UNIVERSITY OF CALIFORNIA, SAN DIEGO

Molecular mechanisms of cadmium detoxification and long distance transport in plants

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for the degree of Master of Science

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by

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Professor Yunde Zhao

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The thesis of Emerald Claire Butko is approved and it is acceptable in quality and form for publication on microfilm:

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ABSTRACT OF THE THESIS

Molecular mechanisms of cadmium detoxification and long distance transport in plants

by

Emerald Claire Butko

Master of Science in Biology

University of California, San Diego, 2008

Professor Julian I. Schroeder, Chair

Cadmium (Cd) is a toxic heavy metal and its remediation from contaminated soil is a matter of public health. Current remediation technologies are expensive and inefficient, and there is interest in the development of alternative strategies such as phytoextraction. In order to use plants to remove Cd from the soil, it must be taken up by the plant and efficiently translocated and stored in harvestable tissue. Thiol peptides such as glutathione (GSH) and phytochelatins (PCs) are known to chelate and sequester Cd

intracellularly and evidence has suggested that PCs may also be invoved in the long-distance translocation of Cd. Their role in detoxification, translocation and sequestration of Cd makes thiol-peptides of interest for the development of Cd phytoextraction technologies. In this thesis, the phloem and xylem saps of *Brassica napus* were analyzed and it was determined that the phloem is the major vascular system for the thiol-mediated translocation of Cd. Manipulation of PC-Cd transporters may facilitate Cd phytoextraction, however no plant PC-Cd transporters have yet been identified. In the second part of this thesis, a method for the screening and characterization of plant PC-Cd transporters is presented and validated with a known yeast PC-Cd transporter. In the third part of this thesis, a strategy is presented for how thiol-Cd transporters might be used to increase the retention of Cd in harvestable tissue. While this approach does not appear to have been successful, alternative approaches are also proposed.

Chapter One:

Identification of high levels of phytochelatins, glutathione and cadmium in the phloem sap of *Brassica napus*. A role for thiol-peptides in the long-distance transport of cadmium and the effect of cadmium on iron translocation.

1.1. Abstract

Phytochelatins (PCs) are oligopeptides that are synthesized from glutathione (GSH) upon cadmium exposure. These peptides have a high affinity for cadmium (Cd) and are known to function in the intracellular chelation and sequestration of Cd. Recently, PCs have also been shown to undergo long-distance transport between roots and shoots, although it is not yet known which of the vascular systems are involved. In this paper, highly pure phloem and xylem saps were extracted from *Brassica napus* exposed to Cd from one day to two weeks. Glutathione and cysteine were the only non-protein thiols in the xylem and the phloem saps prior to Cd exposure. Two weeks after Cd treatment, the concentration of Cd was found to be roughly 20μM in the phloem sap, and 4μM in the xylem sap, indicating a significant role for the phloem in the physiological distribution of Cd. PC_2 and PC_3 were detected in the phloem sap after 1 day, 1 week and 2 weeks of Cd exposure. After 2 weeks of Cd exposure the level of PCs in the phloem sap was found to be sufficient to provide complete chelation of the Cd present. In the xylem sap of Cd exposed plants, the level of PCs present was too low to be quantified, indicating that PCs are not major contributors to Cd translocation though the xylem. These results indicate that the phloem is the major vascular system for the thiol-mediated translocation of Cd.

1.2. Results and Discussion.

For my participation in this study, I assisted in the quantification of thiol-peptides in the phloem (Figures 2 and 4) and xylem sap samples (Figure 7) through the fluorescent labeling of these samples with monobromobimane. In Figure 2, phloem sap of *Brassica napus* not exposed to Cd was found to contain glutathione (GSH) and cysteine, and after 1 day, 1 week, and 2 weeks of Cd exposure, phytochelatins (PCs) were detected. The results shown in Figure 4 show the quantification of the thiols found in the phloem sap, and high levels of phytochelatins were quantified in the phloem sap samples after 2 weeks of Cd exposure. In Figure 7, the xylem sap of Brassica napus not exposed to Cd was found to contain GSH. In some xylem sap samples collected after 1 day, 1 week and 2 weeks of Cd exposure, PCs were detected by mass spectrometry but were present at very low levels.

Thiol peptides are produced intracellularly throughout the entire plant, and confidence in the thiol content detected in the vascular fluids relies on the samples having little contamination from surrounding tissues. For our analysis of thiol content of the vascular systems, we obtained highly pure phloem samples using the method described in Giavalisco et al., 2006. The purity of the phloem sap was determined by measuring the ratio of reducing sugars such as glucose and fructose to non-reducing sugars such as sucrose, which was found to be 1.4%, while in stems this ratio can be up to 72% (Geigenberger et al., 1993; Giavalisco et al., 2006). This indicated that the samples extracted can be considered as phloem sap with neglibible (less than 2%) contamination from the surrounding tissues.

