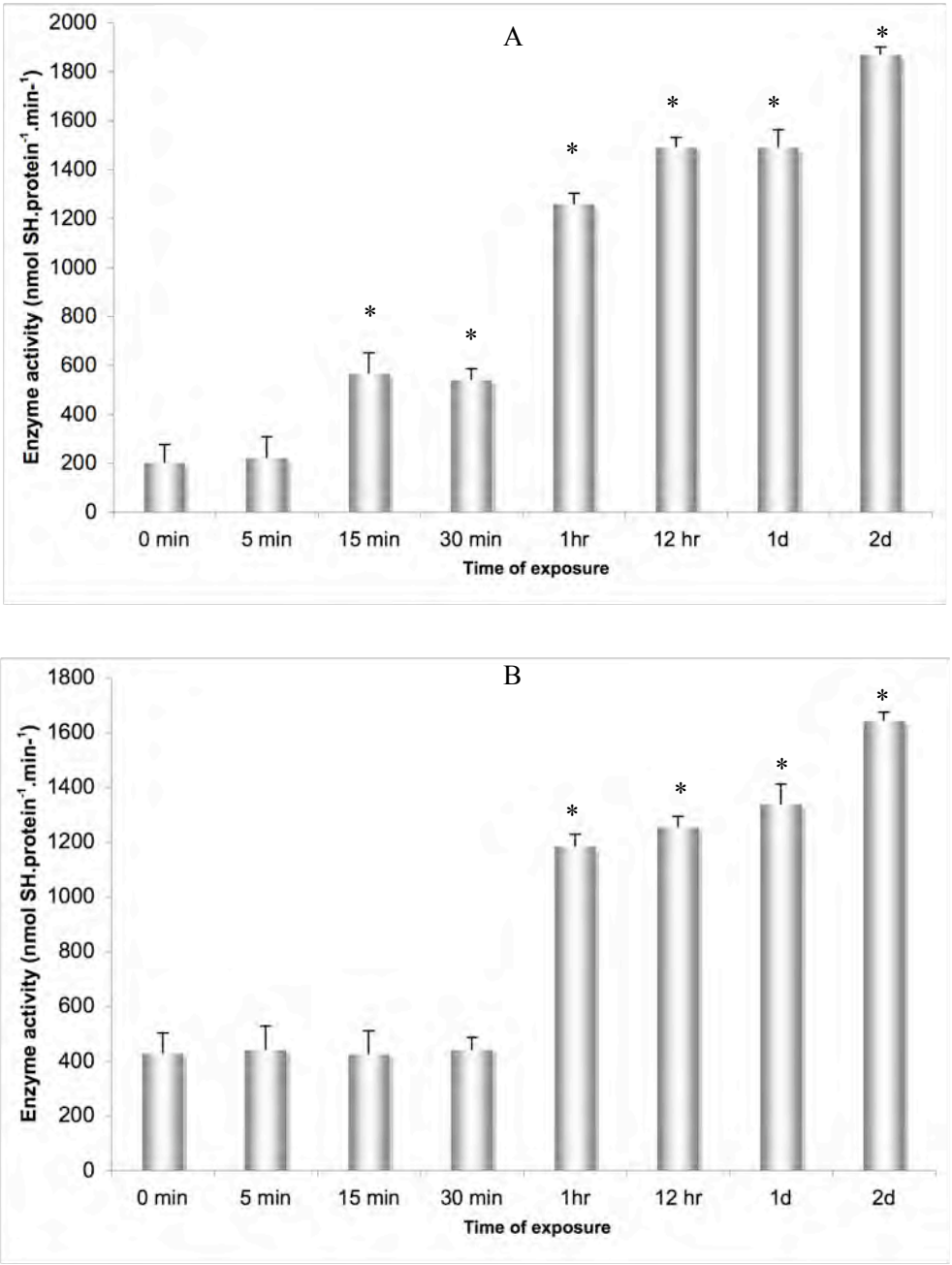


Fig. 3.2.9. Time-course activity of the enzymes γ-ECS (A) and GSHS (B) in *A. thaliana* cells exposed to 100μM Cd during 2 days. Values are the means of at least three replicate observations ±SE. Values significantly different from controls are marked with * ($P<0.05$).

The kinetics of γ-EC and GSH synthesis was monitored throughout two days of exposure to 100 μM Cd, in order to understand their relation to Cd exposure and the timing for PC synthesis. Results are expressed in Fig. 3.2.9. Throughout 1 hour of exposure to Cd, the enzymes involved in GSH synthesis behaved differently. γ-ECS activity was significantly enhanced after 15 minutes of Cd exposure ($P<0.05$), strongly increasing until 1 hour and maintained fairly constant high levels until the end of exposure ($P<0.001$). On the other hand, GSHS activity remained unaltered during the first 30 minutes ($P>0.05$) but was highly increased at 1 hour, and also remained constant high rates until the end of exposure (2 days).

Time-course influence of Cd on PCS activity

Fig. 3.2.10 demonstrates the activity of PCS throughout a 2-day exposure period to 100 μM Cd. PCS activity was not detected at 5 minutes exposure, but was rapidly increased from thereafter, at a higher rate, during the first day of exposure. The timing of PCS activity did not overlap with the timing for Cd absorption in cells (at 5 minutes), but matched the timing for the early Cd binding (15 minutes) and initial intracellular GSH decrease (also 15 minutes).

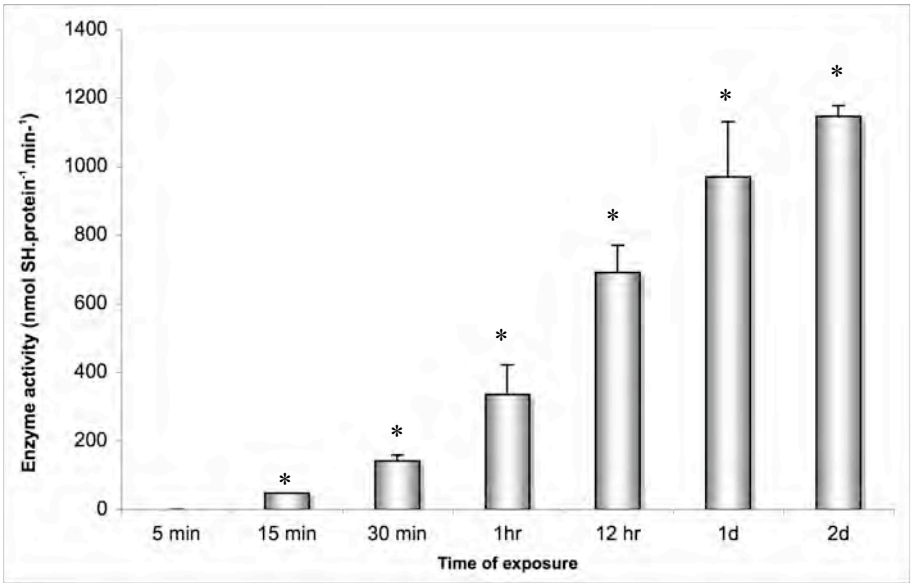


Fig. 3.2.10. PCS activity in *A. thaliana* cells exposed to 100 μM Cd during a 2-days period. Values are the means of at least three replicate observations ±SE. Values significantly different from controls are marked with * ($P<0.05$).

3.3 Discussion

3.3.1. The relation between thiol production and Cd exposure

Several reports have already described that increasing Cd concentrations induce significant growth inhibitions in several plant species, when exposed to increasing metal concentrations (Lozano-Rodriguez et al., 1997; Sandalio et al., 2001; Piechalak et al., 2002). Our data demonstrate that higher metal concentrations yielded a linear and significant increase in total PC content. The aim of this chapter was to identify a specific concentration and time of exposure that would allow a fast and complete synthesis of all types of PCs, but without exerting noticeable deleterious effects on cell viability. The first Cd concentrations of 1 and 10 μM Cd were not high enough to induce all types of PC chains, even after three hours of exposure, whereas the two higher exposures (100, 300 and 500 μM Cd) induced PCs with 2 to 4 olygomeric repeats. The fact that higher metal concentrations are better inducers of longer chained PCs has already been demonstrated by Matsumoto et al. (2004). These authors reported that when the Cd concentrations were adjusted from 0–0.75 mM Cd, the length of synthetic PCs became longer in *Escherichya coli* and *Saccharomyces cerevisiae* expressing AtPCS1. These results support the idea that longer PCs are related to higher tolerance. However, the higher exposures also induced a strong decrease in cell viability. They also induced a pronounced depletion of GSH in the cell, suggesting that the high amount of PCs was formed at the expense of cellular redox homeostasis. These responses substantiate the hypothesis that a high degree of PC synthesis can be detrimental for the cells. Other works have showed that an overexpression of the *Arabidopsis* gene AtPCS1 (Lee et al., 2003**)** induced an increased hypersensitivity to Cd, due to a GSH reduction caused by PC synthesis. In Pomponi et al. (2006), the addition of external glutathione had a positive impact on both tolerance and accumulation of Cd in transgenic tobacco overexpressing AtPCS, supporting the above conclusion. This fact can suggest that the signaling pathways that induce PCS activation are different or at least faster than those inducing the GSH

biosynthesis, and that PC activation is more related to the intracellular metal concentration than GSH producing enzymes.

Because one goal of this chapter was to identify a concentration and a time of exposure that would allow the monitorization of PCS catalytic activation and subsequent PC formation, the concentration of 100 μM Cd for 1 hour of exposure was chosen for the rest of the experiments since this conentration induced all PCs with n ranging from 2 to 4, without any pernicious effects on GSH levels.

3.3.2. The activation of PC formation

Results presented here demonstrate that quantifiable PC production began at around 15 minutes of exposure to 100 μM Cd, 10 minutes after intracellular Cd levels were detected. This rapid response indicates the efficiency of the Cd detection and PCS activation and points the importance of analyzing the dynamics of signal pathway mediators during these early minutes. Interestingly, Cd binding was only observable after 15 min, the timing of the first PCs formed, suggesting that most Cd began to be sequestrated immediately after PC synthesis. Previous reports have shown the quantitative importance of PCs as a metal tolerance mechanism in several plant species, demonstrating that it accounted for 70 to 85% of intracellular bound Cd (Rauser, 2000; Sousa and Rauser, 2004; Lima et al, 2006).

In the present work, the first type of PC chains to be detected were PC_2. Since PC synthase uses GSH or PC_n as substrate, it is expected that the first products would be PC_2 and only later PC_3 and PC_4. However, only after 1 hr of exposure all PCs with three and four olygomeric repeats were observed. These results seem to indicate that during the initial stages of metal chelation, PC synthase does not produce PCs longer than PC_2, hence, during the first initial metal chelation would be a function of PC_2 and GSH alone. Furthermore, since PC_2 are always in higher levels than the other olygomeric repeats, it seems likely that PCS has a higher affinity for GSH when compared

to PC_n. If so, then the concentration levels of GSH in the cell can be an important regulator of PCS.

3.3.3. The dynamics of constitutive thiol levels and PC formation

The time course determination of the thiol concentrations in the cell, namely the constitutive monothiols Cys and GSH and the synthesized PCs provided important clues for the relation between the constitutive monothiols and PCs, as well as their timing. The time course analysis of Cys, GSH and PC levels (Fig. 3.2.6), showed that most of the major alterations occur during the first hour. After the first timing of PC synthesis (15 minutes), the levels of monothiols suffered pronounced modifications. First, the reduction in GSH levels, at around 30 minutes probably corresponds to the first initial GSH depletion to form the first PC_2 peptides, which were also enhanced at this point. Several authors describe that GSH synthesis is increased after its reduction in the cytoplasm for the PC synthesis (Zenk, 1996; Rauser, 2000; Cobbett, 2000). Results suggest that at 30 minutes, the reduction in GSH used for PC_2 formation triggered the GSH biosynthetic pathway, which resulted in a marked increase in GSH concentrations, observed at 1 hour. On the other hand, Cys only began to be consumed for GSH synthesis after this point (1 hour of exposure), in opposite trends to the GSH increase and with the increment of PC_2, PC_3 and PC_4. After 1 hour, the classical increase in PC_2, followed by PC_3 and PC_4 occurs, with the GSH depletion. Taken altogether these results suggest that most of the PC induction pathway is triggered until the first hour of exposure and that it involves a dynamic modulation of the constitutive monothiols, especially GSH. Firstly GSH is consumed for PC synthesis, and the first PC_2 appear. This will induce an increase in GSH synthesis, which will in turn increase Cys consumption.

3.3.4. Cd-induced regulation of enzymatic activities

Mendoza-Cózatl and Moreno-Sánchez (2006) have proposed that PC synthesis is regulated at multiple levels after exposure to Cd. According to these authors, Cd induces the synthesis of PCs from GSH by activating PC

synthase and promotes the synthesis of GSH not only through transcriptional activation of the GSH biosynthetic pathway but also by stimulating the endogenous generation of ROS such as H_2O_2. Under this context, the activities of the enzymes involved in GSH and PC synthesis, γ-ECS, GSHS and PCS respectively, were monitored throughout the initial hour of exposure to Cd, up to 2 days of Cd treatment. Results clearly show that most of these enzymes are highly influenced by Cd, which is consistent with previous reports. Both γ-ECS and GSHS were highly increased after 1 hour of exposure, which coincides with the time that GSH began to be reduced. This suggests that the reduction on GSH levels for PC synthesis could constitute a signal that effectively triggers the activation of both enzymes. In *B. juncea* over-expression of either GSHS or γ-ECS during Cd exposure increased the Cd resistance and accumulation capacity (Zhu et al., 1999) and in *Arabidopsis* increased GSH production by over-expression of γ-ECS also improved arsenic tolerance (Dhankher et al., 2002), which substantiates this hypothesis. However, in this chapter, γ-ECS activity was enhanced after 15 minutes of exposure to Cd while GSHS was not. This increase in enzymatic activity matched the timing of the increase in Cd binding, as well as PC production and suggests that γ-ECS could also be regulated by the presence of Cd in the cell or by earlier events that precede GSH depletion. According to Mohanpuria et al. (2007), γ-ECS could be induced by the oxidative status of GSH. If so, the binding of Cd to the reduced forms of GSH could be enough to trigger γ-ECS. These findings indicate that γ-ECS is as a more important target for earlier Cd signaling events than GSHS.

Without the presence of Cd there was no detectable PCS activity, which indicates that Cd ions are indeed important for PCS activation. When analyzing the PCS activities, results show that the rate of enzyme activity increase is higher during the first hours of exposure, which is similar to the rate of Cd absorption in the cell. The initial increase in PCS activity matches the timing of the increase in Cd binding to SH and is consistent with the

appearance of PC_2 in the cells (15 minutes). During the rest of the exposure, PCS activity was very similar to Cd concentrations in the cell.

3.4 Conclusions

Shortly, the findings presented here indicate that most of the important events on PC synthesis take place during the first 15 min and continued ongoing until 1 hour. After this all enzymatic processes are activated in order to maintain thiol equilibrium while Cd tolerance is achieved. The first moments of PCS activation seem to show a primary targeting of this enzyme, but also of the γ-ECS enzyme. In fact, these two enzymes seem to be the main targets for PC production and also seem to be dependent on Cd accumulation in cells. Therefore, the first initial triggering of the PC-based mechanism should evolve in the following manner:

1. At around 5 min: first binding of Cd to GSH
2. Between 5 and 15 minutes PCS has becomes active and PC_2 is produced, with concomitant decrease in GSH
3. At the same time, γ-ECS is activated, due to either Cd increase or to the GSH depletion/oxidation
4. At 30 minutes GSH is reduced which will trigger γ-ECS and GSHS activities
5. At 1 hour of exposure, longer chained PCs have been formed, and GSH synthesis is enhanced
6. More hours of exposure (12 hours, 1 and 2 days) show the attainance of equilibrium, between the three enzymes activity, and while GSH and Cys were depleted, PCs continued increasing.

Considering results altogether, it seems plausible to conclude that the first hour of exposure to 100 μM Cd is sufficient for triggering and

sustaining the PC mechanism. Essential timings were 5 min, 15 min, 30 min and 1hr of exposure, since they showed key alterations in the enzymes involved in the PC production, but also in their respective products. Important targets for Cd signaling were found to be, γ-ECS , PCS and GSH levels.

Chapter 4

Calcium signalling and PC synthesis

4.1 Introduction

4.1.1 Calcium signaling in abiotic stresses

Calcium (Ca) is one of the most ubiquitous second messengers among eukariots and the modulation of its intracellular levels provides a generic signal for almost every aspect of the plant life cycle, namely growth, development, reproduction, circadian rhythms, immunity, redox status, hormone biosynthesis, early signaling events, and also in responses to biotic and abiotic stresses (Bush, 1995; Clark et al., 2001; Luan et al., 2002 Hong-Bo et al., 2008). Several authors suggest that higher stress tolerance efficiency is related to the signal perception and transduction by calcium and calcium sensors (Kudla et al., 2001; Luan, 2004; Kaur and Gupta, 2005; Hong-Bo et al., 2008**)**. If so, a thorough analysis of the calcium signalling networks during stress acclimation could help us understand the signal mechanisms leading to stress tolerance in environmental conditions.

Stress-induced changes in the cytosolic concentration of Ca occur as a result of influx of Ca^{2+} from outside the cell, or release of Ca from intracellular stores (Kaur and Gupta, 2005; Hong-Bo et al., 2008). These calcium alterations will then target specific proteins that act as calcium sensors and will carry on the signal to other molecules, such as enzymes or transcription factors, which are the tolerance response *per se* (Luan, 2004; Hedrich and Kudla, 2006). In this way, calcium serves as an important second messenger during abiotic stresses and is a major point of signalling and cross-talk, because it can be elicited by numerous stress cues, being

particularly important in osmotic, heat, salt and water stress (Kudla et al., 2001; Cheong et al., 2003; Luan, 2004; Rentel and Knight, 2004; Kaur and Gupta, 2005; Hong-Bo et al., 2008**)**. In the last few years, calcium signaling has been better elucidated by the observation of calcium oscillations across the cell, referred as the calcium signatures (Clark et al., 2001; Luan et al., 2002). According to some authors, each signature represents a cellular expression that confers specificity to calcium signals. In order to fully understand the Ca signaling pathways, we must understand the "combination code" that consists of calcium oscillations, calcium sensors and downstream target proteins (Clark et al., 2001). Until recently, little was known about the *in vivo* targets and the downstream outputs of salt-stress signalling pathways, but some calcium sensor proteins have now been identified and are well characterised in plants. Some of them are well conserved proteins, known in animal tissues, other are novel calcium sensors, that only exist on the plant kingdom (Kudla et al., 1999; Kim et al., 2000; Luan et al., 2002; Luan, 2004).

4.1.2 Calcium signals and calcium sensors in plants

Ca signaling pathways comprise several types of Ca sensors that monitor temporal and spatial changes in Ca concentrations (Luan et al., 2002). Several families of Ca sensors have been identified in higher plants. The best known is calmodulin (CaM) and CaM-related proteins, which typically contain (EF)–hand domains for Ca binding (Zielinski, 1998**;** Snedden and Fromm, 2001). (EF)-hand domains are composed of two pairs of Ca binding sites joined by a linker domain. A binding site is composed of two helices, called the E and F helices, flanking a Ca-binding loop in a structure that somewhat resembles a hand – hence the use of the term 'EF hand' for the Ca binding motif (Fig. 4.1.1).

A second family of Ca sensors from *Arabidopsis* consists of proteins similar to both the regulatory B-subunit of calcineurin (PP2B) and the neuronal Ca sensor in animals (Liu and Zhu, 1998; Kudla et al., 1999). These Ca sensors are referred to as as calcineurin B–like (CBL) proteins (Kudla et al.,

1999). A third major class of calcium sensors are the Ca-dependent protein kinases (CDPKs), which contain CaM-like Ca binding domains and a kinase domain (Roberts and Harmon, 1992**;** Harmon et al., 2000).

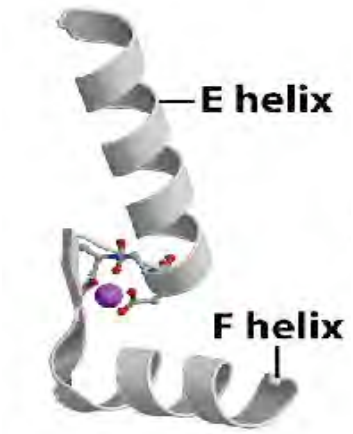


Fig. 4.1.1: Schematic representation of the EF-hand motif

Both CaM and CBL are small proteins that contain multiple Ca binding domains but lack other effector domains, such as the kinase domain in CDPKs. Their role relies on their ability to bind Ca selectively, with micromolar affinity. This binding makes the protein go through a conformational change, that allows CaM and CaM-related proteins to interact with several target proteins and therefore modulate their activity (Chin et al., 2000; Snedden and Fromm, 2001). Some CaM target proteins have been identified in higher plants and include protein kinases, metabolic enzymes, cytoskeleton-associated proteins, and others (Reddy et al., 1996**,** 2002; Snedden et al., 1996; Zielinski, 1998; Snedden and Fromm, 2001). In this way, cytosolic Ca changes perceived by calcium sensors are transduced into altered target activity and, hence, inducing a cellular response.

4.1.4 CBLs: plant exclusive stress-induced calcium sensors

CBLs are encoded by a multigene family of at least 10 members in *Arabidopsis* that have similar structural domains with small variations in the length of the coding regions (Kudla et al., 1999; Kim et al., 2000; Albrecht et al., 2001). Unlike CaM genes, CBLs are only identified in higher plants,

suggesting that CBLs may function in plant-specific signaling processes. As in CaM, CBLs are characterized by a common helix-loop-helix structural motif (the EF hand) that acts as the Ca binding site. Comparing CaM with CBL proteins, the two families do not show significant similarity in their primary amino acid sequences, except for the conserved positions in the EF-hand motifs, however CaMs contain four EF hands whilst CBLs contain three.