Without Cd treatment, both phloem and xylem samples were found to contain thiols in the form of cysteine and glutathione. Phytochelatins (PCs) became detectable and quantifiable in the phloem sap after 1 day, 1 week and 2 weeks of Cd exposure. At 1 week, the ratio of thiols from PCs to Cd was found to be roughly 1.5:1. Among all functional groups, Cd has the highest affinity for thiols and among thiols, Cd has a particularly high affinity for PCs: the K_d for Cd binding to cysteine is 1.28×10^{-10} M, while the K_d for PCs is 7.9×10^{-17} M (Dorcák and Krezel, 2003). Even with the high affinity that PCs have for Cd, a ratio of at least 4 phytochelatin thiol groups for every Cd is necessary to ensure stable chelation of Cd (Dorcak and Krezel, 2003; Pickering et al., 1999). This means that after 1 week of Cd exposure, even though there are more PCs than Cd in the phloem it is likely that Cd is also bound to other thiol-containing compounds in the phloem such as glutathione and cysteine, in addition to phytochelatins. However, after 2 weeks of cadmium exposure, the ratio of PCs to Cd in the phloem was roughly 5:1 (Figure 4), indicating that PCs were stably chelating the Cd in the phloem.

The thiol-containing compounds glutathione and cysteine were detected at quantifiable levels in the xylem before and after Cd exposure. After Cd exposure, PCs were detectable in some samples by mass spectrometry although the levels were too low to quantify accurately (Figure 7). While mass spectrometry confirmed the presence of phytochelatins in some samples, we did not assess the purity of the xylem samples. It is possible that the PCs detected were a result of contamination from phloem sap or from surrounding tissues. Even if the PCs detected were not the result of contamination, their level in the xylem sap was too low to be physiologically relevant in Cd chelation in the xylem sap.

While there were quantifiable levels of glutathione and cysteine present in the xylem after Cd exposure, the total thiol content of the xylem was roughly 50-fold less than the thiol content of the phloem sap (Figure 8; Figure 4). In addition, the slightly acidic conditions of the xylem sap (pH 5.8, n=2) provide a less favorable environment for thiol-Cd interactions and therefore thiol peptides are less likely to be associated with Cd than the phloem sap (pH 7.7, n=2). Indeed, a study of PC-Cd associations found that with a 2:1 stoichiometry of PC thiols to Cd, nearly half of the Cd in solution was present as a free ion at pH 5, while at pH 7.3 only 9×10^{-9} % of Cd was present in an unchelated form (Dorcak and Krezel, 2003). The findings that the thiol content is too low and the pH is too acidic in the xylem for significant thiol chelation of Cd is in agreement with a previous study which reported that in the xylem sap, Cd associated mainly with oxygen and nitrogen containing compounds which were not further characterized (Salt et al., 1995).

In this study, the high levels of PCs, present in the phloem and the more favorable conditions for thiol chelation of Cd suggest PC-mediated redistribution of Cd occurs through the phloem, while Cd is likely to be bound to other compounds during translocation through the xylem.

1.3. Mendoza-Cózatl et al., 2008.

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Identification of high levels of phytochelatins, glutathione and cadmium in the phloem sap of *Brassica napus*. A role for thiol-peptides in the long-distance transport of cadmium and the effect of cadmium on iron translocation

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Summary

Phytochelatins (PCs) are glutathione-derived peptides that function in heavy metal detoxification in plants and certain fungi. Recent research in Arabidopsis has shown that PCs undergo long-distance transport between roots and shoots. However, it remains unknown which tissues or vascular systems, xylem or phloem, mediate PC translocation and whether PC transport contributes to physiologically relevant long-distance transport of cadmium (Cd) between shoots and roots. To address these questions, xylem and phloem sap were obtained from *Brassica napus* to quantitatively analyze which thiol species are present in response to Cd exposure. High levels of PCs were identified in the phloem sap within 24 h of Cd exposure using combined mass spectrometry and fluorescence HPLC analyses. Unexpectedly, the concentration of Cd was more than four-fold higher in phloem sap compared to xylem sap. Cadmium exposure dramatically decreased iron levels in xylem and phloem sap whereas other essential heavy metals such as zinc and manganese remained unchanged. Data suggest that Cd inhibits vascular loading of iron but not nicotianamine. The high ratios [PCs]/[Cd] and [glutathione]/[Cd] in the phloem sap suggest that PCs and glutathione (GSH) can function as long-distance carriers of Cd. In contrast, only traces of PCs were detected in xylem sap. Our results suggest that, in addition to directional xylem Cd transport, the phloem is a major vascular system for long-distance source to sink transport of Cd as PC–Cd and glutathione–Cd complexes.

Keywords: cadmium translocation, iron content in xylem, long-distance transport, heavy metals, thiol-peptides, mass spectrometry.

Introduction

Heavy metal pollution has become a serious environmental and health problem and bioremediation has been proposed as a sustainable technology for the clean-up of sites contaminated with heavy metals and other toxic compounds (Lasat, 2002; Omichinski, 2007). An efficient phytoextraction strategy will require significant amounts of heavy metals to be translocated to the aerial, harvestable, tissues of plants. Genetic manipulation of plants to achieve this goal requires a mechanistic understanding of how heavy metals are mobilized within the plant. The chemical nature of cadmium (Cd) complexes during long-distance transport between shoots and roots remains incompletely understood. It is also unknown which vascular system, xylem or phloem, is more active in transporting this heavy metal. In xylem sap, oxygen- and nitrogen-containing compounds have been identified as ligands able to bind Cd (Salt *et al.*, 1995) but neither the full identity of these compounds nor the relative contributions of the xylem and

phloem to long-distance transport of Cd have been quantified.

Cadmium is an extremely reactive heavy metal with affinity towards the functional groups of biomolecules (i.e. phosphate, carboxy, amino and thiol groups; Sillén and Martell, 1964). Among these molecules, thiols have the highest affinity towards Cd, and this reactivity has led to the hypothesis that, within the cell environment, Cd is more likely to form complexes with biomolecules than to be present as a free ion (Mendoza-Cózatl *et al.*, 2005; Vatamaniuk *et al.*, 2000).

Phytochelatin-mediated heavy metal detoxification is a major mechanism for Cd resistance in plants, some yeast and photosynthetic protists (Grill *et al.*, 1985; Hayashi *et al.*, 1991; for reviews see Clemens *et al.*, 2002; Mendoza-Cózatl *et al.*, 2005). Phytochelatins (PCs) are glutathione (GSH)-derived peptides synthesized by the transpeptidase phytochelatin synthase (Clemens *et al.*, 1999; Ha *et al.*, 1999; Vatamaniuk *et al.*, 1999). Originally, PCs were considered solely as an intracellular mechanism for Cd detoxification by shuttling PC–Cd complexes into plant cell vacuoles. However, recent studies have shown that in transgenic *Arabidopsis thaliana* and in grafted Arabidopsis plants, PCs also have the ability to undergo long-distance transport in the root-to-shoot and shoot-to-root directions (Chen *et al.*, 2006; Gong *et al.*, 2003). As PC synthesis is strictly dependent on the presence of heavy metals, it is conceivable that PCs may be involved in the long-distance transport of Cd, and possibly other heavy metals. However, the physiological relevance and relative contribution of PCs and other thiols to long-distance transport of Cd has not yet been directly examined.

Thiol transport from shoots to roots seems to be a very efficient process. Shoot-specific expression of the key enzyme of GSH biosynthesis, γ-glutamylcysteine synthetase (γ-EC synthetase), in an Arabidopsis GSH-deficient mutant restored the levels of all thiols [γ-glutamylcysteine (γ-EC), GSH and PCs] in roots (Li *et al.*, 2006). However, whether γ-EC, GSH, PCs or any other thiol-peptide can be transported through the phloem of plants has not yet been directly quantified.

In this study, experiments were pursued to determine whether xylem and/or phloem mediates PC, γ-EC and GSH transport and to analyze the role of these thiols in the long-distance transport of Cd. We apply a well-developed technique in *Brassica napus*, a close relative of Arabidopsis, to extract large amounts of pure phloem and xylem sap and determine the concentrations of different thiol species and Cd. The results show that PCs are present in the phloem sap in large quantities that are sufficient to form stable complexes with Cd. The [thiol]/[Cd] stoichiometries also reveal that GSH may contribute to the long-distance transport of Cd. The Cd concentration and abundance of thiols suggest that the phloem plays a major role in the long-distance source-to-sink transport of Cd as thiol–Cd complexes.