Since their discovery (Kudla et al., 1999), CBL expression and subcellular localization were found to be regulated by multiple signals, including light, mechanical stress, heat/cold shock, wounding, osmotic stress, pathogens, and plant hormones (Kudla et al., 1999; Nozawa et al., 2001; Mahajan et al., 2006**)**. Different members of the *Arabidopsis* CBL gene family also show specific expression patterns. Although there is high identity between AtCBLs, different members behave differently in response to various stresses, providing evidence that different AtCBL members perform different functions under various stress conditions. For example AtCBL1 expression was reported to be induced by cold, salt, wounding, and drought, whereas AtCBL9 expression did not respond to these stimuli (Kudla et al., 1999; Kolukisaoglu et al., 2004). Both CBL1 and CBL2 respond to light, but CBL2 lacks the other responses of CBL1 (Nozawa et al., 2001). Furthermore, two of the AtCBLs, AtCBL2 and 3, also show constitutive expression in response to abiotic stresses (Kudla et al., 1999) and PsCBL, a CBL isolated from Pisum sativum an homologue of AtCBL3, was strongly upregulated in response to cold, salinity and wounding (Mahajan et al., 2006). Such expression patterns suggest that CBLs have overlapping and specific functions in certain signal transduction pathways.

4.1.5 Metal stress and calcium signaling

Calcium signalling has been implicated in a number of stresses response and plant acclimation, serving as an important second messenger during abiotic stresses and providing a major point of signalling cross-talk because it can be elicited by numerous stress cues. However, very few reports

focus on the relation between metal stress and Ca signals. Although there is a lack of information on Cd stress signalling and calcium, few reports have begun to show that calcium is important for Cd tolerance. Most of these studies make use of pharmacological modulators of the calcium levels in the cell. For example, Skorzynska-Polit et al. (1998) showed that a calcium deficit increased Cd toxicity, whereas excess Ca reduced its toxic effects. Also He et al (2005) showed that cadmium tolerance in *L. sativa* was enhanced when synchronously treated with lanthanum (La), a calcium channel blocker, or calcium (Ca) and the levels of the expression of phytochelatin synthase gene were altered under Cd, Cd+Ca and Cd+La treatments. These results point out to the need to further investigate the importance of Ca signaling in metal stress and PC synthesis

One other important aspect is the relation between calcium and GSH, the substrate for PC synthesis. Gomez et al. (2004) demonstrated that modulation of glutathione contents interferes with the release of calcium to the cytosol. According to these authors, feeding GSH rapidly and specifically triggers calcium release in *Nicotinana tabacum*, suggesting that the modulation of GSH levels observed upon PC synthesis (see chapter 3), could trigger specific calcium signals.

4.2. Objectives

Calcium serves as an important second messenger during abiotic stresses and is a major point of signalling crosstalk since it can be elicited by numerous stress cues. Until recently, little was known about the *in vivo* targets and the downstream outputs of Cd stress signalling pathways, but some calcium sensor proteins have been identified and are well characterised in plants. The exclusivity of some of these sensors, such as CBLs suggests their importance in plant-specific processes, such as the case of the PC-based mechanisms.

Although few reports focus the importance of calcium signalling in metal stress, some findings suggest that calcium can be important for GSH

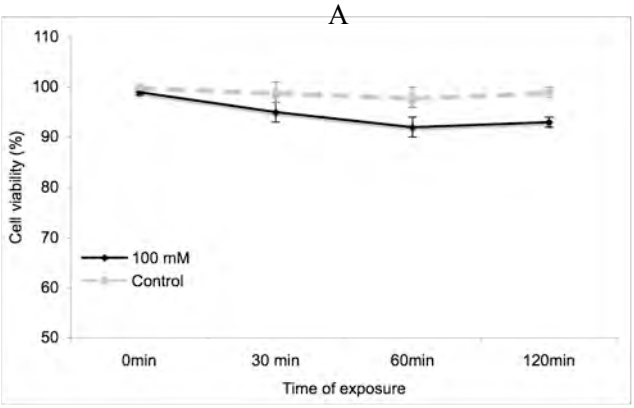
and PC synthesis. Nonetheless, the way this mechanisms is related to PC signalling pathway is not understood, nor the calcium sensors that might be associated with this pathway. Under this context, the goal of this chapter was to understand the importance of calcium levels and cellular calcium sources on the pathways that lead to Cd accumulation and PC synthesis. With this aim, several pharmacological approaches were used in order to modulate intracellular calcium levels during Cd stress. The effects of these agents on Cd tolerance and on PC and GSH synthesis were analyzed. Also, the identification of the calcium sensors such as CBLs that recognize and transmit the calcium signal, under Cd stress, was also pursued. Results should bring important insights on the role of calcium in Cd stress and on the identification of CBLs as important signal transducers in Cd stress.

4.2. Results

4.2.1 Calcium modulating assays

Calcium modulation influences Cd tolerance

A



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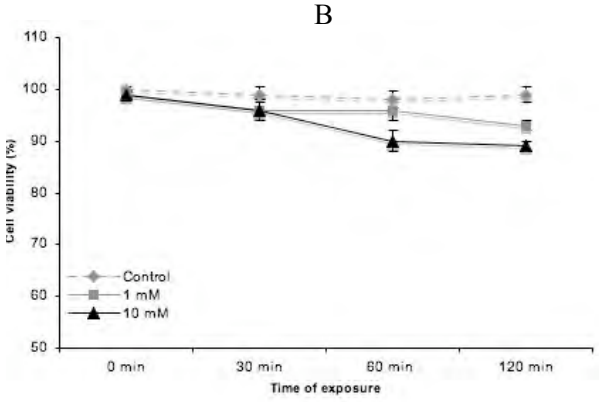


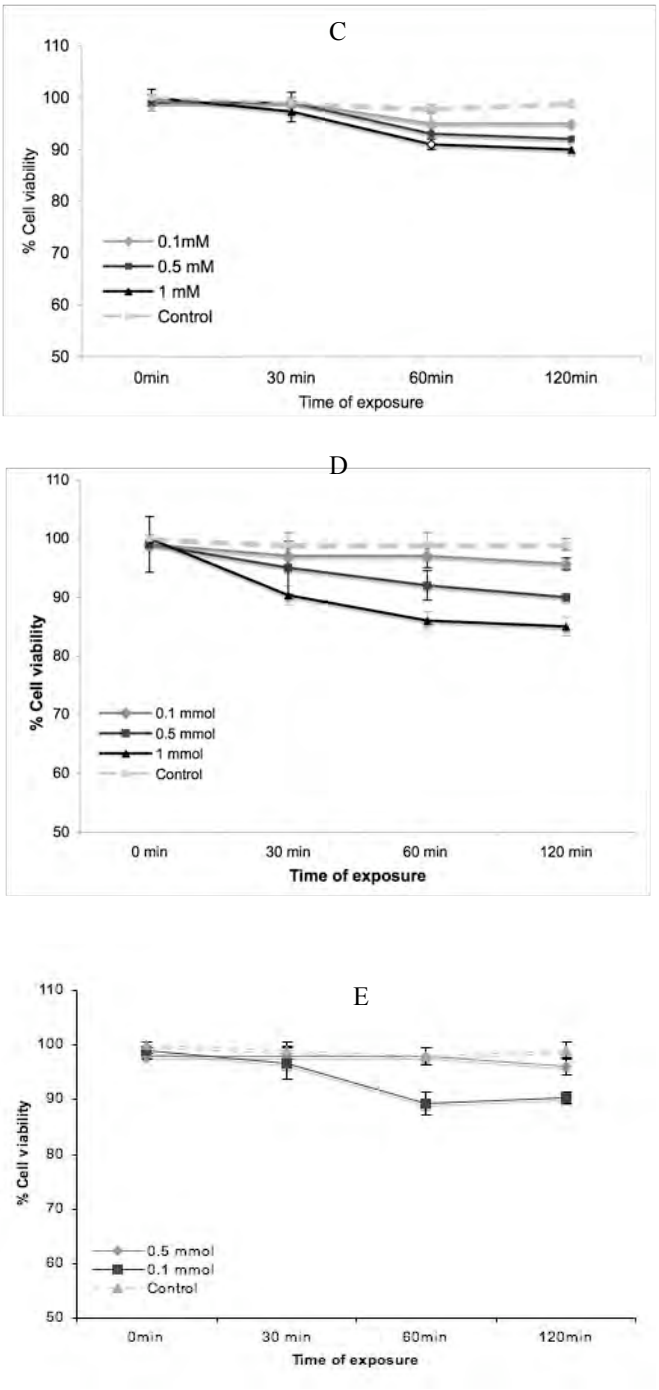
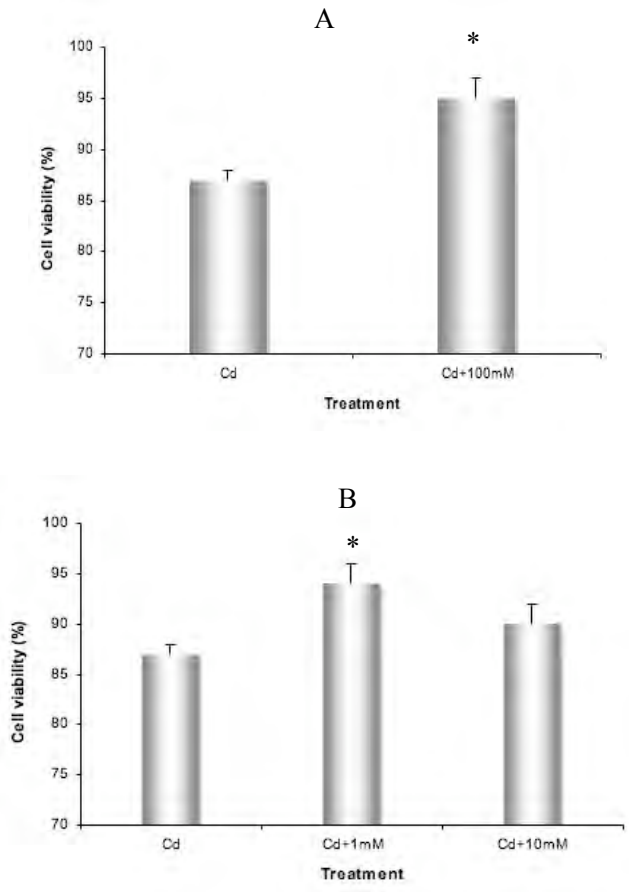
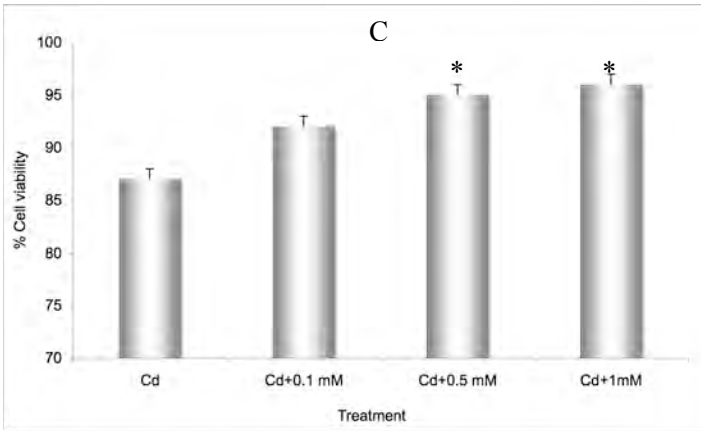


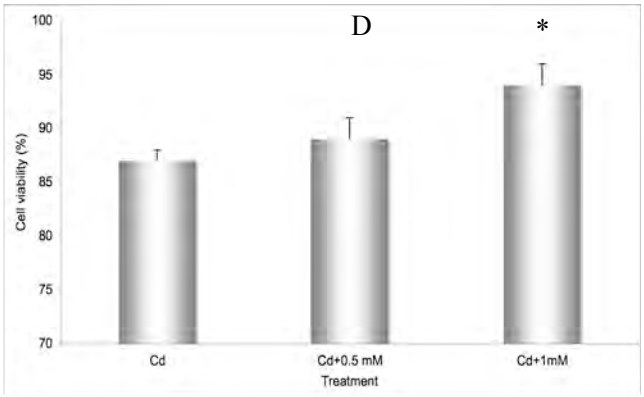
Fig. 4.2.1. The effect of different pharmacological calcium modulators: calcium (A); caffeine (B); EGTA (C); lanthanum (D); and ruthenium red (E) on *A. thaliana* cell viability, during a two-hours exposure period. Cell viability is expressed as a percentage of controls. Values are the means of at least three replicate experiments ±SE.

In order to understand the role of Ca in Cd tolerance and PC synthesis, several pharmacological Ca modulators were used, in different concentrations. In the presence or absence of Cd, cells were challenged with excess $CaCl_2$ (Ca); caffeine (Caff), an inducer of calcium release from intracellular stores; EGTA, and extracellular Ca blocker; lanthanum (La), a

calcium channel blocker; and ruthenium red (Rut Red), a known inhibitor of calcium release from intracellular stores. The effect of the addition of these modulators on cell viability is expressed in Fig. 4.2.1. For Cd tests, cells were pre-exposed to these modulators 1 hour prior to Cd treatments (of also1 hour). Therefore, their effect on cell viability during a two hours-exposure was assessed, under control conditions. Different concentrations of each blocker were tested in order to assure that their exposure during the Cd test would not interfere with the cellular viability during the two hours of the experiment. Results show that some calcium modulators were more toxic than others, however most did not exert severe viability reduction. Ca, caffeine and EGTA, did not alter significantly cell viability (P>0.05), but such was not the case for lanthanum and ruthenium red. In the absence of Cd, these two Ca blockers caused a significant reduction on cell viability, (P>0.05), particularly lanthanum. Nonetheless, none of the concentrations reduced cell viability bellow 85% of controls.







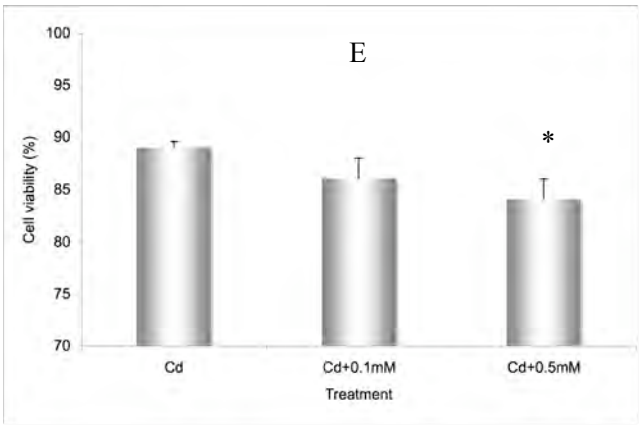


Fig. 4.2.2. The effect of different pharmacological calcium modulators: calcium (A); caffeine (B); EGTA (C); lanthanum (D) and ruthenium red (E) on *A. thaliana* Cd tolerance, during a two-hours exposure period. Cell viability is expressed as a percentage of controls. Values are the means of at least three replicate experiments ±SE. Values significantly different from controls are marked with * ($P<0.05$).

The effect of calcium modulation on Cd tolerance was further analysed. Because the presence of Cd modulators in controls did not exert a high toxicity, the higher exposure concentrations for each Ca modulator were chosen. Cd-tolerance alterations in the presence of calcium inducers and calcium inhibitors on Cd tolerance are demonstrated in Fig. 4.2.2. In the

presence of Cd, the addition of Ca (Fig. 4.2.2.A) or caffeine (Fig. 4.2.2.B) significantly increased Cd tolerance, when compared to Cd treatments ($P<0.05$). The calcium blockers EGTA (Fig. 4.2.2.C) and La (Fig.4.2.2.D) induced similar responses to calcium induction, producing an increase in cell viability ($P<0.05$), when compared to Cd alone. This increment in Cd tolerance was also higher with increasing EGTA and La concentrations. In fact, in the presence of Cd+EGTA (Fig. 4.2.2.C) the percentage of viable cells was higher than in controls+EGTA (Fig. 4.2.1.C). This trend was not observed with La treatments. On the contrary, the addition of ruthenium red under Cd exposure significantly reduced cell viability ($P<0.05$) when compared to Cd control.

4.2.2. Calcium modulation influences GSH and PC synthesis

The effect of calcium modulation on the constitutive monothiols, as well as on PCs was also tested, in order to assess whether PC synthesis can be regulated by calcium modifications. Fig.4.2.3 shows the effect of Ca addition on total thiols in *A. thaliana* cells during 1 hour of Cd exposure.

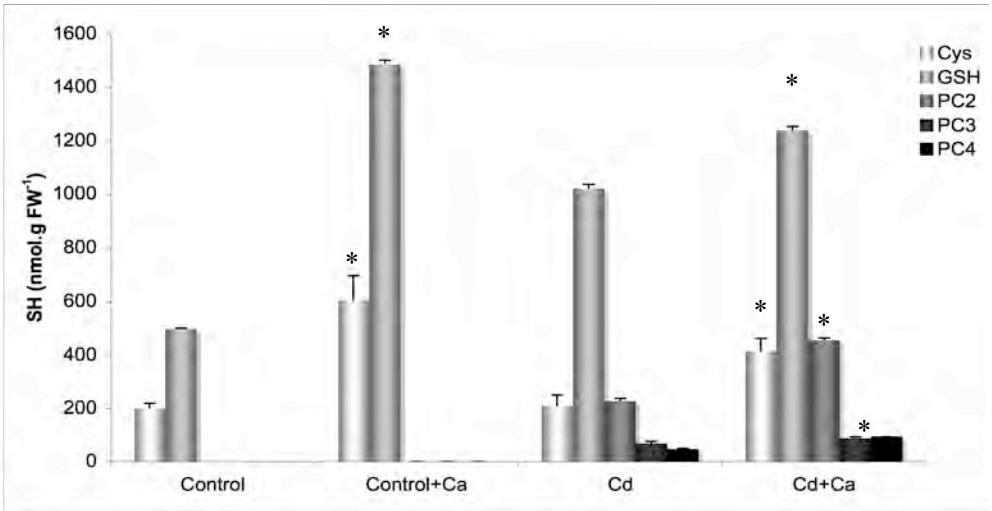


Fig. 4.2.3. The effect of 100 mM Ca addition on *A. thaliana* PC synthesis after 1hour of 100 μM Cd-exposure. Cell were incubated with Ca 1 hour before Cd exposure.Total thiol concentrations are expressed in the histogram (E). Values are the means of at least three replicate experiments ±SE. Values significantly different from controls are marked with * ($P<0.05$).

HPLC analysis demonstrates that under control conditions, Ca addition to the growth media tripled GSH production ($P<0.05$). Under Cd exposures, this

increase was also significant (P<0.05), when compared to Cd alone. Furthermore, PC accumulation was also enhanced in Ca-Cd treatments, mostly due to an increase in PC_2.

The effect of Ca release from intracellular stores, stimulated by caffeine is demonstrated on Fig. 4.2.4. The addition of caffeine induced similar effects to those observed with the addition of Ca. Without the presence of Cd, caffeine promoted a significant increase in GSH concentrations (P<0.05) nearly doubling control levels. Under Cd exposure, caffeine also induced an increase in GSH, which was even more prominent than with Cd alone. In contrast, PC levels were similar to controls.

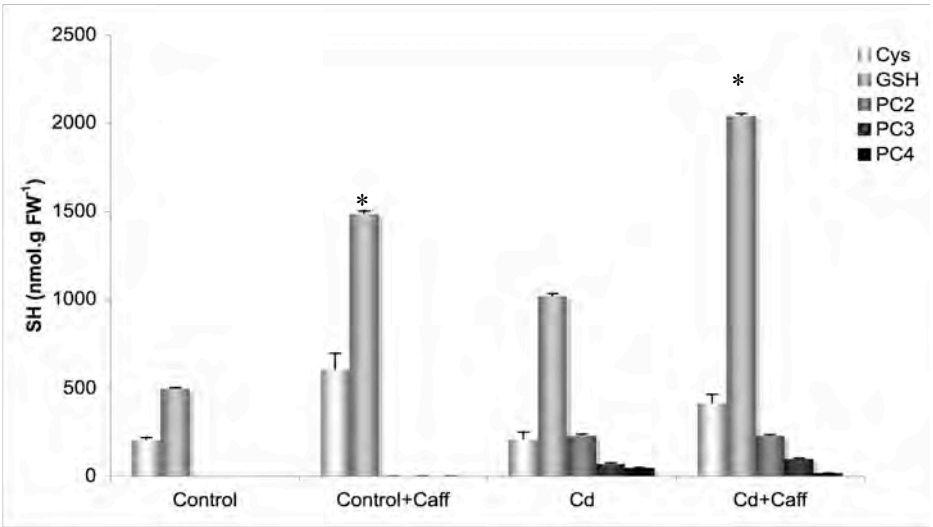


Fig. 4.2.4. The effect of 10 mM caffeine addition on *A. thaliana* PC synthesis after 1hour of 100 μM Cd-exposure. Cell were incubated with caffeine 1hour before Cd exposure. Values are the means of at least three replicate experiments ±SE. Values significantly different from controls are marked with * (P<0.05).

The effect of each Ca inhibitor on PC synthesis, as well as on the constitutive monothiols was also tested. Fig. 4.2.5 shows the levels of mono and polythiols in cells under the different EGTA exposures and Cd exposures. Because EGTA is also a known extracellular Cd chelator, the two different EGTA concentrations were used, in order to understand if the differences induced under Cd treatments were influenced by Cd chelation. Under control conditions extracellular Ca blocking induced a significant decrease in GSH content (P<0.05), which was more pronounced in the highest EGTA

concentration. Nonetheless, this chelator had no significant effect on Cys content ($P>0.05$). Under Cd exposure, EGTA also decreased GSH content ($P<0.05$) and a total lack of PC synthesis was observed. Different EGTA concentrations induced similar levels of mono and polythiol decreases, both in the presence and absence of Cd.