Results

Analysis of purity of phloem sap

Brassica napus was chosen as a model, because the method for obtaining large quantities of highly pure phloem is well established (Giavalisco *et al.*, 2006). The Cd concentration used in the present study (75 μM), induced strong phytochelatin synthesis but allowed the plants to flower for phloem sampling (Figure 1a). As a first step, the purity of phloem sap used for thiol and Cd measurements was evaluated. This was determined by measuring the ratio of reducing sugars (glucose, fructose) to total sugars present in the sap. The levels of glucose, fructose and sucrose in phloem sap samples are shown in Figure 1(b). From these values, a reducing sugars/total sugars ratio of 1.8% was calculated. This low ratio was indicative of a highly pure phloem sap, as the same ratio in the surrounding stem tissues can be up to 72% (Geigenberger *et al.*, 1993; Giavalisco *et al.*, 2006).

Identification of thiol in the phloem sap

Thiols in the phloem sap were separated and quantified by fluorescence HPLC (Figure 2). In phloem sap obtained from

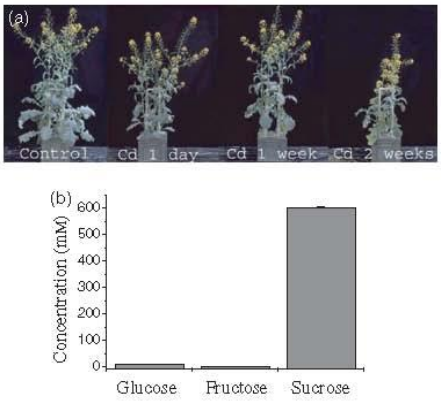


Figure 1. Phloem and xylem sap samples were obtained from *Brassica napus* grown in hydroponic cultures.
(a) Plants were grown under greenhouse conditions. After 9 weeks plants were exposed to 75 μM $CdSO_4$ in the hydroponic solution for 1 day, 1 week or 2 weeks before phloem and xylem sap sampling.
(b) Sugar content in phloem sap samples was measured enzymatically to assess the purity of the sap. The ratio of [reducing sugars]/[total sugars] obtained from these concentrations was 1.8%, showing highly pure phloem sap. Values shown are means ± SE ($n = 5$).

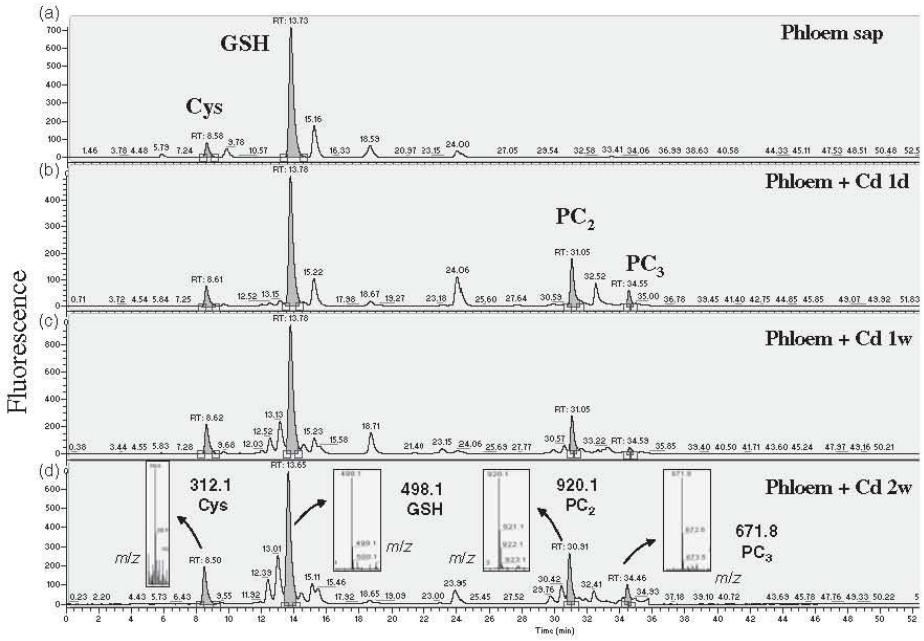


Figure 2. Cadmium exposure results in the appearance of phytochelatins (PCs) in the phloem sap of *Brassica napus*. Thiols from (a) control and plants exposed to 75 μM $CdSO_4$ for (b) **1** day, (c) **1** week and (d) 2 weeks were labeled with monobromobimane, separated by reverse phase HPLC and detected by fluorescence. Thiols were identified with a mass spectrometer coupled to the HPLC. Phytochelatins appeared after **1** day of cadmium exposure and their concentrations increased with the time of exposure. Note the difference in the *Y*-axis scales in panels (c) and (d). The insets in (d) show the mass spectrum of the corresponding monobromobimane-labeled thiols. Bimane label accounts for the addition of **190** Da per thiol to the mass of the unlabeled compounds.

control plants (non-Cd treated), GSH was the most abundant thiol (Figure 2a, $n = 4$ experiments; two to three samples measured per experiment). Cysteine (Cys) was also present, although at a much lower level (≈10% relative to GSH; Figure 2a). No PCs were detected in phloem sap obtained from control plants (Figure 2a). Interestingly, PCs were clearly detected in the phloem sap 1 day after the onset of Cd exposure and after 1 and 2 weeks of Cd treatment. (Figure 2b–d, $n = 3$–4 experiments; two or three samples measured per experiment). The identity of the PCs was directly determined by parallel mass spectrometry analyses of the thiol-labeled samples (Figure 2d, insets). The bimane label increases the mass of the thiol compounds by 190 Da per thiol labeled. Therefore, PC_2, which has a molecular mass of 539.1 Da and contains two thiols per molecule, was detected as a singly charged ion at *m*/*z* 920.1. Phytochelatin PC_3 was detected as a doubly charged ion at *m*/*z* 671.8 (Figure 2d). Phytochelatin PC_4 would be observed as a doubly charged ion at *m*/*z* 882.9, as determined by the use of a PC_4 standard, but no PC_4 was detected in any of the phloem sap samples analyzed ($n = 3$ experiments for phloem sap from 1 day and 1 week of Cd exposure and $n = 4$ experiments for plants exposed to Cd for 2 weeks). Glutathione was the most abundant thiol at all time points measured, even during Cd exposure (Figure 2a–d, $n = 3$–4).

After 1 week of Cd exposure, additional thiols with retention times close to GSH and PC_2 were also detected (Figure 3). The peak eluting after GSH was identified as γ-EC (*m*/*z* 441.1, singly charged ion; Figure 3). The peaks eluting before GSH were identified as GSH-related peptides. The thiol compound at *m*/*z* 569.1 corresponded to γ-glutamyl-cysteinyl-glutamine (γ-ECQ), often called homoglutathione, where glutamine substitutes for the glycine at the C-terminus (Kubota *et al.*, 2000). The glutamine substitution at the C-terminus was confirmed using tandem mass spectrometry (Figure S1). The thiol compound at *m*/*z* 528.1 showed the expected mass for hydroxymethyl-glutathione (γ-ECS), where serine substitutes for the glycine in the GSH molecule. The same amino acid modifications were found for the compounds near PC_2 (Figure 3). The *m*/*z* 863.0 ion was

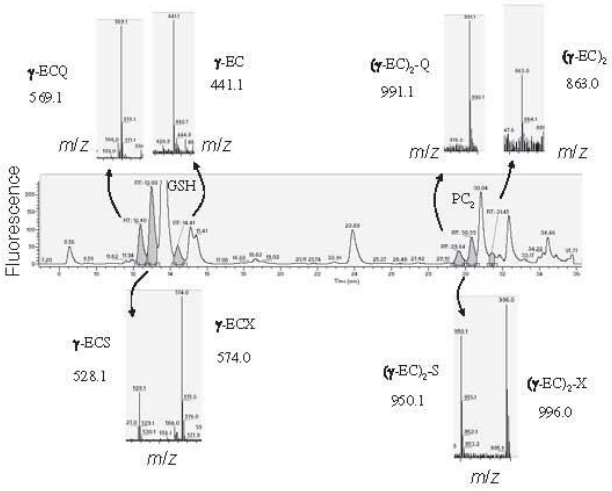


Figure 3. Glutathione (GSH) and phytochelatin (PC) related peptides were induced after 1 week of Cd exposure.
Thiol-containing peptides with retention times close to GSH and PC_2 were detected after Cd treatment. In the upper part of the chromatogram from left to right the compounds were identified as: γ-glutamyl-cysteinyl-glutamine (γ-ECQ; 569.1 Da), γ-glutamylcysteine (γ-EC; 441 Da), glutamine-PC_2 [(γ-EC)$_2$-Q; 991.1 Da), desGly-PC_2 [(γ-EC)$_2$; 863.0 Da). The mass spectra corresponding to hydroxymethyl-GSH (γ-ECS; 528 Da) and hydroxymethyl-PC_2 [(γ-EC)$_2$-S; 950.1 Da) are shown below the main chromatogram. These ions were accompanied by an ion that is 46 Da heavier. In the case of the ion with a mass of 574.0 Da, tandem mass spectrometry confirmed that this compound is a γ-EC peptide with a modified amino acid residue (133 Da) at the C-terminus.