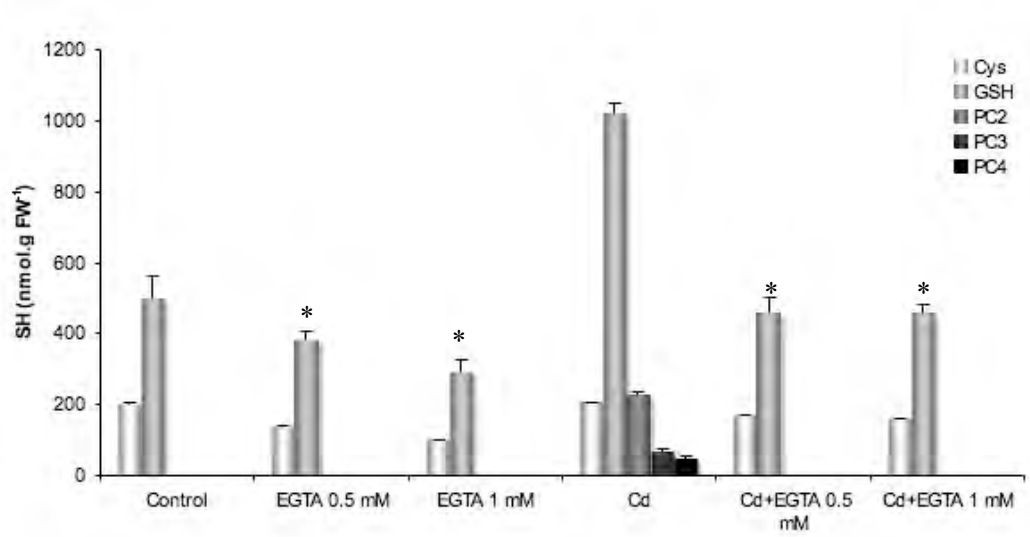


Fig. 4.2.5. The effect of different EGTA concentrations on *A. thaliana* PC synthesis after 1hour of 100 μM Cd-exposure. Cell were incubated with different EGTA concentrations 1hour before Cd exposure. Values are means of at least three replicate experiments ±SE. Values significantly different from controls are marked with * ($P<0.05$).

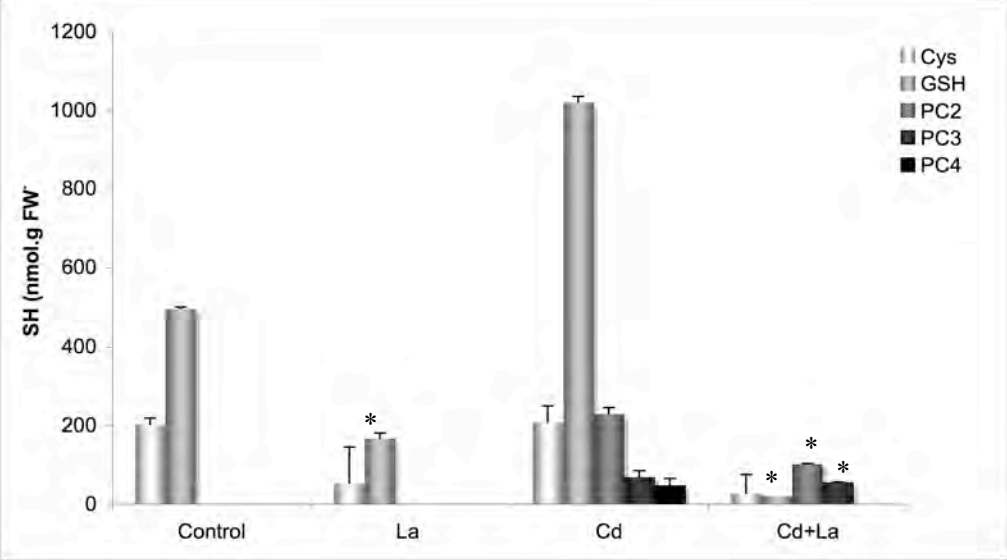


Fig. 4.2.6. The effect of LA on *A. thaliana* PC synthesis after 1hour of 100 μM Cd-exposure. Cell were incubated with 1 mM La, 1hour before Cd. Values are the means of at least three replicate experiments ±SE. Values significantly different from controls are marked with * ($P<0.05$).

Fig. 4.2.6 shows the effect that La addition (1 mM) induced on the mono and polythiols under control and Cd exposures. With La, GSH and Cys levels were significantly reduced to about half of the levels observed in controls ($P<0.05$). In the presence of Cd+La, Cys and GSH levels were even lower than controls ($P<0.05$). PC levels were also reduced and PC_4 was not detected under Cd+La exposure.

The effect of ruthenium red on PC, GSH and Cys levels was also analysed and is expressed on Fig. 4.2.7. The presence of this Ca inhibitor induced a significant decrease in GSH levels, when compared to controls ($P<0.05$). This reduction was more pronounced under the presence of Cd. However, in the presence of ruthenium red+Cd, PC synthesis was not significantly different from Cd alone.

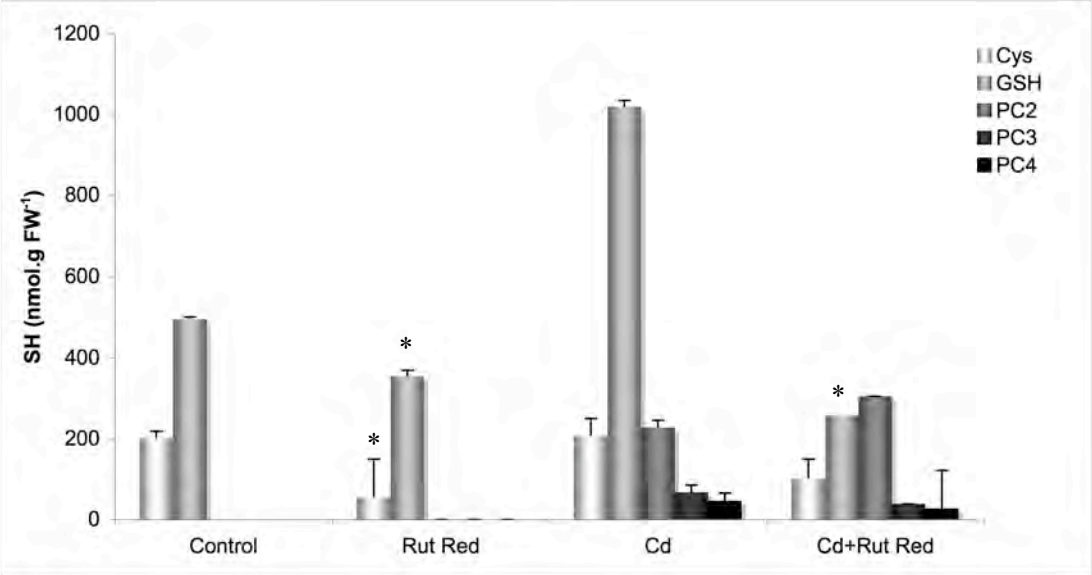


Fig. 4.2.7. The effect of ruthenium red on *A. thaliana* PC synthesis after 1hour of exposure to 100 μM Cd. Cell were incubated with 0.5 mM Rut Red, 1hour before Cd. Total thiol concentrations are expressed in the histogram (E). Values are the means of at least three replicate experiments ±SE. Values significantly different from controls are marked with * ($P<0.05$).

4.2.3. Calcium modulation affects Cd absorption

Since Ca modulation exerted a strong impact on PC and GSH synthesis, its influence on Cd absorption was also investigated. Fig. 4.2.8

shows the levels of intracellular Cd in *A. thaliana* cells after 1hr exposure to 100 μM Cd.

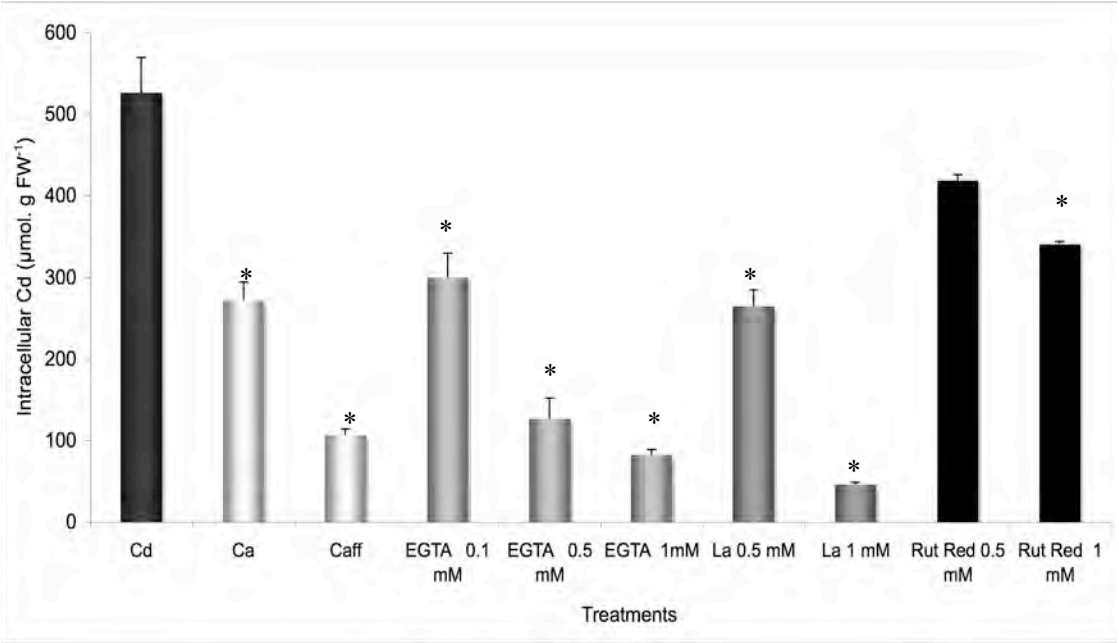


Fig. 4.2.8. The effect of of Ca and Caff, and the the Ca blockers EGTA, LA and Rut Red on *A. thaliana* Cd absorption after 1hour of 100 μM Cd-exposure. Cell were incubated with each treatment 1hour before Cd. Values are the means of at least three replicate experiments ±SE. Values significantly different from controls are marked with * ($P<0.05$).

The different calcium modulators altered very significantly the amount of Cd absorbed by cells ($P<0.001$). In Ca and Caff treatments, Cd uptake was markedly reduced ($P<0.05$), particularly with caffeine. With EGTA treatments, Cd absorption was also lower than controls, and a dose-response was observed ($P<0.05$). La treatments also induced a significant reduction in Cd absorption in cells ($P<0.05$). On the other hand, although it significantly reduced Cd absorption, the influence of ruthenium red on Cd uptake by cells was less pronounced.

4.2.4. Calcium modulation alters the enzymatic PC synthesis pathway

Due to the alterations observed in both the constitutive monothiols and the induced polythiols, the effect of the individual Ca modulations on the enzymes responsible for GSH and PC synthesis was studied, in the presence of each Ca modulator. Figure 4.2.9 shows the influence of this modulation on γ-ECS, GSHS and PCS.

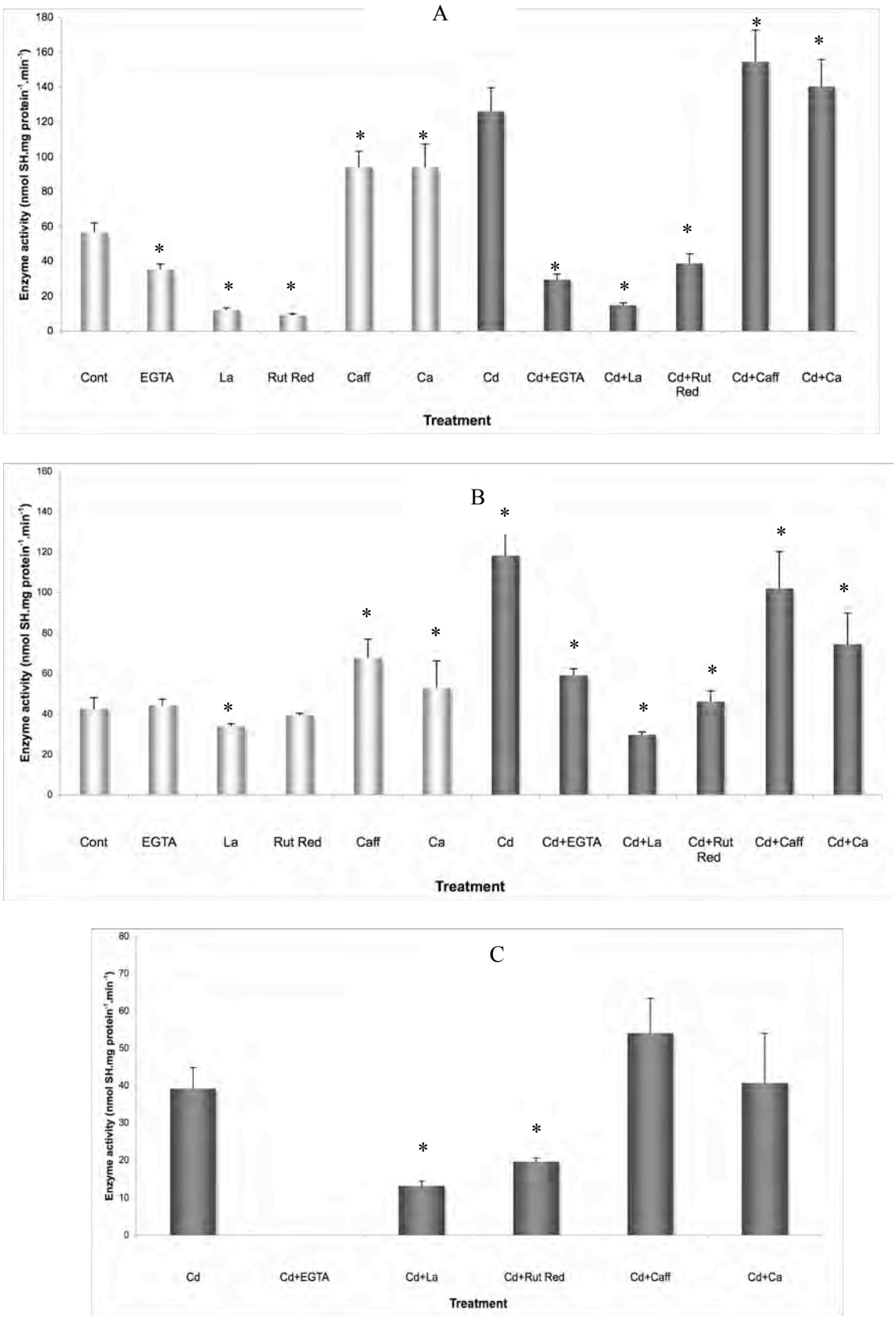


Fig. 4.2.9. The effect of calcium modulation on *A. thaliana* ECS (A), GSHS (B) and PCS (C) after 1hour of 100 μM Cd-exposure. Cells were incubated with on 100 mM Ca, 10 mM caffeine, 1 mM EGTA, 1 mM lanthanum and 1 mM ruthenium red 1hour before Cd exposure. Values are the means of at least three replicate experiments ±SE. Values significantly different from controls are marked with * ($P<0.05$).

Calcium modulations induced very significant alterations in the activities of all the enzymes analyzed, although not in the same manner. Ca and caffeine addition induced a significant increase in γ-ECS ($P<0.05$), both in control conditions as in Cd treatments. In the presence of the other Ca blockers (EGTA, lanthanum and ruthenium red), the activity of this enzyme was very significantly reduced ($P<0.05$). GSHS activities were similarly altered by Ca modulations, however, not as pronouncedly as those observed with γ-ECS. In the absence of Cd, Ca and caffeine also induced higher GSHS activities ($P<0.05$). With exception of La, Ca blockers (EGTA and ruthenium red) did not significantly reduced GSHS activities ($P>0.05$). In the presence of Cd, all Ca modulations prompted significant reductions on GSHS activity ($P<0.05$), inducing significant reductions ($P<0.05$). Concerning PCS activities, results show that the three calcium blockers significantly inhibited PCS activity ($P<0.05$), particularly EGTA, whereas Ca and Caffeine did not significantly altered PCS activity ($P>0.05$).

4.2.5. Calcium sensors are modulated by Cd exposure

The knowledge of the individual calcium binding proteins (CaBPs) that detect each specific calcium oscillation is the key to understand signal specificity. As an attempt to understand which CaBPs are involved in Cd signalling, cell calcium sensors were isolated by affinity chromatography, during a time-course Cd exposure. Fig. 4.2.10 shows the alterations on the amount of CaBPs detected on *A. thaliana* cells during cadmium exposure and after stress recovery. Shortly after Cd exposure (15 minutes), the total amount of CaBPs was significantly increased ($P<0.05$) and kept rising until 1 hour of exposure. This trend was then reversed and total CaBPs concentration have decreased significantly at 3 hours ($P<0.05$) of exposure and remained unaltered until 24 hours of Cd treatment ($P>0.05$), but still keeping higher levels than controls. After stress recovery, CaBPs reached values similar to those of controls.

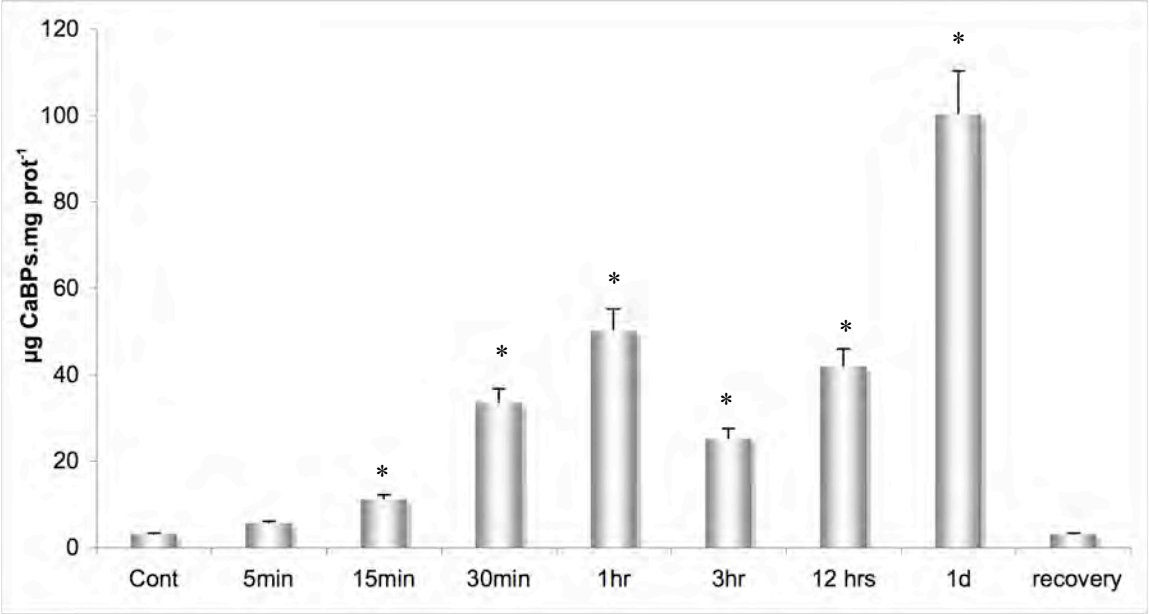


Fig. 4.2.10. Cadmium-induced alterations on the amount of calcium-binding proteins in *A. thaliana* cells, throughout a 1day-exposure to 100 µM Cd and after 3 days of recovery. Values are the means of at least three replicate experiments ±SE. Values significantly different from controls are marked with * (P<0.05).

The CaBPs eluted from the chromatography column were further analyzed by SDS-PAGE electrophoresis, as shown in Fig. 4.2.11. Specific alterations occurred after Cd exposure, both in a quantitative and a qualitative manner. Several specific calcium sensors appeared shortly after Cd exposure (5 min), whereas others seem to disappear.

The overall trend was a reduction of the number in different CaBPs from 5 minutes to 30 minutes of exposure, and after 1 hour, a higher diversity of CaBPs was again obtained. Specific calcium sensors increased their expression with Cd stress. More specifically, two proteins of higher molecular masses of 90 and 70 KDa were early very expressed (5 minutes) and increased. Another one, of estimated mass of 50 KDa was increased during 5 minutes and 15 minutes of exposure. Finally, a protein of 26 KDa was also highly induced after Cd exposure, but was more enhanced at 30 minutes and 1 hour. This protein was not detectable in controls, suggesting its expression was induced by Cd.

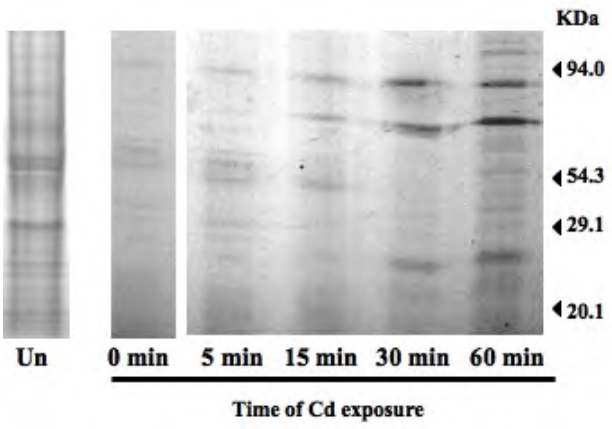


Fig. 4.2.11. Electrophoretic profiles of the Cd-induced calcium binding proteins in *A. thaliana* cells, throughout 1 hour exposure to 100 μM Cd. Different lanes contain unpurified controls (Un) and calcium-binding proteins purified by affinity chromatography, after different times of exposure.

4.2.6. CBLs are important calcium signalling transducers in Cd exposure

The molecular weight estimations of the proteins isolated through affinity chromatography suggested that one protein highly induced by Cd (26 KDa) could be a CBL. In order to identify whether CBLs are important for Cd stress we investigated their expression in particular, through immunoblotting analysis, as demonstrated in Fig. 4.2.13.

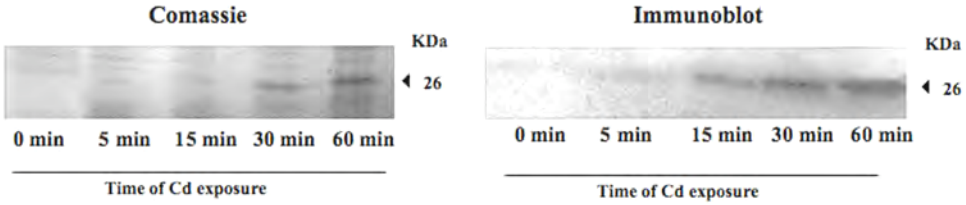


Fig. 4.2.13. Time course of CBL expression during Cd stress. Calcium-binding proteins were isolated through affinity chromatography and the gel subjected to western blot analysis with CBL antibody.

Immunoblots showed that CBLs were poorly expressed in controls, but were increasingly overexpressed in the presence of Cd. This response was influenced in a time-dependent manner, showing higher intensity at 30 and 60 min of exposure.

4.3 Discussion

Although calcium is a critical component in the signal transduction pathways and there is a considerable amount of work discussing the importance of calcium signalling in abiotic stresses in plants, its importance on metal stress has been neglected. The present work shows the importance of calcium and calcium signal sensors in Cd tolerance and PC synthesis, opening doors to unravel a Cd-induced signalling pathway.

4.3.1. Calcium influences Cd uptake but also PC synthesis

In plants, two kinds of calcium stores are believed to contribute to calcium modulation: extracellular (apoplastic) stores in the cell wall and intracellular stores in the vacuole and endoplasmic reticulum (Bush, 1995). In this work, Cd-induced stress was combined with chemical agents known to modulate cellular Ca concentration, such as Caffeine, which causes the release and depletion of Ca from internal stores; lanthanum, a Ca channel blocker, EGTA an extracellular Ca chelator, and ruthenium red, an inhibitor of Ca release from internal stores. (Subbaiah et al., 1994**;** Muir and Sanders 1996; Shimazakiet al. 1999; Cessna and Low 2001; Navazio et al. 2001; Taylor et al. 2001). Our pharmacological tests suggest that Ca levels, either by intracellular release or by extracellular sources, induces Cd tolerance, possibly by reducing Cd uptake. Previous works have already demonstrated that Cd can compete with Ca for membrane transporters and also for Ca-binding proteins (Rivetta et al., 1997), suggesting that calcium can reduce Cd absorption. In the report of Perfus-Barbeoch et al. (2002), patch-clamp studies with *V. faba* guard cell protoplasts showed that Ca channels were permeable to Cd. Furthermore, Cd uptake is inhibited by several Ca channel blockers (Blazka and Shaikh 1991). Concordantly, in other works, Ca was also been reported to alleviate Cd toxicity, in radish **(**Rivetta et al., 1997), rice roots (Kim et al., 2002), and *Arabidopsis* seedlings (Suzuki, 2005) by reducing the Cd uptake.

Nonetheless, results in this work demonstrate that even with reduced Cd uptake, Ca had a strong influence on both GSH and PC synthesis. Under

control conditions, blocking the extracellular Ca with EGTA reduced the amount of GSH in the cells. This trend was more pronounced with higher EGTA concentrations and is consistent with a scenario where Ca is important for GSH synthesis. The increase in Cd tolerance, together with the reduction in PC synthesis in EGTA+Cd treated cells is certainly related to the chelation properties of EGTA, that is known to bind Cd extracellularly, hence reducing its absorption. This also explains why control cells were more affected by EGTA exposures than cells under Cd stress, since in Cd exposures, Cd ions would bind EGTA, reducing the intracellular concentrations and hence Cd toxicity. On the other and, Cd ions compete with Ca for EGTA, reducing Ca blocking. This hypothesis is substantiated by the amounts of GSH obtained in Cd exposures, which were higher than those observed with EGTA alone. Nonetheless, the total lack of PCs in EGTA+Cd treatments suggests that another cation, can also be important for PCS activity synthesis, since with La treatments, a more specific Ca blocker, PCS activity was not affected.

When Ca channels were blocked with lanthanum both GSH levels and Cd absorption were also very reduced. The La-induced reduction in Cd absorption was more increased with higher La concentrations, corroborating that Ca channels are important for Cd absorption in cells. If Ca depletion inhibits GSH synthesis, as suggested by our results, GSH would be completely consumed without being synthesised *de novo*, which is consistent with our results. This suggests that although Ca stimulates GSH production, it may have no effect on PCS, since PC synthesis remained active in the presence of La.

Even though La and EGTA treatments suggest that Ca is related to GSH synthesis, its effect on PC synthesis itself cannot be excluded because of the reduced Cd absorption observed with these modulators. Nonetheless, the inhibition of Ca release from intracellular sores by ruthenium red treatments showed that Ca is indeed important for Cd tolerance. Because ruthenium red blocks Ca release from intracellular stores, it did not interfere as much with extracellular Cd absorption as did the other Ca blockers. Results show that in

Rut Red+Cd treatments, there was a more pronounced reduction in cell viability, when compared to Cd controls, corroborating that Ca is indeed important for Cd tolerance, but not only by reducing Cd uptake. Furthermore, ruthenium red also inhibited GSH synthesis in controls, but presented a PC synthesis similar to controls. This result may suggest that extracellular Ca sources may be more important for GSH production than the intracellular stores.

4.3.2.Calcium modulation affects the enzymatic activities of the PC synthesis pathway

The activities of the enzymes GSHS, γ-ECS and PCS were tested in the presence of the Ca modulators in order to substantiate the hypothesis that Ca is important for GSH synthesis. Results showed that in the presence or absence of Cd an increase in Ca concentrations enhanced both γ-ECS and GSHS activities whereas blocking Ca flow to the cytoplasm prominently reduced these enzymes activity, which explains the reduction in the PCS substrate concentrations. Nonetheless, Ca modulation was more efficient in affecting γ-ECS activities than GSHS. In fact, under control conditions, GSHS was generally not inhibited by Ca blockers, and in the presence of Cd, its inhibition was lower than γ-ECS. γ-ECS was already demonstrated to be more sensitive to Cd alterations (see chapter 3) and is known to be the key step in GSH synthesis (May et al., 1998; Noctor et al., 1998). These results point out Ca as a modulator of the activity of γ-ECS .

The PCS inhibition in the presence of calcium blockers can be explained by the reduction of Cd absorption and as a consequence of intracellular Cd levels, and not as a direct effect of Ca on PCS *per se*. With ruthenium red treatments, the reduction in GSH levels and not as pronouncedly on Cd levels would expectedly be responsible for the reduction of PCS activity. These results seem to suggest that GSH is more important than Cd alone for PCS activation, since with Rut Red+Cd treatments intracellular Cd levels were still high, but with reduced GSH levels, PCS activity also decreased. This implies that GSH conjugation with Cd could be

the main factor for this enzyme activation and not Cd concentrations and is consistent with the model of PCS activation proposed by Vatamaniuk et al. (2002) (see chapter 3), where PCS is more activated by bisglutathionate conjugates than with Cd alone.

4.3.3 Calcium sensors are modulated by Cd stress and CBLs are metal stress signalling intermediates

In this work, calcium-binding proteins were isolated through affinity chromatography and provided important insights on the role of individual calcium sensors in Cd stress. Results clearly show an important modulation of Ca-asssociated proteins throughout Cd stress, which is mostly prominent during the first hour of Cd induction. This timing of CaBP induction matched the timing of initial PC production and the subsequent increase of GSH production, as demonstrated in chapter 3. These results corroborate that Ca is a second messenger for Cd stress and is important for PC synthesis. The fact that CaBPs are more induced at 30 and 60 minutes also substantiates that CaBPS are more important for the increase in GSH synthesis than for PC synthesis, which was already active at 15 minutes.

The electrophoretic separation of the eluted calcium sensors provided more information on the nature of the induced CaBPs. Results demonstrated that during the Cd exposure, there was a time-course induced alteration on the quality and quantity of the induced CaBPs. In fact, several calcium associated proteins were reduced until 1 hour of exposure, which was the timing for the establishment of PC synthesis. Few individual calcium sensors are overexpressed during Cd challenge, suggesting a probable role in Cd signalling. One of these specific proteins was only induced after 30 min of Cd stress and was identified as a Calcineurin-B-like protein (CBL). It is generally believed that cellular Ca levels hold the key to the activation of stress response pathways. He et al (2005) proposed that the levels of some calcium sensors may serve as a threshold (rate-limiting) factor for the responsiveness of plants to stress signals. In some studies with CBLs, their basal level is usually low under normal conditions and is induced by stress signals

(Cheong et al., 2004; Luan et al., 2004). A similar effect was found in the present work, corroborating a similar mode of action of CBLs in metal stress. The stress-induced increase in CBLs levels could probably serve as a feedback mechanism for increasing the sensitivity to the stress signals. The fact that CBLs are associated with salt and oxidative stresses, which are known to induce GSH-mediated tolerance mechanisms, may suggest that this calcium sensor can be involved in a GSH inducing pathway, which can open new possibilities for more research studies on calcium sensors and GSH synthesis in abiotic stresses.

4.4 Conclusions

Overall, results presented here introduced novel findings on the role of Ca signalling in Cd stress and PC synthesis. Firstly, they corroborate the notion that Cd uptake in cells can be processed through Ca channels. Secondly they demonstrates that Ca can have an important modulator effect on PC synthesis, probably not by a direct action on PCS, but most likely by an increase of the GSH synthesis pathway, especially in γ-ECS activity, which would allow the formation of more GS-Cd substrates to activate PCS. The analysis of calcium binding proteins corroborated that Ca signaling pathways are activated by Cd stress, since Cd modulated their expression. CCBLs were shown to be altered by Cd exposure, suggestin a possible role as mediators of Cd signaling and similarly to other stresses, as threshold parameters in calcium-mediated signal transduction in PC synthesis.

These results can be of significant importance for phytoremediation studies, since it demonstrates that Ca modulation in soils can be used for enhancing plant tolerance to Cd, as well as reducing heavy metal uptake and therefore its transfer to the food chain.

Chapter 5

Protein phosphorylation and PC signalling

5.1 Introduction

5.1.1. Plant phosphatases

The reversible phosphorilation of proteins regulates most aspects of cell life and is also an important part of signal transduction. It is well recognised that protein phosphorilation/dephosphorilation is a key step in most stress signal transduction pathways. This role is played both by kinases and by plant phosphatases. A typical protein kinase catalyzes the phosphorylation of the hydroxyl group on amino acid residues including serine, threonine, and tyrosine.

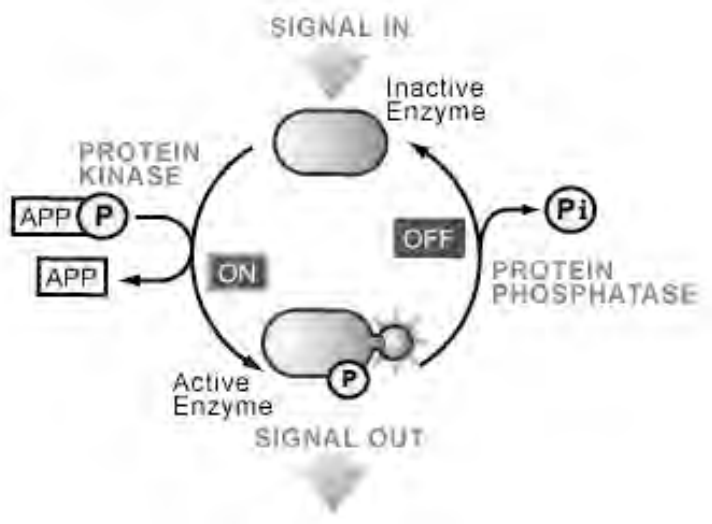


Fig. 5.1 An example of protein activity regulation by protein phosphorilation mediated by kinases and phosphatases

Protein kinases and protein phosphatases, are therefore key controlling elements, which are in turn regulated by a myriad of extracellular and intracellular signals, being the basis of almost all signal transduction pathways. Unlike the protein kinases that all belong to a single gene family, the protein phosphatases are divided into several distinct and unrelated protein/gene families (Bradford, 1995; Neel, 1997). Overall, they can be divided into two major classes: protein tyrosine phosphatases (PTPs) and protein serine/threonine phosphatases. Protein tyrosine phosphatases include PTPs and dual-specificity phosphatases (DSPTPs) (capable of dephosphorylating Ser, Thr and Tyr residues). The protein serine/threonine phosphatases are classified into the PPP and PPM gene families. The PPP family includes the so-called signature phosphatases [types 1 (PP1), 2A (PP2A) and 2B (PP2B)], whereas the PPM family includes type 2C (PP2C) and pyruvate dehydrogenase phosphatase (Cohen, 1989, 1997; Luan, 2002, 2004).

Protein phosphatases have been found in all eukaryotes and they represent one of the largest functional groups of proteins, being likely involved in all signaling processes in eukaryotic organisms. Although we know less about protein phosphatases in higher plants, evidence for their functional involvement is overwhelming. The serine/threonine protein phosphatases were traditionally classified into four subgroups (PP1, PP2A, PP2B and PP2C), due to their biochemical and pharmacological properties. PP1 and PP2A are inhibited by okadaic acid and calyculin A; PP2B is Ca^{2+}/calmodulin-dependent; and PP2C requires Mg^{2+} for activity. Most PPases are composed of more than one catalytic or regulatory subunit. Enzymes from the PPase family share high sequence similarity (except for PP2C), but they are very different from protein tyrosine phosphatases (PTPases) (Luan, 2002, 2004). Also, several studies in plants have uncovered the unique structure and function of these enzymes in plant-specific developmental and physiological processes.

5.1.2 PP1, PP2A and plant stress

Using substrates for mammalian phosphatases and the same pharmacological agents (e.g., okadaic acid), PPP family enzymes such as PP1 and PP2A have been detected in several plant species *(*Mackintosh et al., 1989, 1991; Luan 2002, 2004*)*. In addition to substrate specificity and pharmacological properties, the primary structure of plant phosphatases is also highly similar to that of the mammalian enzymes. Nevertheless, different structural domains and unique functions have been identified through studies on plant enzymes. In recent years, a great deal of research has been concentrated on identifying genes and elucidating signal transduction pathways involved in the plant response to abiotic stresses, mostly salt, osmotic, temperature, wounding and pathogen attack. Although one family of plant PPs, the PP2C have been extensively studied in plant responses to stress (Luan, 2004), the roles of PP1 and PP2A in abiotic stress signaling has been largely neglected. These PPPs are the most ubiquitous protein phospahatases in eukaryots and have been identified as important signal transducers of several metabolic pathways. Our knowledge of PP1 and PP2A functions in higher plants comes mostly from studies using specific inhibitors such as okadaic acid and others (Mackintosh et al., 1989, 1991; Shii et al., 2008). Because it is difficult to distinguish PP1 from PP2A by using pharmacological inhibitors, most studies defined the inhibitor–sensitive process as involving PP1/PP2A. Generally, inhibitor analysis heve indicated that PP1/2A is involved in ion channel regulation, gene expression, and developmental processes. Also, expression of cold responsive genes is enhanced at normal temperature by the protein phosphatase 1 and 2A inhibitor (Monroy et al., 1997, 1998; Sangwan et al., 2001). These inhibitors also caused an increase in freezing tolerance at normal growth temperature (Sangwan et al., 2001). Moreover, low temperature caused a rapid and dramatic decrease in protein phosphatase 2A (PP2A) activity, which is dependent on Ca^{2+} influx. Studies with an unspecific inhibitor of PP1 and PP2A also suggest a role for PP1 and PP2A in regulation of ABA signalling

and expression of ABA and cold-responsive genes in *Arabidopsis* (Wu et al., 1997).

5.1.3. Protein phosphorilation and heavy metal stress.

Since PP1 and PP2A have been related to abiotic stress signalling, it could be likely that they are also associated with metal signalling (see chapter 1). Nonetheless, the only reports focusing on protein phosphorylation and Cd studied protein kinases and not phosphatases. Few recent reports on the importance of protein phosphorylation in heavy metal stress have increased the interest in this area. The work of Jonak et al (2007) showed that several MAP Kinases (mitogen activated kinase) are induced upon metal signal transduction, and that they are differently involved with different metals (Nakagami, 2004, 2005). Rios Barrera et al. (2009) also showed that in *Euglena gracilis* the inhibition of MAPKs induced a reduction in PC synthesis after Cd expousure. However, the pathway suggested by these authors was activated by $CuCl_2$ but not by $CdCl_2$. This fact allow us to infer that the diversity of signal transducers might be of significative importance for signal specificity in exposures to different metals. Since plant PPs are much fewer in number, their importance shall be much easier to investigate than kinases. Therefore, if protein phosphorilation can regulate PCS, then a valid approach would be to test which plant PP is involved. On the other hand, post-translational control of the enzymes responsible for GSH synthesis has been suggested to be dependent upon covalent modification or protein phosphorylation. There is some evidence to suggest that γ-ECS is regulated by phosphorylation (Sun et al., 1996), however this type of regulation has never been demonstrated in plants. All of these reports suggest that protein phosphorylation may be important for PC synthesis and Cd signalling. Overall, these works sugest that protein phosphorylation might be important for metal tolerance and PC synthesis. However, information available is contradictory and demands for further investigation.

5.1.4 Objectives

Up until now, the role of protein phosphorilation on metal signalling and PC production is poorly investigated, however few studies stress out the importance of understanding the role of protein phosphorylation in PC synthesis. From the seryne/threonine PPPs, PP1 and PP2A are two of the most important phosphatases, and seem to play important key roles in cell metabolism, as well as in abiotic stress signalling. Under this context, this chapter describes an attempt to understand the importance of plant protein phosphorylation in Cd signalling and PC production. With this goal, we set out to identify the importance of PP1/PP2A activity in Cd stress signalling by monitoring Cd tolerance and PC synthesis after their inhibition. Results shall bring new findings on the role of protein phosphorilation in PC synthesis and provide evidence of the involvement of PP1 or PP2A in PCS activation. This should bring new insights on the activation process of PCS, as well as on the Cd-induced signalling pathway.

5.2. Results

5.2.1 Protein phosphatases are involved with Cd tolerance

In order to verify the importance of protein phosphorylation for PC synthesis and Cd tolerance, several concentrations of cantharidin (Can), a PP1 and PP2A inhibitor, was added to the growth media and cell behaviour was monitored under controlled conditions to assure that this inhibitor would not significantly interfere with the cellular metabolism (Fig. 5.2.1).

As in chapter 4, cells were subjected to a two-hour exposure, because for Cd treatments they were subjected to a 1 hour of pre-exposure. Increasing concentrations of cantharidin induced a reduction of cell viability ($P<0.05$). After two hours the 1 μM Cant concentration had reduced viability to below 85% when compared to controls.

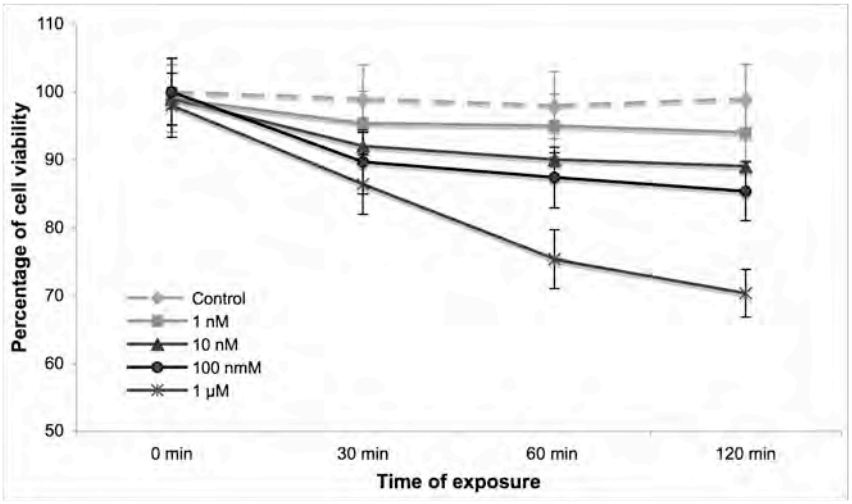


Fig. 5.2.1: Cell viability studies of *Arabidopsis* cells exposed for two hours of exposure to 1nM, 10 nM,100 nM and 1µM of protein phosphatase inhibitor cantharidin (Can). Values are the means of three to five replicates ±SE.

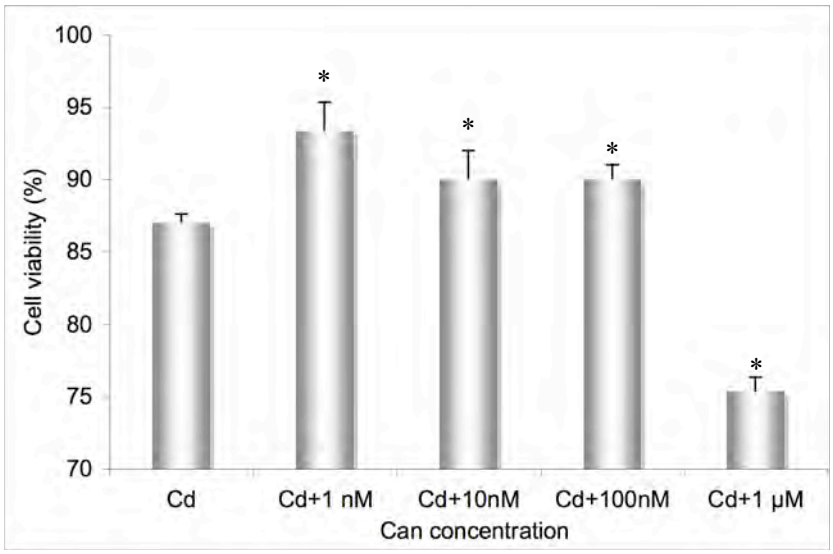


Fig. 5.2.2. The effect of PP1/PP2A inhibitor cantharidin (Can) on Cd tolerance of *Arabidopsis* cells exposed for 1 hour to 1nM, 10 nM, 100 nM and 1µM of cantharidin (Can). Can exposures were performed during 1 hour prior to exposure to 100 µM Cd. Values are the means of three to five replicates ±SE. Values significantly different from controls are marked with * ($P<0.05$).