identified as desGly-PC_2 [(γ-EC)$_2$], the *m/z* 991.1 ion corresponded to glutamine-PC_2 [(γ-EC)$_2$-Q] and the *m/z* 950.1 ion was assigned as hydroxymethyl-PC_2 [(γ-EC)$_2$-S]. Interestingly, both serine-containing thiol-peptides (hydroxymethyl-GSH and hydroxymethyl-PC_2) were accompanied by an ion that was 46 Da heavier (*m/z* 574 and *m/z* 996; Figure 3). The presence of the b_2 ion (corresponding to γ-EC) in the tandem MS of the *m/z* 574 ion confirmed that this compound is a γ-EC-related peptide with a modified amino acid at the C-terminus that differed from a serine, glutamic acid, glutamine or β-alanine (Figure S2). The precise chemical structure of this modified amino acid was not determined in this study (see Discussion). These analyses directly demonstrated the presence of PCs in the phloem sap during Cd exposure and led us to further quantify the different thiol compounds and the Cd levels in the phloem sap during Cd exposure.

Thiols and heavy metal content in the phloem sap after Cd exposure

The quantification of acid-soluble thiols from the phloem sap is shown in Figure 4. Levels of Cys and GSH remained constant over the duration of Cd exposure (Figure 4a,c; n = 3-4). The γ-EC and PCs were undetectable under control conditions and increased dramatically in response to Cd exposure (Figure 4b,d). Interestingly, the glutathione- and PC-related peptides (see above, Figure 3) were induced only upon Cd exposure and their levels increased with the time of Cd exposure (Figure 4e,f; n = 3–4). However, canonical GSH and PCs were more abundant at all times than their corresponding modified peptides (Figure 4c–f).

The levels of cadmium, iron, zinc and manganese in the phloem sap were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES) and are shown in Figure 5. The Cd content of the phloem sap was similar after 1 and 2 weeks of continuous Cd exposure (Figure 5a, n = 3–4), reaching a concentration of 20 μM after 2 weeks of treatment. Whereas zinc and manganese remained constant both before and during Cd exposure (Figure 5c,d), iron showed a dramatic decrease after 1 and 2 weeks of Cd exposure to 36% of the iron content in the phloem sap under control conditions (Figure 5b, n = 3–4). This decrease in iron was found to be independent of the level of the phytosiderophore nicotianamine (NA), as determined by NA measurements performed by fluorescence HPLC. In fact, Cd induced a significant increase (P < 0.01) in NA levels in phloem sap obtained from plants exposed to Cd for 2 weeks (132.65 ± 25.6 nmol NA mg^{-1} protein; mean ± SD, n = 3) compared with control plants (54.2 ± 10.2 nmol NA mg^{-1} protein; mean ± SD, n = 3). Therefore, NA is not responsible for the decreased levels of iron found in the phloem sap during Cd exposure.

Iron content in leaves and roots during cadmium exposure

To further establish whether the decrease in iron content in the phloem sap was due to a decreased uptake of iron into roots or an impaired translocation from roots to shoots, the iron content in roots and leaves from *B. napus* was

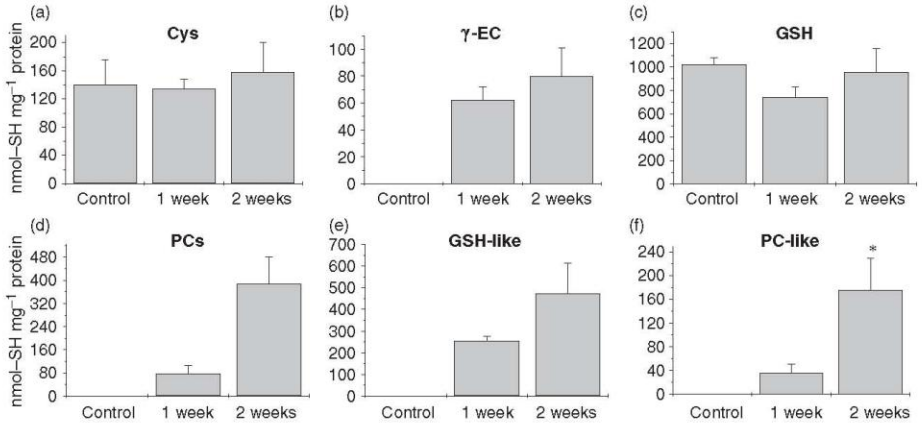
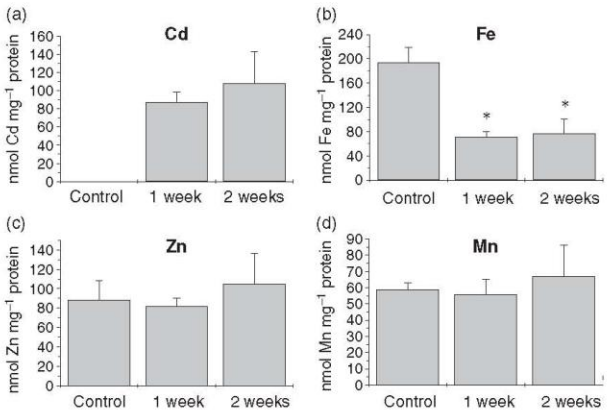