The influence of cantharidin on Cd tolerance was observed, after 1hr exposure, and is demonstrated in Fig 5.2.2. The lower concentrations of Can induced a significant tolerance increase ($P<0.05$) to Cd and only the highest Can concentration induced a reduction of viable cells lower than Cd alone. Since this concentration induced a percentage of viable cells lower than 85%, even without the addition of Cd, it was excluded from the rest of the experiments.

5.2.2. PC synthesis under protein phosphatase inhibition

The inhibitory effect of protein phospahtases PP1 or PP2A on PC synthesis and on the levels of the constitutive monothiols was analysed and is expressed on Fig. 5.2.3.

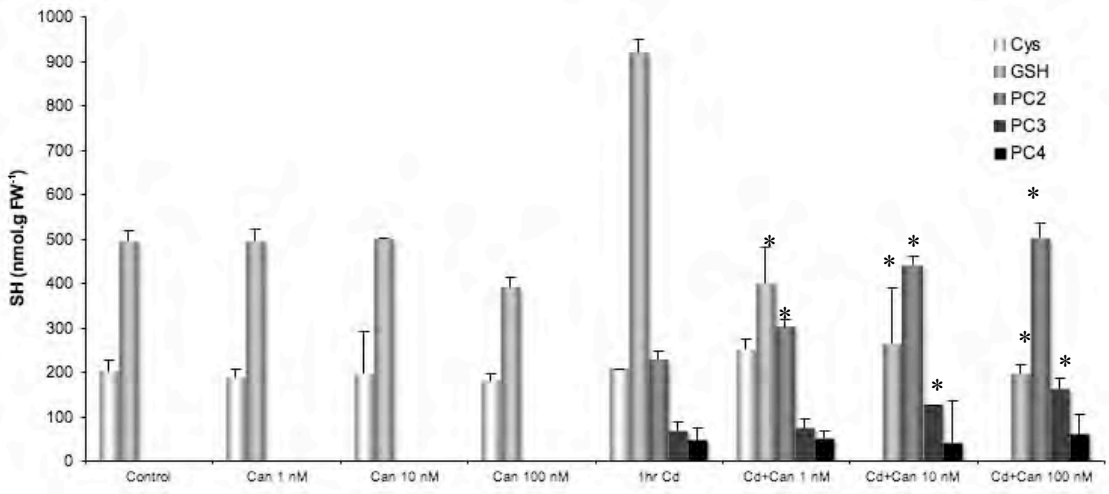


Fig. 5.2.3. The effect of different concentrations of the PP1 and PP2A inhibitor cantharidine (Can) on *A. thaliana* PC synthesis after 1hour of 100 μM Cd-exposure. Cell were incubated Can 1 hour before Cd exposure. Values are the means of three to five replicates ±SE. Values significantly different from controls are marked with * ($P<0.05$).

Although Can induced a reduction in cell viability (Fig. 5.2.1), the constitutive monothiol pools, Cys and GSH were not significantly affected by the presence of this inhibitor (Fig. 5.2.3). However, in the presence of Cd, results unexpectedly showed that exposure to cantharidin significantly increased PCs levels ($P<0.05$), in a dose-dependent manner. Despite of the increase in PC synthesis, Cys and GSH levels were significantly reduced under Cd+Can exposures, when compared to Cd alone.

Fig.5.2.4. shows that all Can concentrations induced higher levels of polythiols than Cd alone ($P<0.05$) (Fig. 5.2.4), at the expense of GSH (Fig. 5.2.3).

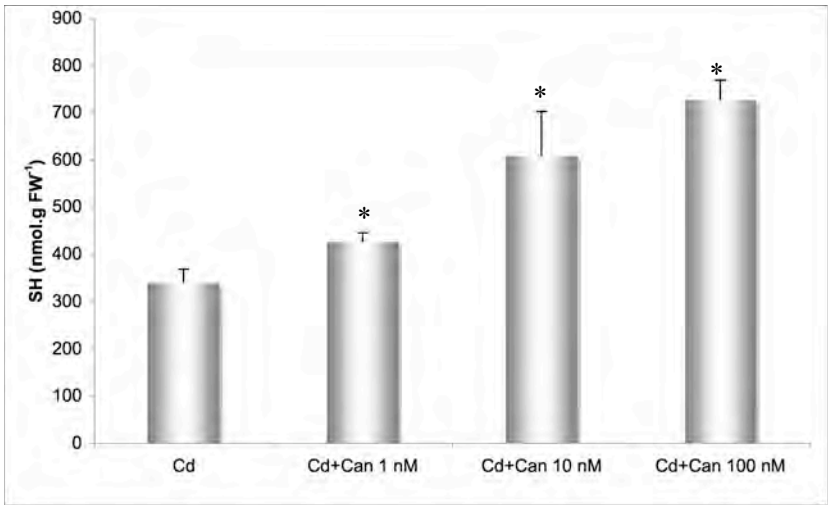


Fig. 5.2.4: The effect of protein phosphatase inhibitor cantharidin on total polythiol synthesis in *Arabidopsis* cells after 1hour of 100 μM Cd exposure. Cells were treated with different Cant concentrations 1hour previously to Cd exposure. Values are the means of three to five replicates ±SE. Values significantly different from controls are marked with * (P<0.05).

In order to understand if PC synthesis alterations induced by phosphatase inhibition were associated with the Cd uptake, the intracellular metal concentrations were analysed in the presence of the different Can concentrations. Results are shown in Fig 5.2.5. Can exposure increased Cd absorption in a dose-response pattern. However, only at 100 nM this increase was significant (P<0.05).

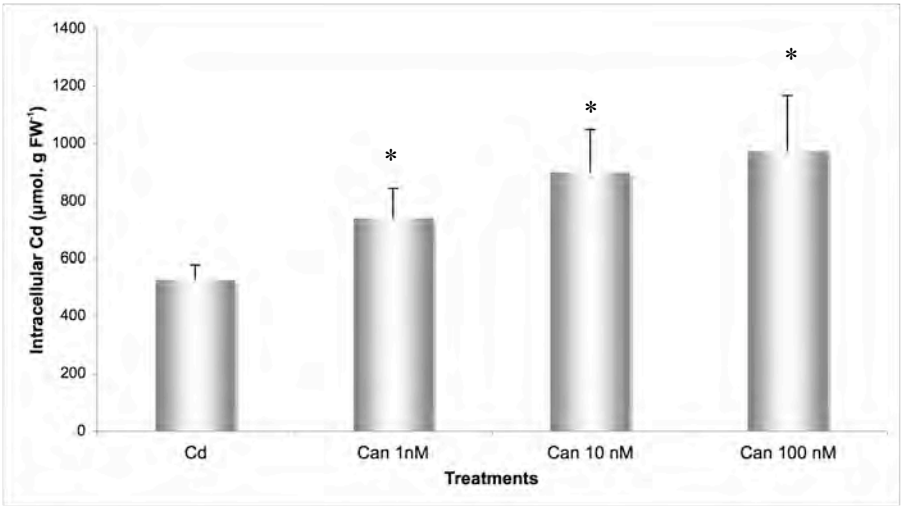


Fig. 5.2.5. Cd uptake in *A. thaliana* pre-exposed to different concentrations of the protein phosphatase inhibitor cantharidine. Cells were incubated with each treatment 1 hour before exposure to 100 μM Cd. Values are the means of three to five replicates ±SE. Values significantly different from controls are marked with * (P<0.05).

5.2.3. Protein phosphatases can modulate the enzymatic pathways of GSH and PC synhesis

The effect of cantharidine on the enzymes involved in the GSH and PC biosynthetic pathway was investigated. Figures 5.2.6 and 5.2.7 show the effects of the different Can concentrations on the activities of γ-ECS, GSHS and PCs.

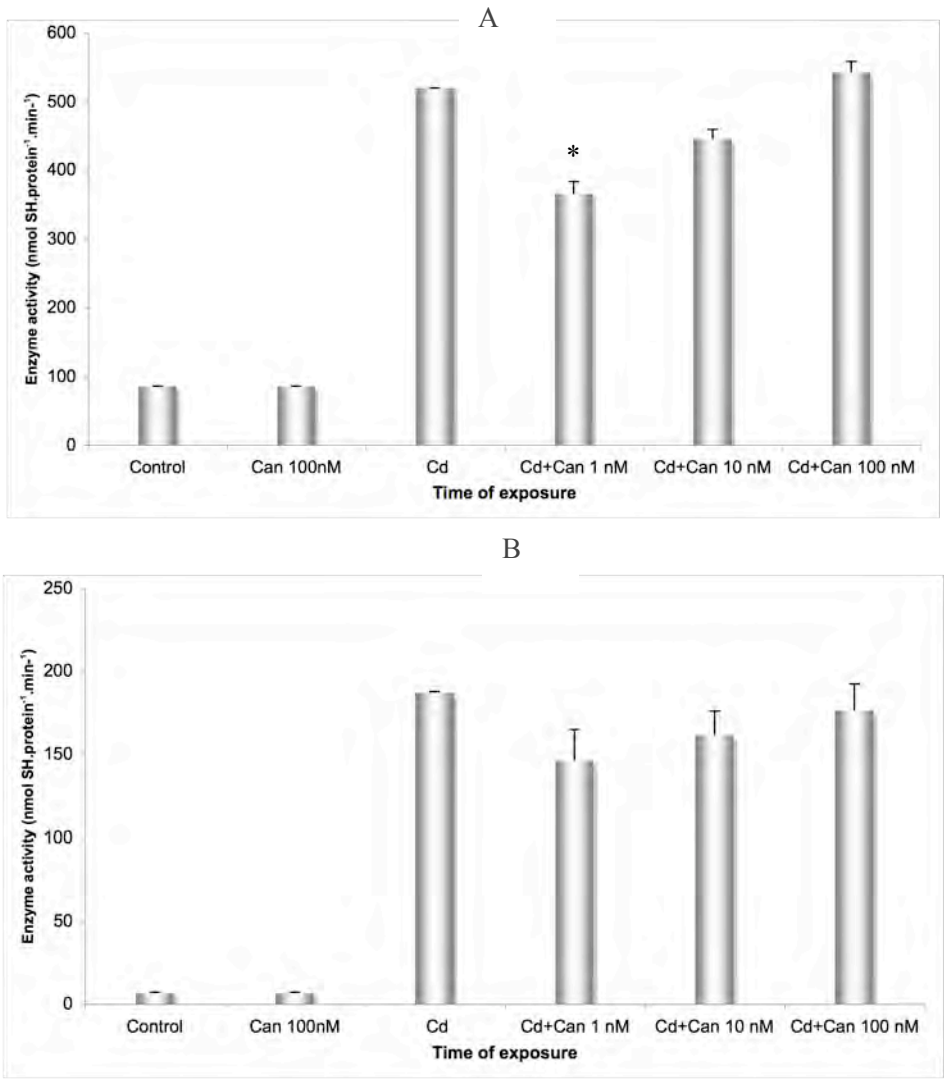


Fig. 5.2.5. The effect of the protein phosphatase inhibitor cantharidin on *A. thaliana* γECS (A) and GSHS (B). Cells were incubated with Can for 1hour before exposure to 100 μM Cd. Values are the means of three replicate experiments ±SE. Values significantly different from controls are marked with * ($P<0.05$).

In controls, the presence of Can did not influence significantly γ-ECS or GSHS activities ($P>0.05$). In Can+Cd exposures, γ-ECS enzymatic activities

were only significantly lower than Cd treatments in the 1nM Can concentration ($P<0.05$), whereas in 10 and 100 nM, γ-ECS activities were similar to controls ($P<0.05$). Can treatments did not induce any significant alterations in GSHS activities ($P<0.05$), neither in controls nor in the presence of Cd. The influence of protein PP1/PP2A inhibition on PCS was further addressed.

Fig. 5.2.7 shows the PCS activity levels in the presence of increasing Can concentrations. Can addition enhanced PCS enzymatic activity when compared to Cd treatments, that was significant higher can concentrations ($P<0.05$).

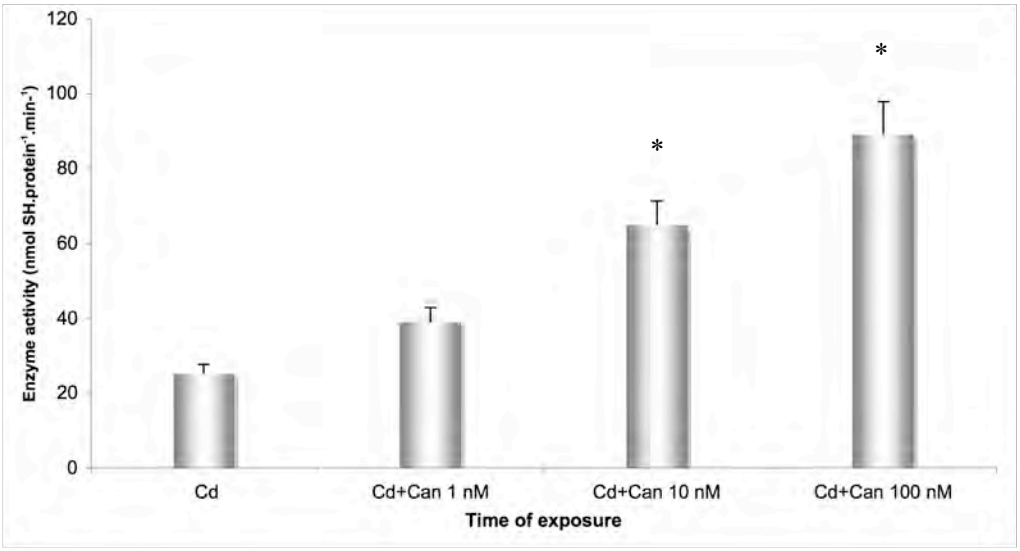


Fig. 5.2.7. The effect of the protein phosphatase inhibitor cantharidin on *A. thaliana* PCS after 1hour of 100 μM Cd-exposure. Cells were incubated with Can 1hr before Cd exposure. Values are the means of three replicate experiments ±SE. Values significantly different from controls are marked with * ($P<0.05$).

In order to understand if Can had a direct effect on PCS or was acting indirectly, the PCS activity was monitored *in vitro*, with the addition of Can, with or without the presence Cd (Fig 5.2.8). The Cd chelator EDTA was used in order to understand if Can can alter PCS activity in the absence of Cd. Results show that Can addition *in vitro* also induced a significant increase in PCS activity ($P<0.05$). However this effect was suppressed by EDTA, indicating that this effect was Cd-dependent.

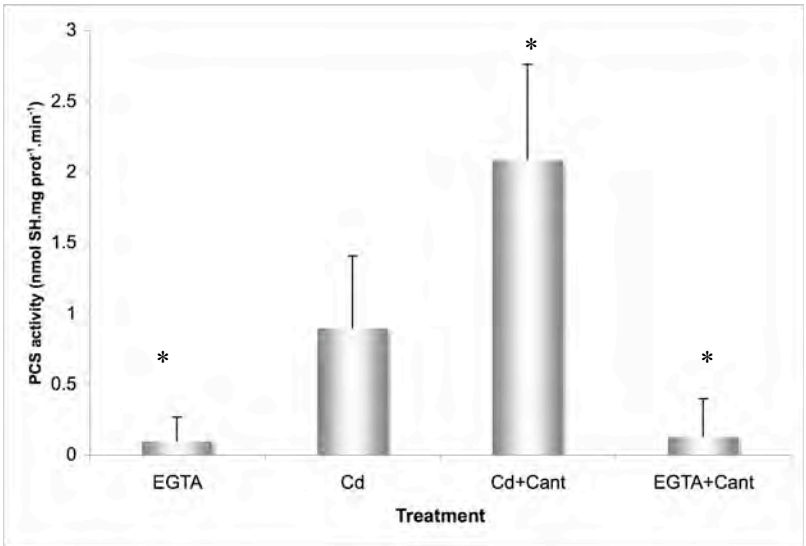


Fig. 5.2.8. The effect of the protein phosphatase inhibitor cantharidin on *A. thaliana* PCS in vitro in the presence or absence of Cd. Values the means of at least three replicate experiments ±SE. Values significantly different from controls are marked with * (P<0.05).

5.2.4 Cd affects PP1 expression

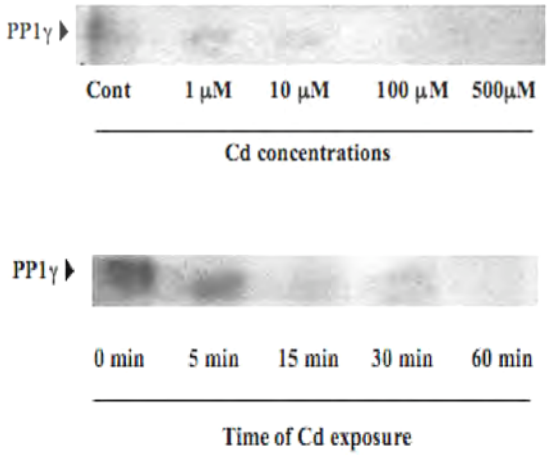


Fig. 5.2.9. The effect of Cd exposure on PP1 expression. *A. thaliana* cells were exposed to a range of increasing Cd concentrations during 1 hour or were submitted to 100 μM Cd and collected through the time of exposure. Total protein extracts were separated by SDS-PAGE and subjected to western blot analysis with PP1 antibody.

Since PC induction was mediated with the higher concentrations of Can, we hypothesized that PP1 inhibition was the responsible for enhancing PCS activity. Thus, we set out to analyze the PP1 expression patterns under different Cd exposures. Fig 5.2.9 shows the influence of different Cd concentrations on PP1 expression, as well as different times of exposure to 100

μM Cd. The analysis of PP1 expression levels during exposure to increasing Cd concentrations shows that Cd inhibited PP1. This inhibition was more pronounced with higher Cd concentrations. Furthermore, during an exposure to 100 μM Cd PP1 levels were gradually reduced with time, showing that Cd-induced PP1 inhibition was time-dependent, being more pronounced at 30 minutes.

5.3 Discussion

Posttranslational modification of proteins by phosphorylation is a universal mechanism for regulating diverse biological functions. Recognition that many cellular proteins are reversibly phosphorylated in response to external stimuli or intracellular signals has generated an ongoing interest in identifying and characterizing plant protein kinases and protein phosphatases that modulate the phosphorylation status of proteins (Smith and Walker, 1996). The work presented here showed the importance of protein phosphatases for PC synthesis and consequently Cd tolerance in *A. thaliana* cells.

5.3.1. Protein phosphatase inhibition increases Cd tolerance by enhancing PC synthesis.

Pharmacological agents that selectively affect stimulus-response coupling are particularly valuable in identifying the unique features of a transduction pathway. This approach has led to the identification of the role of protein phosphatases in several response pathways in plants (Luan et al., 1993; Neuhaus et al., 1993). The availability of cell membrane permeant inhibitors like okadaic acid (Tachibana et al., 1981) and cantharidin (Honkanen, 1993) has facilitated the study of the functional role of PP 1 and PP 2A. Using these inhibitors, PPs have gained interest in recent years as potentially important regulators of cellular function. In this work, the inhibition of PP1/PP2A enhanced Cd tolerance, not because of a reduction in Cd uptake, but due to an effective elevation of PC synthesis, particularly of its dimeric forms. These results are of significant importance, since they suggest

that PC synthesis can be regulated by protein phosphorylation. More importantly, either PP1, PP2A or both can block PC synthesis. It has been widely accepted that phytochelatin synthase is a constitutive enzyme that may be controlled by post-translational modifications (Vatamaniuk et al., 2004). Thus, there should be an efficient mechanism that blocks PCS activity during normal conditions, that can be switched off in the presence of metals such as Cd. Results presented suggest that PP1/PP2A, or both, can be the responsible for maintaining PCS inactive, when metallic ions are maintained low.

If PPs are blocking the PC synthesis, then in order to trigger their production under Cd stress, a specific pathway, with a specific kinase is expected. So far, the only few reports on protein posphorylation involvement in metal stress do seem to suggest the involvement of MAP Kinases. Exposure of *Medicago sativa* seedlings to excess copper or cadmium ions was also shown to activate four distinct mitogen-activated protein kinases (MAPKs.) Rios Barrera et al. (2009) also found that p38 MAPK-like activity was stimulated by acute or chronic heavy metal exposure, and its inhibition by a p38 MAPK inhibitor slightly diminished the accumulation of PCs. However, p38 MAPK has been reported to participate in the induction of γ-glutamyl transferase, the enzyme responsible for GSH degradation (Pandur et al. 2007; Zhang et al. 2005), which can explain that the decrease in PCS activity was due to a GSH depletion (Sun et al., 1996). These reports show that much more work should be done in order to understand how protein phosphorylation is involved in Cd tolerance, since it is not clear which step of the PC synthesis pathway requires phosphorylation to induce PC synthesis. In this work, the effect of phophatase inhibition on both γ-ECS and GSHS activities as well as on PCS was analyzed. With Can addition a high GSH depletion was observed, concomitantly to the PC increase. This suggests that phosphatase inhibition is related to PCS and not to GSH synthesis. Since PC synthesis was rapidly enhanced, the GSH pool would be increasingly depleted, without enough GSH production to balance its consumption. This assumption was indeed substantiated by γ-ECS and GSHS activity analysis, since Can had no effect on

their activities. Therefore, it seems that PP1/PP2A have no relation to GSH synthesis, but are strongly related to PCS activity, which was highly induced by PP1/PP2A inhibition in a dose-dependent manner. These results unequivocally demonstrate that PCS activity can be increased through phosphorilation, most specifically, serine/threonine phosphorylation. Indeed as this thesis was being written, a publication reported the importance of serine threonine phopsphorylation in PC synthesis (Wang et al., 2009**).** These authors showed that PCS activity increased following phosphorylation by casein kinase 2 (CK2) and decreased following treatment with alkaline phosphatase. Taken together our results and those reported by Wang et al., (2009), elucidate the route leading to the activation of PC synthesis. These findings are of extreme importance for the understanding of the PCS activation because they demonstrate that PCS is not only triggered by GSH conjugates and by Cd ions, but it is also influenced by protein phosphorylation. The *in vitro* studies of PCS demonstrate unequivocally that PCS activity is increased by PP1/PP2A inhibition, in a Cd-dependent manner. This means that although phosphorilation is important for PCS activity, it can only occur in the presence of Cd. One possible model for the above observations is that Cd binds to the C-terminal domain and triggers a conformational change that exposes the phosphorylation site on the N-terminal domain (Wang et al., 2009)

5.3.2. Cd stress induces PP1 inhibition

According to some authors, although Can is an inhibitor of PP2A and PP1, we can differentiate which phosphatase is being inhibited by the concentration of Can that is inducing that inhibitory response, since cantharidine is 10- to 100-fold more effective in inhibiting PP2A than PP1 (Biolajan and Takai, 1988**).** This means that while PP2A should be inhibited at low Can concentrations, PP1 is only reduced at higher concentrations of Can. Since the increase in PC synthesis is mostly obtained with 100 nM Can, we hypothesised that it is the PP1 the responsible for maintaining PCS inactive. Under this context, the expression patterns of PP1 were analysed during

different Cd exposures. Results clearly show that Cd induces indeed a reduction of PP1 protein levels, corroborating the hypothesis that PP1 inhibition was the one leading to the increase in PC synthesis. Nonetheless, these results do not exclude the importance of PP2A on PCS activation.

PP1 is generally thought to be one of the most important protein phosphatases, being ubiquitously expressed and involved in the regulation of a wide variety of cellular signal transduction mechanisms. Their significant structural diversity can be generated by the presence of unique regulatory and targeting domains or by the attachment of regulatory subunits to the catalytic subunit. These regulatory domains or regulatory subunits may localize the protein complexes to a specific subcellular compartment, modulate the substrate specificity, or alter catalytic activity. Therefore, it is possible that further studies on the regulatory subunits that control PP1 activity under metal stress may bring novel findings on signal specificity.

5.4. Conclusions

Our results provided important novel findings of the signal transduction pathways that are triggered during Cd stress by demonstrating that protein phosphorylation is important for PC synthesis regulation and Cd tolerance. In short, PP phosphatase inhibition induced a prominent increase in PC synthesis, by influencing PCS activities, but not GSH synthesis. Results suggest that PP1 is strobgly modulated by Cd and can be directly or indirectly associated with maintaining PCS inactive in the cells, under Cd-free conditions and that its inhibition during Cd imposure can enhance PCS activity and hence PC production and metal tolerance. This result can be of specific importance for phytoremediation studies, since PP1 pharmacological inhibitors could be used as a way to enhance plant's specific tolerance responses. These findings also open new doors for further studies on Cd tolerance regulation in plants. Since PP1 activity and specificity is controlled by several protein regulatory subunits, it is possible that future studies can

identify which regulators are involved in Cd stress and in PP1 inhibition in the presence of Cd.

Chapter 6

ROS signalling and PC synthesis

6.1 Introduction

6.1.1 ROS in plants

In plants, reactive oxygen species (ROS) are continuously being produced by several metabolic processes, mostly in chloroplasts, mitochondria and peroxisomes. The term ROS is generic, embracing not only free radicals such as superoxide (O_2^-) and hydroxyl radicals but also H_2O_2 and singlet oxygen. While it is generally assumed that the hydroxyl radicals and singlet oxygen are so reactive that their production must be minimized (Jako and Heber 1996; Foyer and Harbinson 1994), O_2^- and H_2O_2 are usually synthesized, even under optimal conditions. All of these molecules are scavenged by different antioxidative defense components that are often confined to particular compartments (Alscher, 1989).

Plants have evolved a number of mechanisms to protect themselves from ROS-mediated damage. The term antioxidant can be considered to describe any compound capable of quenching ROS without itself undergoing conversion to a destructive radical (Noctor and Foyer, 1998). Antioxidant enzymes (e.g. catalase, ascorbate peroxidases or superoxide dismutases) catalyze such reactions, or are involved in the direct processing of ROS. Metabolites such as nonprotein thiols can function as antioxidants, scaveging ROS and keeping other redox-active molecules in a reduced state (Noctor and

Foyer, 1998). Glutathione is the major thiol compound present in plants and plays a pivotal role in ROS scavenging. It is usually found in the reduced form (90%– 99%; Foyer et al., 1997), providing a store for reducing power within the cell and acting as a redox buffer. The antioxidant properties of reduced glutathione (GSH) lie in the thiol group, which can reduce ROS molecules rendering them harmful to the cell and producing oxidized glutathione (GSSG), a dimmer connected by a disulfide bound. Consistently, oxidative conditions increase GSH synthesis in *Arabidopsis* cell suspension cultures (Griffith and Meister, 1979), whereas depletion of glutathione by treatment with L-buthionine-[S,R]-sulfoximine (BSO), an inhibitor of glutathione synthesis, renders them susceptible to oxidative damage (May and Leaver, 1998).

6.1.2 ROS can be signaling transducers

Whereas plants struggle to combat increased ROS levels during some abiotic stress conditions, in other circumstances they appear to purposefully generate ROS as signaling molecules, as a way to signal and to control several processes including pathogen defense, programmed cell death and stomatal behavior (Foyer and Noctor, 2003). Relatively little is known about the signalling events triggered by oxidative stress, that in turn lead to mechanisms against themselves, but some works have began to unravel specific targets that are regulated by ROS under abiotic stresses. Using cDNA microarray technology to carry out a transcriptomic analysis, Desikan et al. (2001) provided evidence that the expression of some genes is up-regulated by H_2O_2, whereas others are repressed. These genes include heat shock proteins, heat shock transcription factors, mediators for calcium signal transduction, such as calmodulin, protein tyrosine phosphatases (PTPs), a blue copper-binding protein which is an essential catalyst for redox reactions, a mitochondrial uncoupling protein, pyruvate decarboxylase, and transcription factors. Fig.6.1.1 demostrates a model of ROS-signalling induction.

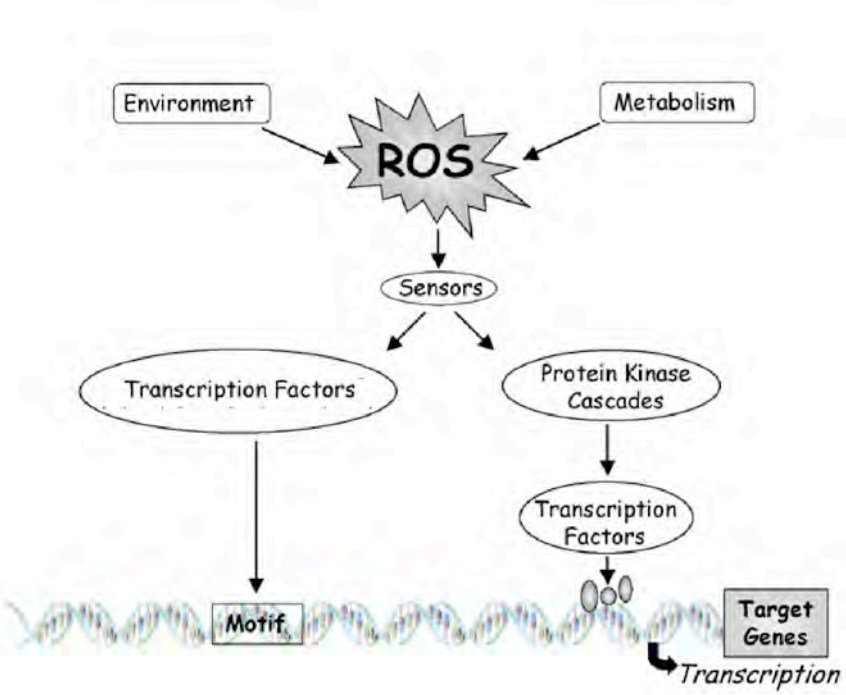


Fig. 6.1.1. A model for ROS signaling in plants.

6.1.3. Metal stress-induced ROS: a possible signaling pathway?

It is still unknown if Cd ions directly induce ROS because the Cd ion is unable to catalyze Fenton–Haber–Weiss reactions. Even so, an increasing body of evidence suggests that metal-induced phytotoxicity is attributed, at least in part, to oxidative damage, mostly lipid peroxidation (Semanea et al., 2007; Maksymiec, 2007). The presence of ROS induction under Cd stress suggests that they can also be involved in signaling the cell for Cd stress, similarly to other abiotic stresses. So far, very few works have investigated a possible role of ROS in metal stress signaling, but many studies show the relation of ROS with important intermediates of the PC-inducing pathway, such as GSH, GSTs, calcium and protein phosphatases.

ROS can influence Ca signaling

Some works have demonstrated that ROS are important for calcium signaling, an important stress modulator (see chapter 4). In *Commelina communis* guard cells, low concentrations of superoxide and H_2O_2 triggered rapid increases in cytosolic calcium concentrations (McAinsh et al., 1996).

H_2O_2 challenge of tobacco (*Nicotiana tabacum*) seedlings expressing recombinant aequorin, also resulted in a rapid, transient elevation of calcium that was inhibited by lanthanum chloride ($LaCl_3$), a calcium channel blocker, and ruthenium red, an inhibitor of Ca release from internal stores (Price et al., 1994). Activation of Ca-permeable channels in response to ROS treatment was also demonstrated in *Arabidopsis* guard cells by patch-clamping (Pei et al., 2000). This study showed that application of H_2O_2 stimulated Ca influx through a hyperpolarization-activated Ca-permeable channel, followed by partial stomatal closing. However, a link between H_2O_2- triggered rises in Ca and H_2O_2-induced gene expression has not been demonstrated. Yang and Poovaiah (2002) also demonstrated that cytosolic Ca elevation activates the calcium sensor calmodulin and subsequently passes the signal to a downstream targetting of catalase. This finally down-regulates H_2O_2 levels by stimulating the plant catalase activity. All of these findings provide evidence indicating that H_2O_2 can influences Ca signaling in plants, which in turn has been demonstrated as an important PC synthesis modulator (see chapter 4).

GHS and ROS: partners in plant signaling?

The present understanding about the stress signaling in plants is that the changes in the GSH/GSSG ratio is indicative of the cellular redox balance and may be involved in ROS perception (Foyer and Noctor, 2003). For example, the bacterial OxyR protein can be activated either directly by hydrogen peroxide or alternatively by changes in the intracellular GSH/GSSG balance, suggesting that the increase in H_2O_2 is sensed in this manner (Droge, 2002). It has been reported that the formation of Cd–PC complexes reduces the Cd-free concentration in the cytosol and lead to the depletion of glutathione content, causing a loss in the cellular antioxidative response (Dixit et al., 2001; Cho and Park, 2000; Lima et al., 2006). Concordingly, high PC synthesis is known to lead to Cd hypersensitivity, due to a high GSH depletion (Lee et al., 2003). These facts strongly suggest that alterations and GSH fluctuations may influence ROS signalling.

The role of GSTs

The GSH metabolism involves several reactions where GSH is synthesized, degraded, conjugated or oxidized. In one of these pathways, the glutathione S-transferase (GSTs, E.C.2.5.1.18), a family of multifunctional isozymes that catalyze both GSH-dependent conjugation and reduction (Ketterer et al. 1993) can catalyze the nucleophilic attack of the sulfur atom of the tripeptide GSH on the electrophilic group of a specific substrate, usually a xenobiotic, which is either conjugated with reduced glutathione. It can salso be reduced or isomerized with the concomitant production of GSSG (Vuilleumier and Pagni, 2002; Kim et al., 2001).

The role of plants GSTs has been well characterized in various types of abiotic stresses, but it has been poorly investigated in metal stress. However, according to Marrs (1996) plant glutathione S-transferases may also be induced by heavy metals, besides other types of abiotic stresses. This fact has been demonstrated in several plant species (Marrs and Walbot 1997, Adamis et al., 2004). Several authors (Kumar et al., 2003; Sun et al., 2005; and Singla-Pareek et al., 2006) suggested that the protection from metal induced oxidative stress is also mediated by GSTs by direct quenching the excess of metal ions as GSH-metal ion adduct and sequestering them to the vacuoles (Kumar et al., 2003). Furthermore, Rentel and Knight (2004) have examined the characteristics of the Ca elevations in *Arabidopsis* seedlings upon H_2O_2 treatment and demonstrated that inhibiting or enhancing the height of the cytosolic Ca leads to alterations in ROS-induced GST gene expression. Conversely, GSH inhibition also lead to enhancement of GST induction. These findings suggest that GSTs may be activated buy the crosstalk between GSH levels, ROS production and Ca signals.

6.1.4 Objectives

So far, only few and unclear data are known about the molecular events affecting the course of heavy metal-induced ROS signals (Maksymiec, 2007). Increased accumulation of H_2O_2 is usually connected with changes in

the cellular redox status, alerting the plant cell against oxidative stress, probably enhancing the plant's antioxidant response through calcium signaling , GSH and altered expression of GSTs (Foyer and Noctor 2003; Rentel and Knight 2004). Taken altogether, these findings seem to point out for a possible role of ROS signaling in Cd stress, since they are both regulated and regulate the Ca and GSH signals. However, the influence of these signal mediators on PC synthesis remains to be analyzed. Under this context, this chapter will focus on understanding the role of H_2O_2 in Cd signaling and PC synthesis.

With this in mind, the Cd-induced ROS formation was analyzed, as well as its relation to GSH and GST activity. The impact of ROS induction in PC synthesis, and on the activity of the enzymes involved on the PC synthesis pathway was also investigated. Finally, the influence of different pharmacological calcium modulators on ROS accumulation was also evaluated in order to test the influence of Ca on ROS signaling in Cd stress. Results should provide important findings on the role of GSTs in Cd stress an introduce H_2O_2 as an important link in the crosstalk of the GSH and PC signaling pathways under Cd stress.

6.2 Results

6.2.1. ROS accumulation is affected by Cd exposure

In order to understand the relation between ROS induction and Cd stress, increasing Cd concentrations were used during 1 hour of exposure and the H_2O_2 levels were analyzed, as demonstrated in Fig. 6.2.1. Increasing Cd concentrations elicited a linear and significant increase in cellular H_2O_2 concentrations, in a dose-dependent manner ($P<0.05$).

The time-inducing effects of Cd on cell ROS buildup, upon Cd stress, the H_2O_2 production during 1 hour of exposure to 100 μM Cd was also monitored, and is described in Fig. 6.2.2. ROS production was not linear throughout time. In fact, results demonstrate a significant increase ($P<0.05$) in H_2O_2 accumulation during the first 30 minutes of exposure, followed by a

decrease at 1 hour to basal levels. Nonetheless, after 3 hours of metal exposure, H_2O_2 levels were highly increased after 1 day of exposure.

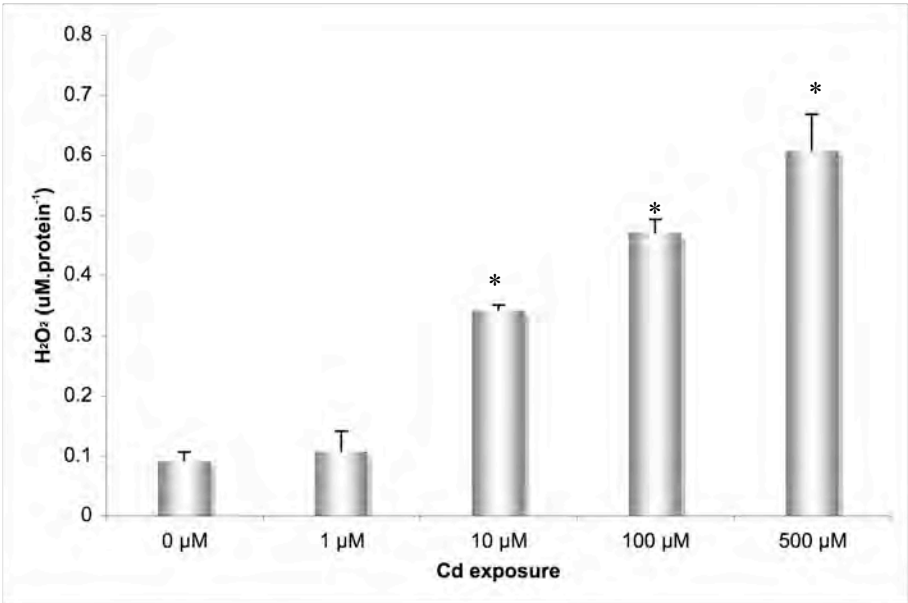


Fig.6.2.1. H_2O_2 accumulation in *A. thaliana* cells, after 1hour exposure to different Cd concentrations. Values are the means of at least three replicate measurements ± SE. Values significantly different from controls are marked with * ($P<0.05$).

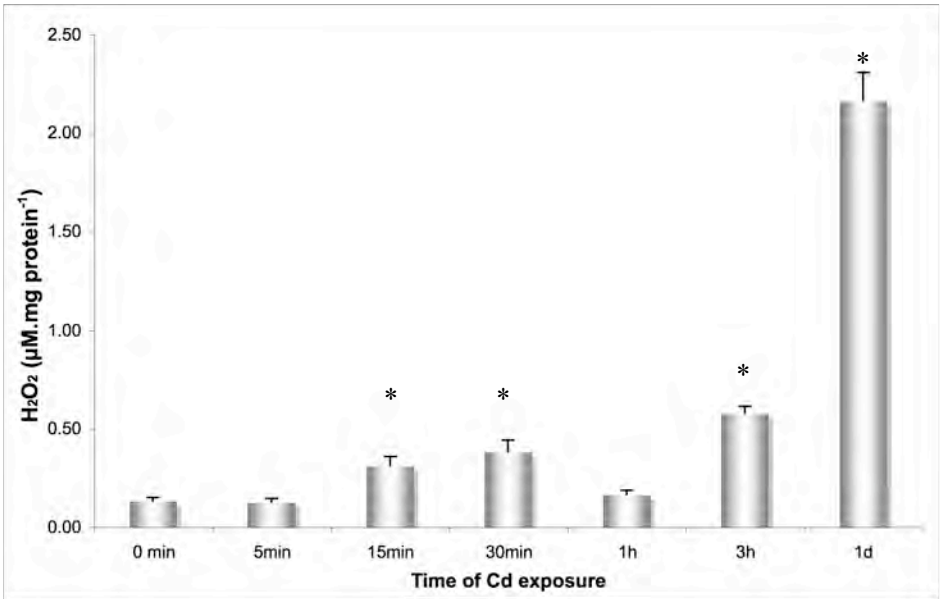


Fig.6.2.2. H_2O_2 induction in *A. thaliana* cells, during 1 hour exposure to 100 µM Cd. Values are the means of at least three replicate measurements ± SE. Values significantly different from controls are marked with * ($P<0.05$).

Fig. 6.2.3 shows the relation between H_2O_2 induction and GSH accumulation in *A. thaliana* cells under Cd exposure.

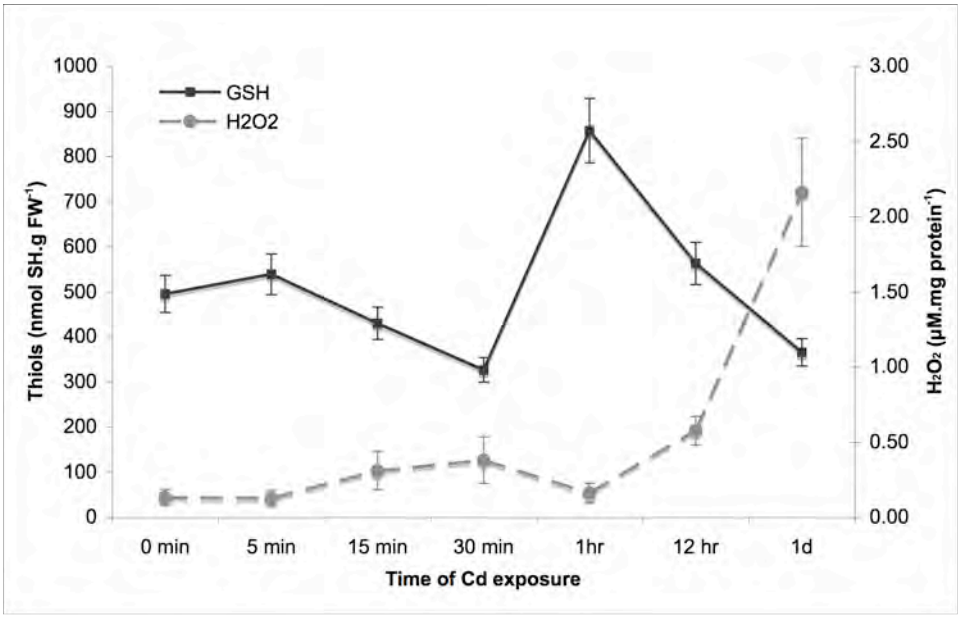


Fig.6.2.3. H_2O_2 induction and GSH accumulation (total PCs and GSH) in *A. thaliana* cells, during exposure to 100 µM Cd. Values are the means of at least three replicate measurements ± SE.

When comparing the ROS accumulation with GSH levels throughout time, it is noticeable that ROS are accumulated in an opposite manner to GSH, *i.e.*, when GSH levels are reduced, ROS increase and vice-versa.

6.2.2. ROS addition assays

Excess ROS affects Cd tolerance

In order to understand the influence of ROS on Cd tolerance, the effect of the addition of H_2O_2 was analyzed in controls and in Cd exposures. The influence of the H_2O_2 addition on cell viability was tested under control and Cd exposures, for a two-hours, in order to ascertain that H_2O_2 was not inducing a severe stress to cells (Fig. 6.2.4). This period was chosen two hours is the total duration of the subsequent treatments (1 hour of H_2O_2 exposure and 1 hour of Cd9. After two hours, the presence of hydrogen peroxide induced a significant reduction on cell viability ($P<0.05$) in all concentrations, when compared to controls. Viability reductions were consistently higher with higher concentrations of this ROS, reaching a 40% reduction at 100 mM.