Figure 4. Thiol content in the phloem sap of *Brassica napus*. The contents of (a) cysteine (Cys), (b) γ-glutamylcysteine (γ-EC), (c) glutathione (GSH), (d) phytochelatins (PCs), (e) GSH-like peptides, and (f) PC-like peptides were measured in the phloem sap of non-treated and Cd exposed plants (see Results).
(a, c) Cysteine and GSH levels remained constant independently of the length of Cd treatment.
(b) The level of γ-EC in phloem sap samples increased dramatically upon Cd exposure. The GSH-like (sum of γ-glutamyl-cysteinyl-serine and γ-glutamyl-cysteinyl-glutamine, γ-ECQ) and PC-like (sum of hydroxymethyl-PC_2 [$(\gamma\text{-EC})_2$-S] and glutamine-PC_2 [$(\gamma\text{-EC})_2$-Q]) peptides were also induced only after Cd exposure (d–f). Levels of PCs and their homologs are expressed in thiols mg^{-1} of protein. The phloem sap contained on average 0.174 ± 0.09 mg protein ml^{-1} (mean ± SE, n = 4); this value was used to express GSH in concentration (196 μM after 2 weeks of Cd exposure), allowing direct comparison with the xylem sap measurements.
The asterisk in the content of PC-like peptides after 2 weeks of Cd exposure denotes a significant difference compared with the levels of PC-like peptides after 1 week of treatment. Values shown are n = 3–4 experiments; two or three samples were measured per experiment.

Figure 5. Heavy metal content in the phloem sap of *Brassica napus*. The levels of (a) cadmium, (b) iron, (c) zinc and (d) manganese were measured in the phloem sap from control and Cd exposed plants.
(a) Using the relationship 0.174 ± 0.09 mg protein ml^{-1}, a Cd concentration of 15.5 μM for 1 week of Cd exposure and 20 μM for 2 weeks of exposure was calculated. Cadmium content in the phloem sap after 1 day if Cd exposure was 35 nmol Cd mg^{-1} protein (n = 2).
(b) Iron concentration dramatically decreased during Cd exposure (*, P < 0.01) with respect to plants not exposed to Cd.
(c, d) No significant differences were found in the zinc and manganese content. Values shown are n = 3–4 experiments; two or three samples were measured per experiment.



determined (Figure 6, n = 3–4). The iron content in leaves from plants exposed to Cd decreased dramatically after 1 and 2 weeks of Cd treatment to 40% of the iron content from leaves grown without Cd (Figure 6a). Interestingly, iron content in roots increased with the time of Cd exposure (Figure 6b). After 2 weeks of Cd treatment iron concentrations showed a three-fold increase in roots relative to plants grown without Cd. These results suggest that the decrease in iron in the phloem sap (Figure 5b) is more likely to be related to an impaired vascular loading and root to shoot translocation of iron, rather than due to a Cd-induced blocking of iron uptake into roots.