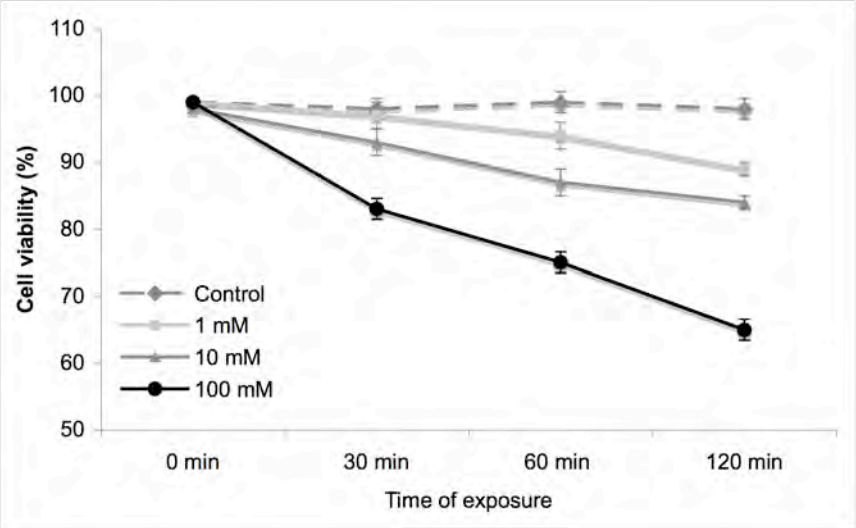


Fig.6.2.4. ROS-induced alteration on cell viability of *A. thaliana* cells, during a 2-hours exposure to different H_2O_2 concentrations, 1mM, 10 mM and 100 mM. Values are the means of at least three replicate measurements ± SE.

Fig. 6.2.5 shows the influence of H_2O_2 on Cd tolerance in *A. thaliana* cells after 1hour exposure to Cd.

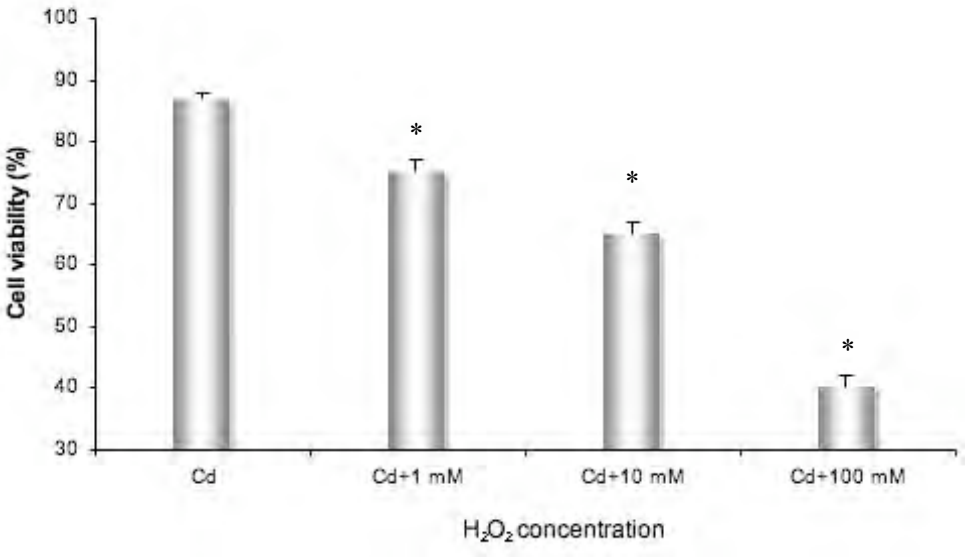


Fig.6.2.5. The influence of different H_2O_2 concentrations on Cd tolerance in *A. thaliana* cells, during a 1 hour expoure to 100 μM Cd. Cells were pre-exposed to 10 mM H_2O_2 for 1hour prior to Cd application. Values are a percentage of viable cells per volume and are a media of three replicate measurements ± SD. Values significantly different from controls are marked with * ($P<0.05$).

Hydrogen peroxide induced a marked reduction on cell viability when in the presence of Cd, which increased with increasing ROS concentrations and was much more pronounced compared to of H_2O_2 alone.

6.2.3. ROS affect thiol maintenance under Cd stress

The influence of ROS-induced alterations on Cd tolerance was further assessed by analysing their effects on thiols pools in cells. Figure 6.2.6 shows the kinetics of thiol levels, namely the constitutive Cys and GSH and the synthesised polythiols, with different chain lengths. Both in controls and Cd exposures, hydrogen peroxide induced a significant increase in GSH contents ($P<0.05$), which was higher under Cd exposures whereas the PCs and Cys levels were similar between H_2O_2+Cd and Cd treatments ($P>0.05$).

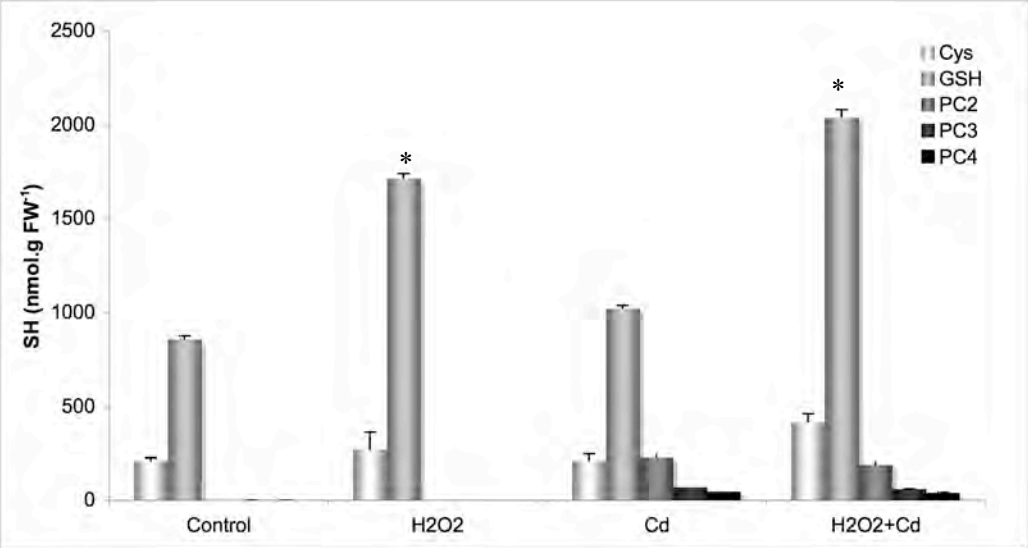
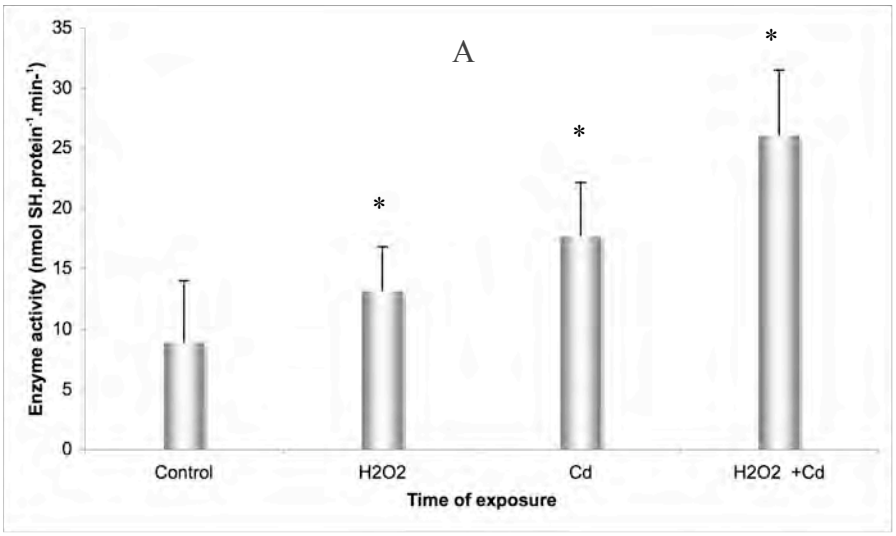
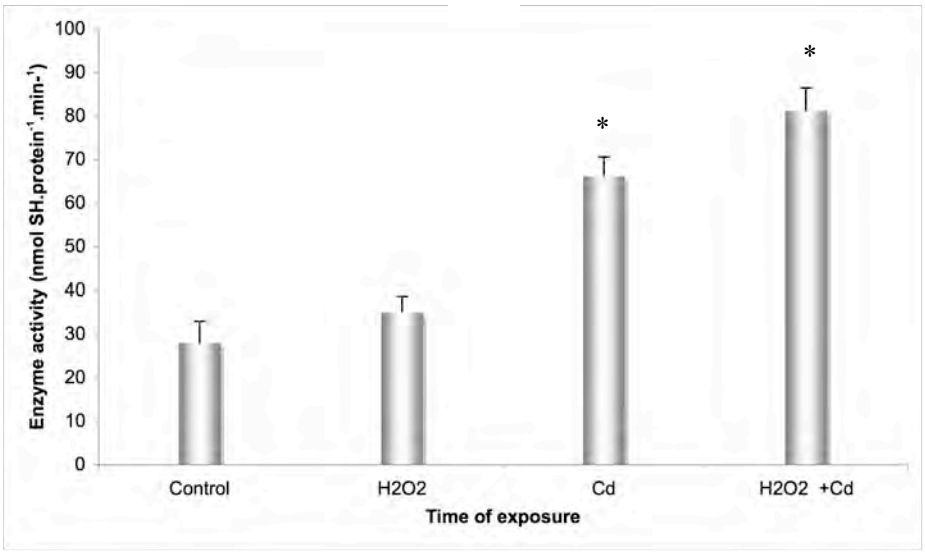


Fig. 6.2.6. The effect of H_2O_2 addition on *A. thaliana* thiol levels. Cells were incubated with 10 mM H_2O_2 1 hour before 100 μM Cd exposure. Values are the means of at least three replicate experiments ±SE. Values significantly different from controls are marked with * ($P<0.05$).

6.2.4. ROS affect the enzymatic pathway of PC synthesis

The effect of H_2O_2 on the enzymes responsible for GSH and PC synthesis, γ-ECS, GSH and PCS, was analysed in order to understand if ROS can influence GSH or PC synthesis (Fig. 6.2.7). The presence of ROS induced significant but different alterations in the activities of the three enzymes analyzed. H_2O_2 and Cd alone or together significantly increased the activities of γ-ECS and GSHS ($P<0.05$). This increase was significantly higher under Cd+H_2O_2 treatments. On the other hand, the presence of ROS reduced PCS activity significantly ($P<0.05$).





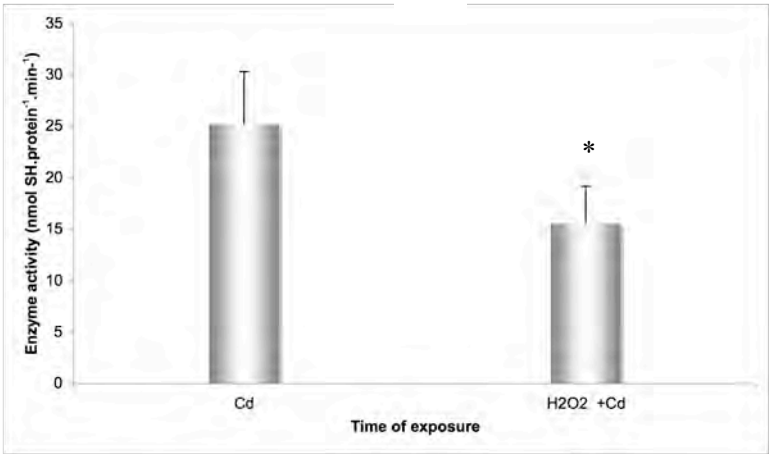


Fig. 6.2.7. The effect of H_2O_2 addition on *A. thaliana* γ-ECS (A), GSHS (B) and PCS (C). Cells were incubated with 10 mM H_2O_2 1 hour before 100 μM Cd exposure. Values are the means of at least three replicate experiments ±SE. Values significantly different from controls are marked with * ($P<0.05$).

6.2.5. ROS accumulation is affected by Ca modulation

Since ROS signaling has been discovered to be associated with calcium levels in the cell, the effect of calcium modulators as well as GSH inhibitors on ROS accumulation were analyzed, in the presence and absence of Cd (Fig 6.2.8).

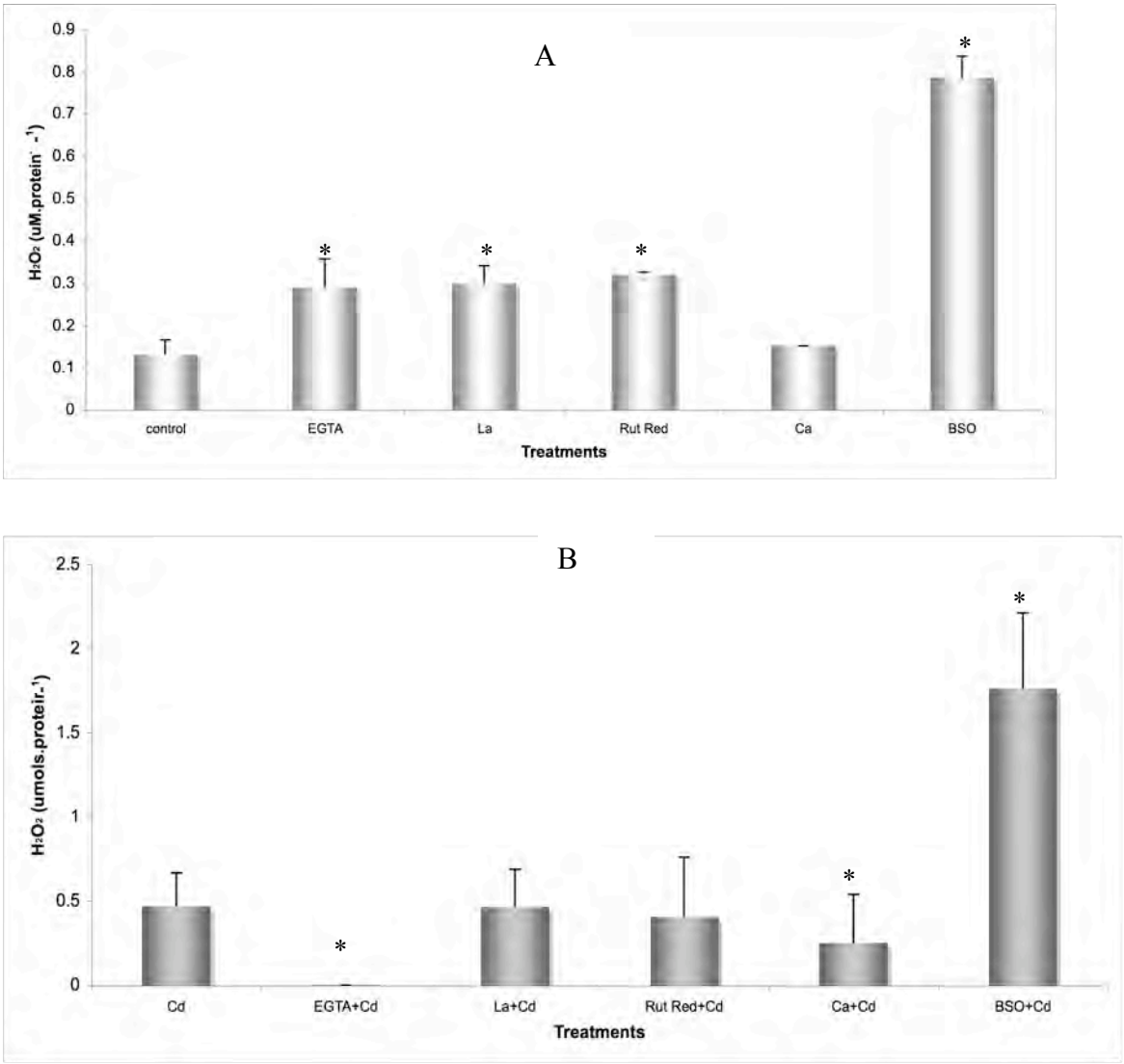


Fig. 6.2.8. H_2O_2 alterations induced by different calcium and GSH modulators, with (B) or without (A) the presence of 100 μM Cd. Values are the means of at least three replicate experiments ±SE. Values significantly different from controls are marked with * ($P<0.05$).

In the absence of Cd, all the Ca inhibitors (EGTA, lanthanum and ruthenium red) increased H_2O_2 levels in the cell ($P<0.05$), nearly doubling controls. Ca addition did not influence significantly ROS levels, and the GSH depletion by BSO induced a prominent increase in ROS levels, even higher

than with Ca blockers. In the presence of Cd, these responses differed. In the presence of the calcium inhibitors, only EGTA induced alterations in H_2O_2 accumulation ($P<0.05$). The presence of Ca reduced significantly H_2O_2 levels ($P<0.05$) and BSO addition caused a very prominent enhancement of intracellular ROS levels ($P<0.05$).

6.2.6. GSTS are induced by Cd and are modulated by ROS

In order to test whether GSTs are induced by metal stress, their activity levels were analysed in the presence of increasing Cd concentrations and throughout a time-dependent exposure to 100 μM Cd. Fig. 6.2.9 shows the alterations induced on GSTs activity in *A. thaliana* cells exposed to different Cd concentrations. Results demonstrate that enhancing Cd concentrations significantly increased GST activity, in a dose-dependent manner ($P<0.05$).

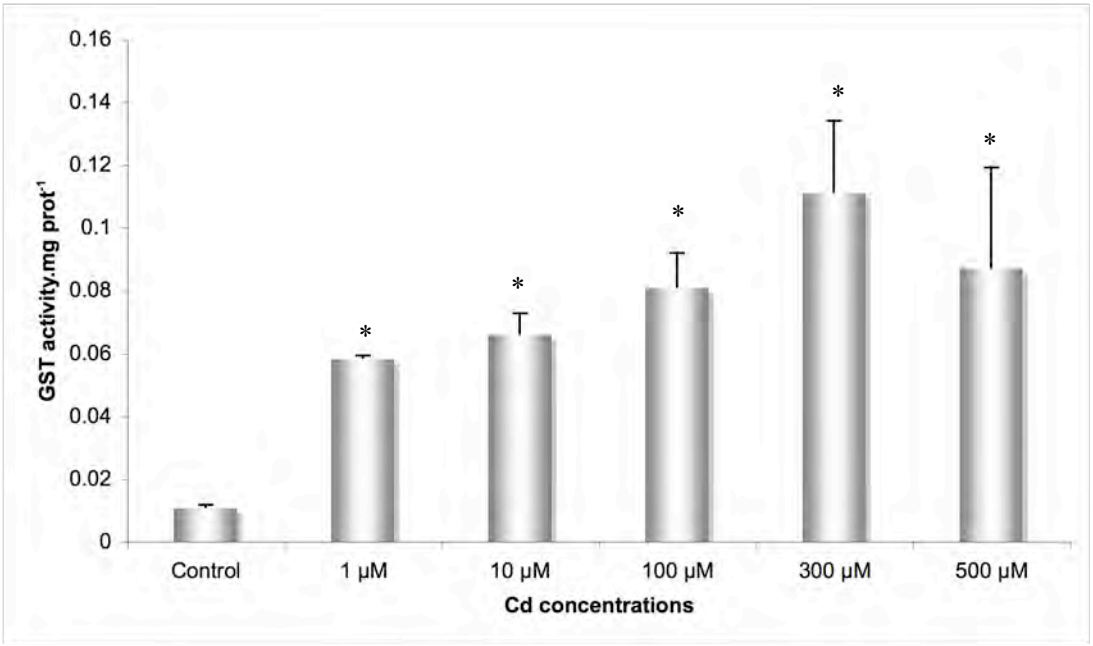


Fig. 6.2.9. GST activity in *A. thaliana* cells exposed to 1μM, 10μM, 100μM, 300μM and 500 μM Cd. Values are the means of three replicate observations ±SD. Values significantly different from controls are marked with * ($P<0.05$).

In order to understand the alterations in GST activity throughout the time-course of Cd exposure the enzymatic activity was also monitored throughout a 2-days exposure to 100 μM Cd, as described in Fig. 6.2.10.

Results show that Cd induces GST activity in a time-dependent manner. A clear and significant increase in GST activity ($P<0.001$) was observed from 15 min to 1 hour of exposure. The GST activity reached a maximum at 30 and 60 min, but after 1 day, it returned to control values.

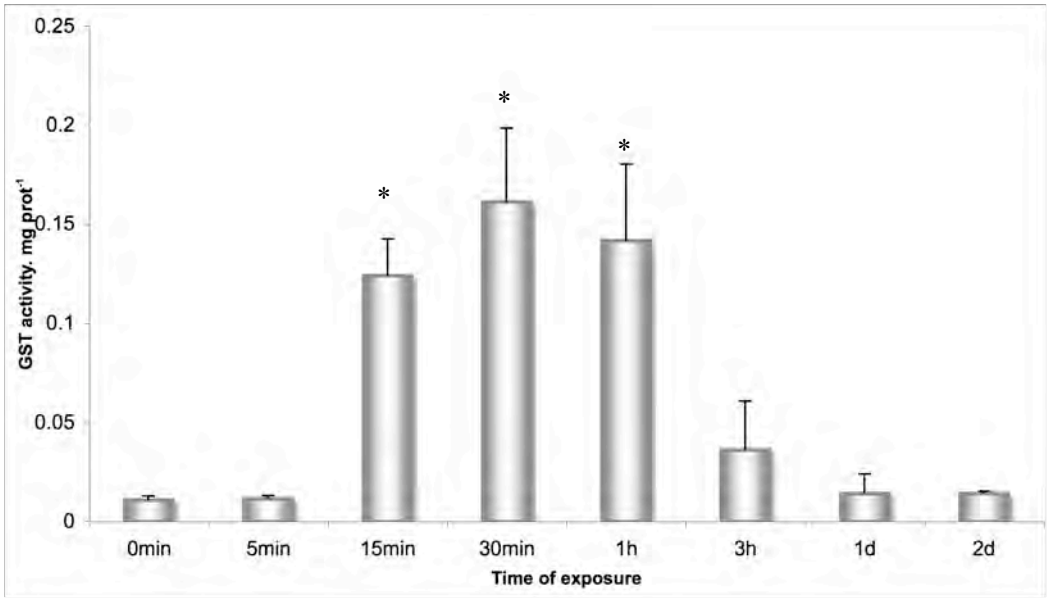


Fig. 6.2.10. GST activity in *A. thaliana* cells exposed to 100μM Cd throughout a period of 2 days. Values are the means of at least three replicate observations ±SD. Values significantly different from controls are marked with * ($P<0.05$).

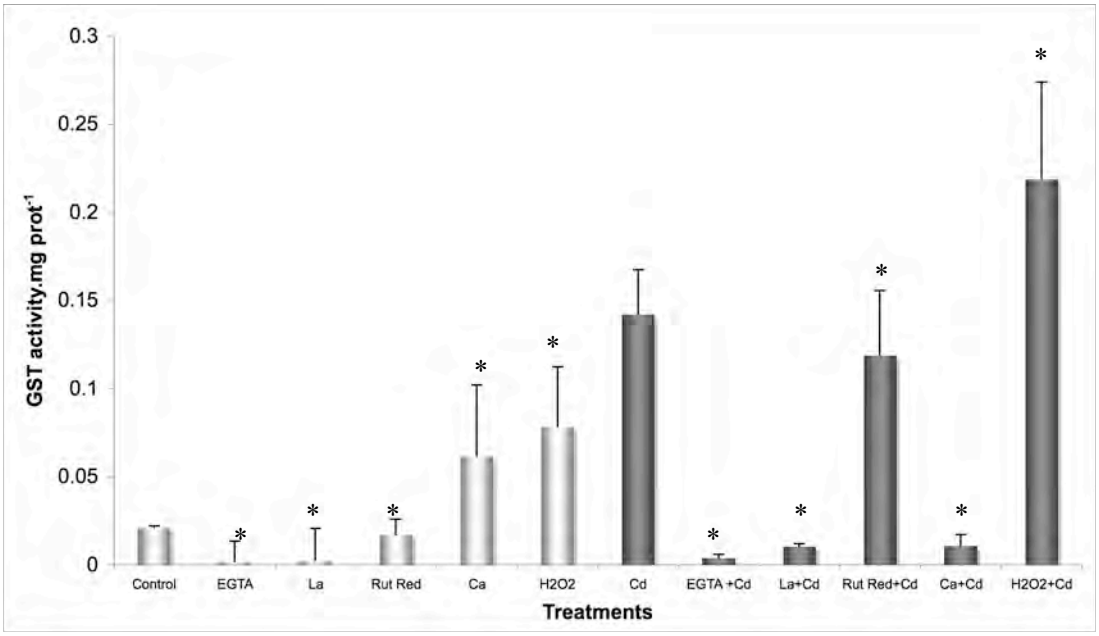


Fig. 6.2.11. Effects of different calcium modulators H_2O_2 ROS on GST activity of *A. thaliana* cells exposed to 100μM Cd. Values are the means of three replicate observations ±SD. Values significantly different from controls are marked with * ($P<0.05$).

The influence of ROS and calcium modulators on GST activity was further addressed in order to understand if ROS signalling can influence the enzymatic activity of GSTs (Fig. 6.2.11). Independently of the Cd presence, EGTA and La reduced GST activity ($P<0.05$), ruthenium red did not exert a significant effect ($P<0.05$) whereas H_2O_2 incremented this enzyme activity. However, the influence of Cd changed the influence of Ca on GST activity, increasing it in its presence or reducing it in its absence.

6.3 Discussion

6.3.1. ROS are induced by GSH signals

Although some works have monitored ROS accumulation under metal stress (Cho and Seo 2005; Foyer et al. 1997, Neill et al. 2002). Few works analyzed the build-up of this reactive oxygen species throughout the timing of the initial Cd signaling induction. In this work, the accumulation of H_2O_2 was enhanced with increasing Cd concentrations, but during the first minutes of Cd exposure there was is a small ROS pulse after the first Cd entrance in the cell, which was increased only during the first 30 minutes. A second enhancement of H_2O_2 was only observed much later, after 3 hours of exposure. These results could indicate that ROS are involved in an early signaling event during Cd stress, since the first ROS enhancement was much less pronounced and could not have been due to an oxidative burst. When comparing ROS accumulation with Cd absorption and thiol levels in the cell, results suggest that the first increase in ROS were most likely due to the modulations on GSH levels (Fig. 6.2.3). The timing of these fluctuations are accompanied by exact opposite trends in ROS levels. GSH has been proposed as a signal transducer for abiotic stresses. In plants, treatments that affected the GSH/GSSG ratios had also opposite effects on the H_2O_2-triggered rise in the cytosolic Ca concentration (Price et al., 1994). These authors reported that a pretreatment with BSO heightened the rise in Ca whereas the inhibition of ascorbate peroxidase which increases the GSH/GSSG ratio by blocking the

ascorbate-glutathione cycle, lowered the cytosolic Ca. These observations suggested that it may not be H_2O_2 accumulation *per se* that is sensed by the plant but its effect on the GSH/GSSG ratio.

6.3.2. ROS reduce Cd tolerance by inhibiting PC synthesis

Several woks point out that the toxicity of heavy metals is partially due either to the inactivation of functional proteins, including enzymes, caused by direct binding of the metal ion, or to oxidative damage caused by the accelerated generation of reactive oxygen species (ROS) (Hall, 2002; Boominathan and Doran, 2003). Our results indicate that ROS addition significantly reduced Cd tolerance Nonetheless, the addition of ROS was found to enhance significantly GSH production, both in control and in Cd treatments. This induction was not observed on Cys levels neither on synthesized PCs, which remained similar to those observed under Cd treatments alone. Xiang and Oliver (1998) have demonstrated that H_2O_2 does not induce accumulation of GSH metabolic gene transcripts such as *gsh 1* and *gsh 2*, although this GSH increase was detected. Therefore, increases in γ-ECS and GSHS activities were more likely to be due to post-transcriptional regulation via stimulation of H_2O_2 generation**.** Our results demonstrate that ROS induced a high GSH production, through an increase of the activity levels of both GSHS and γ-ECS activity.

6.3.3. Ca modulations affect ROS

In the present work, several pharmacological calcium modulators were used, in order to understand its effect on H_2O_2 accumulation. Results provided interesting observations. Under control conditions, all calcium inhibitors induced an increase in ROS levels, whereas excess Ca reduced them. In the presence of Cd, Ca also reduced ROS, but the fact that Ca blockers did not enhanced ROS under Cd stress is probably due to the reduction of Cd absorption observed with EGTA and La (see chapter 4). This explains why in Rut Red+Cd treatments ROS accumulation was similar to Cd treatments. These results are consistent with a scenario in which Ca

stimulation enhances GSH levels in the cell, which in turn reduces ROS levels by direct scavenging.

In chapter 4 it was shown that γ-ECS and GSHS activities were modulated by Ca. Since it has been reported that increasing ROS can enhance Ca signals, results presented here suggest that the alterations in GSH levels can be associated with ROS accumulation. If Ca signals are induced after ROS increase, then a signaling pathway for GSH synthesis may involve both Ca and ROS. Nonetheless, PCS activities were reduced with ROS addition. It is possible that the addition of ROS would consume most reduced GSH in the cells, decreasing the substrate for PCS and therefore reducing PC synthesis. This is consistent with the results obtained with the GSH inhibitor, BSO, that showed enhanced increase in ROS formation.

If calcium is an inducer of GSH synthesis, it is possible that the presence of ROS might increase calcium levels in the cell, which would in turn enhance GSH production. This could be another part of the signaling pathway for GSH production where the Cd-induced ROS would trigger GSH synthesis. On the other hand, although Ca probably has no effect on PCS (see chapter 4), the increase in GSH activates PCS activity, as demonstrated in chapter 3. Xiang and Oliver (1998) have proposed the idea that PC synthesis is regulated at multiple levels in the presence of Cd. In this model, Cd increases PC synthesis by activation of PCS and promotes the synthesis of GSH not only through transcriptional activation of the GSH biosynthetic pathway, but also through stimulation of endogeneous generation of ROS such as H_2O_2. Nonetheless, results presented here demonstrate that PCS activity is reduced in the presence of ROS.

6.3.4. GSTs are induced by ROS and are modulated by calcium

PCS has a clear preference for the bisglutathionate conjugate compared to GSH (Vatamaniuk et al., 2004). These reports suggest that conjugated glutathione activates PCS, which indicates the importance of GSTs for PC synthesis. In the present work, we demonstrated that GSTs are important in

Cd tolerance. Nonetheless, the observed increase in the activity of GSTs with increasing Cd concentrations demonstrates that similarly to other types of abiotic stresses, GSTs are involved in Cd detoxification, possibly by direct binding of Cd to GSH **(**Rentel and Knight, 2004). When observing the time-course exposure to 100 μM Cd it became more evident that this role might be important during the initial time of stress, but became less important afterwords. Results presented show that GST activity is several times increased after 15 minutes, reaching a higher peak at 30 minutes and decreasing therein. These findings seem to suggest that GST activity might be important during the initial binding of Cd with GSH, when intracellular Cd is still in low concentrations, becoming less important afterwards.

It has also been reported that increased accumulation of H_2O_2, usually connected with changes in the cellular redox status, alerts the plant cell against environmental stresses (Ghoshroy et al. 1998; Foyer and Noctor 2003; Rentel and Knight 2004), and may enhance the plant's antioxidant response through calcium signaling in the expression of glutathione transferase gene (Rentel and Knight 2004). Therefore, in this work we analyzed the activities of GST enzymes in the presence of ROS and calcium modulators. Our results clearly show that ROS increased GST activity, but also did calcium. These results are consistent with the findings of Rentel and Knight (2004) and allow us to speculate that GSTs can be important for PC synthesis, and that they can also be triggered by calcium and ROS signaling. However GST importance is only present in the first initial stages of Cd stress. It is possible that he cell would respond initially to Cd stress by enhancing GSH binding to this metal and these conjugates would then activate PCS, inducing the PC-based mechanism.

6.4. Conclusions

Results presented here provided important insights on the pathways induced by Cd stress, that ultimately can lead to enhanced PC synthesis. This work suggests that Cd and PC-induced alterations in GSH levels can be

possible signal mediators, which can trigger ROS signals in cells. These signals are also associated with Ca levels in the cell and it was proposed that ROS enhancement induced by GSH depletion can trigger a Ca signal, that can in turn enhance GSH synthesis. If true, this hypothesis suggests that GSH synthesis can be indirectly activated by Cd and PC production through Ca and ROS signaling pathways. The Ca and ROS increases also induced GSTs, which in turn were more active during the first initial stages of PCS activity. This suggests a possible role of assisting in PC synthesis by providing more GS-Cd conjugates to the cell. Not only these findings came to offer new information on possible points of crosstalk between the GSH synthesis and the PC synthesis pathway, but can also be extremely useful for understanding other abiotic stress signaling pathways, such as salt, oxidative and temperature stress, where GSH levels and ROS are known to be important signal mediators.

Chapter 7

Concluding Remarks

The importance of signal transduction mechanisms in plants has been emerging as an important research subject in plant sciences. The increasing number of new reports has brought to light several novel discoveries that helped to improve our knowledge on signal transduction pathways, not only in plants, but also in animals, because most signaling intermediates are highly conserved throughout all organisms. The importance of studying metal-tolerance mechanisms in plants has also become another important field of research, for more than 3 decades, as a way to prevent the dangers of metal-contaminated crop and to allow the sustainable development of phytoremediation techniques.

Metal tolerance in plants is mostly achieved by phytochelatins. The ability to induce PCs and effectively chelate metal ions is a recognized key factor for plant survival in metal contaminated soils and is pointed out as the basis for the differences in heavy metal tolerance between different plant species. Since signal transduction mechanisms control almost every aspect of the cell life, this work aimed to understand the possible pathways of PC synthesis and Cd tolerance activation in order to understand how these regulatory pathways might be used for improving plant metal tolerance in environmental metal contaminations. Results obtained yielded important insights of PC synthesis activation, either directly through activation of GSH synthesis or by an increase of PCS activity.

First, the timing of Cd-triggered alterations was determined. Most of the important signalling pathways took place between the first 15 min of Cd exposure and 1 hour, after which all enzymatic processes were fully triggered for maintaining the equilibrium in GSH pools, whilst Cd chelation was achieved. For both PCS and γ-ECS, enzyme activation was shown to be a primary point of PCs production, being

both apparently influenced in a Cd-dependent manner and associated with different regulatory pathways, that might interact with each other (Fig. 7.1).

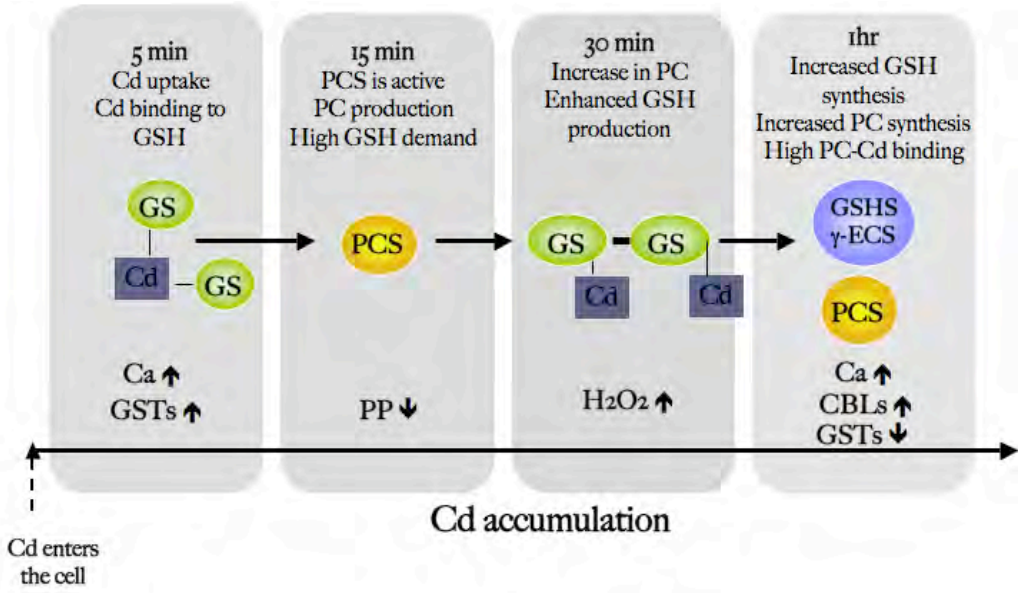


Figure 7.1. Timing of the major events triggered during Cd stress and phytochelatin synthesis. After Cd entrance in the cell, GSTs are mostly active after 5 minutes and calcium concentrations can be possibly altered. At 15 minutes PCS becomes active, and an inhibition of PP1 occurs and PCs begin to accumulate in cells. This is accompanied by a GSH depletion, and also an induction in ROS production. After this, γ-ECS and GSHS catalytic activities are enhanced, which can be modulated by Ca, and produce more substrate for PC synthesis and a higher Cd chelation.

In summary, most important alterations and signalling events found in this work were:

1. Cd is uptaken by calcium channels and is regulated by calcium levels. Because of strong evidence that Cd ions compete with Ca, it is possible that Ca levels are altered by Cd entrance in the cell.

2. Calcium was also shown to be one important regulator of GSH synthesis enzymes, but probably not of PCS (at least directly). During Cd-mediated Ca signalling, several different calcium sensors were involved, being one of them a calcineurin–B-like protein (CBL). Calcium sensors were highly expressed matching the timing of the activation of γ-ECS and GSHS, suggesting they could be involved in the signaling pathway of GSH synthesis.

3. Protein phosphatases were shown to be able to regulate PCS activity. Particularly PP1 protein phosphatase seems likely to be able to keep PCS inactive in the absence of Cd. The Cd inactivation of PP1 remains to be elucidated, but is possibly one key for enhancing PCS activity.

4. H_2O_2 was also presented as an signal transducer in Cd stress. Results suggest that GSH depletion, induced by Cd and PC synthesis is associated with a transient H_2O_2 accumulation, suggesting GSH can also be an important signal mediator. The H_2O_2 accumulation is is known to be associated with Ca production, stimulating a Ca signal in the cells, which in turn can influence GSH synthesis.

5. GSTs were pointed out as possible assistants in Cd chelation by GSH, since they were mostly induced during the initial stages of Cd imposure and were regulated by ROS (H_2O_2) and Ca signals.

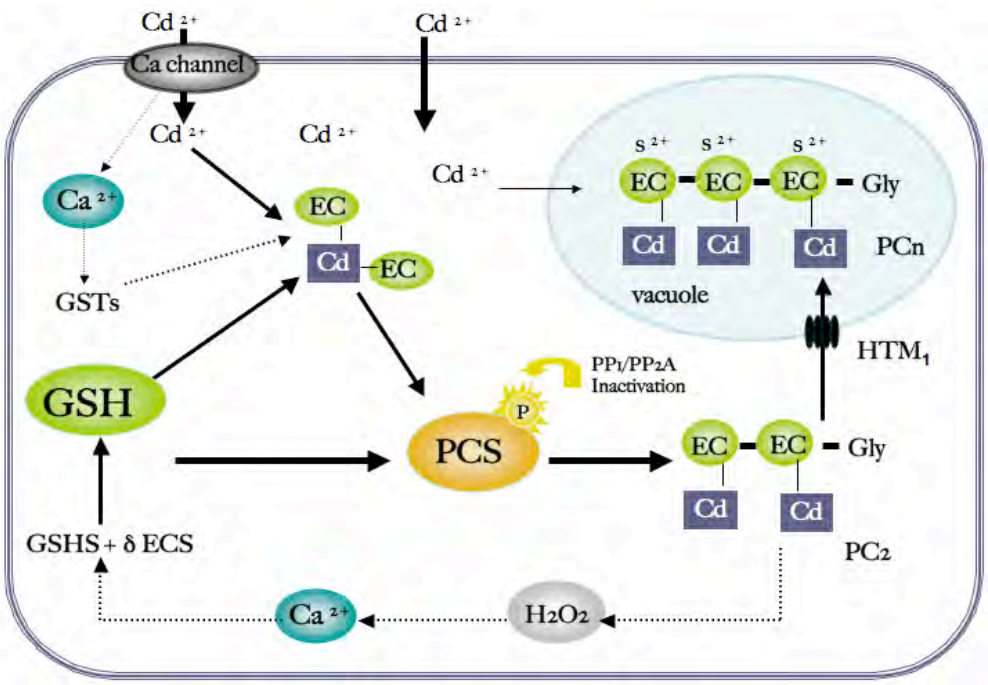


Figure 7.2. A model for Cd-induced signalling pathways, which can regulate PC synthesis. The entrance of Cd through Ca channels will induce alterations in Ca concentrations in the cell. These alterations can be involved with GST activity which can assist in GSH binding to Cd ions. The build-up of GS-Cd conjugates will activate PCS, that suffers a phosphorylation during activation, after PP1/PP2A inactivation. The increase in PCs will yield a high Cd binding and GSH depletion. This depletion will allow the increase of ROS, which enhances intracellular Ca. The increase in Ca can in turn enhance GSH synthesis, which will be used for PC synthesis. During PC synthesis, PC-Cd complexes are formed in the cytosol and are transported to the vacuole, were more Cd ions and sulphide are added, forming more stable complexes. Solid arrows represent events previously describe in other

works, whereas dashed arrows represent proposed events in the signalling of PC formation in plant cells under Cd stress.

Overall, it is therefore possible that Cd entrance in the cell stimulates a Ca signal, which can in turn activate GSTs for enhancing GSH-binding of the metal cations. Bisglutathionate conjugates and Cd will in turn activate PCS, which will ultimately reduce GSH levels. This decrease will in turn activate ROS signalling pathways, which will again induce Ca signatures that may enhance GSH production, via γ-ECS and GSHS. (See fig. 7.2).

Certainly most of the signals reported here would not be the sole regulators of PC synthesis, since other factors are known to activate PCS and the enzymes involved in GSH production, such as the well-known GSH-negative feedback induction of γ-ECS. Nonetheless, in this work, we recognized the importance of key mechanisms that allow plants to detect metal accumulation, such as Ca, ROS and protein phosphatases. These regulators showed to be important factors in modulating both PC synthesis and Cd accumulation in cells.

Taken altogether, the work presented here provides important insights on plant signal transduction of metal stress and bring novel perspectives on Cd tolerance regulation. Further study of these pathways and their signal transducer intermediates will possibly bring to light other stress factors already known to be stimulated by Cd such as MAP kinases and ethylene, among others. It is also possible that the regulation of these pathways, either by different calcium signatures, or different PP1 regulatory proteins can be involved in metal specificity. This information will open new doors to the field of heavy metal tolerance in plants and metal contamination managing techniques with promising new discoveries on how to regulate plant tolerance to heavy metals.

Nonetheless, our results already provided novel and important information, and can be of important use in sustainable agricultural practices. The modulation of Cd uptake in plants may lead to higher crop productivity and result in an efficient way to reduce Cd absorption in plants by modulating Ca signalling. This could decrease the amount of metal entering in the food chain and consequently also

decreasing the health risk to humans. The knowledge that results from the analysis of the molecular mechanisms underlying metal tolerance can also be extremely useful in the development and implementation of more effective techniques of phytoremediation, in which specific pharmacological agents, that can inhibit the PP1 activity associated with PCs, can be used for enhancing the plant's ability to produce PCs and hence develop higher tolerance levels to Cd stress. In this case, the increase in GSH production would have to be assured, since a high increase in PCS without the complementary GSH production would be detrimental to the plant.

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