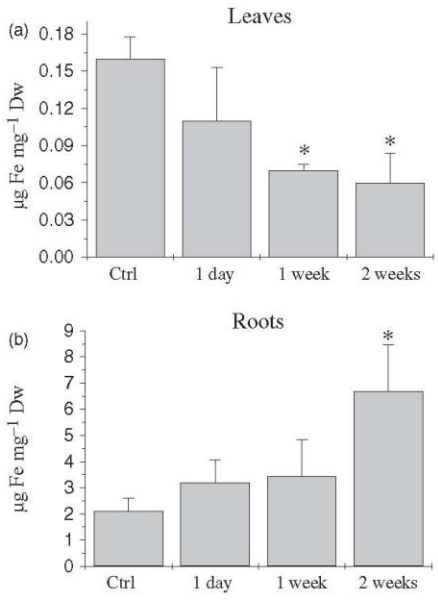


Figure 6. Iron levels in leaves and roots of *Brassica napus* were affected by Cd exposure. Iron levels in (a) leaves and (b) roots from non-exposed and Cd-treated plants were measured by inductively coupled plasma optical emission spectroscopy. Iron content in leaves was dramatically reduced after 1 and 2 weeks of Cd exposure ($P < 0.05$; a), whereas Fe showed significant overaccumulation in roots after 2 weeks of Cd exposure ($P < 0.05$; b). Asterisks denote statistically significant differences compared with non-treated plants. Values shown are $n = 3$–4 experiments; two or three samples were measured per experiment.

Thiols are more abundant than cadmium in the phloem sap

With the Cd content and thiol measurements shown in Figures 4 and 5 it was possible to establish the [GSH]/[PCs] and [PCs]/[Cd] stoichiometries present in the phloem sap. These ratios are important for understanding which stable complexes can be formed in the phloem sap and to determine whether thiols can function in the chelation and long-distance transport of Cd. Phloem sap obtained from Cd-exposed plants contained a higher concentration of thiols than Cd ([thiols]/[Cd] ratio > 10; Figures 4 and 5). Of the different thiol species, GSH and PCs were the most abundant (Figure 4). The ratio of [GSH]/[PCs] after 1 week of Cd exposure was 8:1, whereas the [GSH]/[PCs] ratio after 2 weeks of treatment was 2:1. Furthermore, the ratio of [PCs]/[Cd] was 1.5:1 after 1 week of Cd treatment and 5:1 after 2 weeks of Cd exposure.

To determine the contribution of thiols from proteins to the total thiol content in the phloem sap, the thiols derived from proteins were also measured in the phloem sap. In control plants, thiols from proteins account for 18% (271 nmol –SH mg^{-1} protein; $n = 2$) of the total thiols present in the phloem, whereas in the phloem sap from plants exposed to Cd for 2 weeks this percentage increases to 28% (917 nmol –SH mg^{-1} protein). This increase in protein-thiols appears to indicate a toxic interaction between Cd and protein thiol moieties, leading to an increased demand and consequently an increased loading of these proteins into the phloem sap. It is worth noting that in both samples, non-protein soluble thiols (Cys, GSH and PCs) accounted for more than 70% of the total thiols present in the phloem sap.

Thiol and heavy metal content in the xylem sap

The concentration of thiol compounds in the xylem sap was also determined (Figure 7; $n = 3$-4 experiments, two or three measurements per experiment). Glutathione was the most abundant thiol found in the xylem sap (Figures 7 and 8). Fluorescence HPLC was unable to detect measurable amounts of PC_2 in the xylem sap after 1 day, 1 week and 2 weeks of Cd exposure (Figure 7b and data not shown; $n = 3$–4). However, mass spectrometry showed the characteristic ion corresponding to PC_2 at the retention time expected in four of the seven xylem sap samples measured (Figure 7b inset; m/z 920 at 31 ± 1 min). This peak accounted for <1% of the thiols compared with the GSH peak (Figure 7). The Cys concentration was significantly increased in the xylem sap after 2 weeks of Cd exposure, while the GSH content showed a moderate but statistically significant decrease ($P < 0.05$) after 2 weeks of treatment (Figure 8a,b; $n = 3$–4). Cadmium was found in the xylem sap after the first day of Cd exposure and the concentration was significantly lower after 2 weeks of Cd treatment, decreasing to a concentration of 4 µM (Figure 8c). Of the different essential heavy metals measured in the xylem sap (iron, zinc, manganese) only the iron content was significantly affected (Figure 8d). A similar decrease in the iron content during Cd exposure has also been found in maize and has been linked to an inhibition by Cd of the phytosiderophore-iron complex loading of the xylem, disrupting iron homeostasis in the whole plant (Meda *et al.*, 2007). Interestingly, the ratio of [thiols]/[Cd] in the xylem sap was <1 after 1 day (0.63:1) and 2 weeks (0.9:1) of Cd exposure. These ratios suggest that thiols in the xylem sap are not present in sufficient quantities to bind most of the Cd (see Discussion) and that other molecules, such as histidine, may be involved in the root-to-shoot transport of Cd through the xylem sap (Kerkeb and Kramer, 2003).

It has been reported for other plant species that the pH of the xylem sap is more acidic than that of the phloem sap (Shelp, 1987). As pH is an important factor for metal–ligand complex formation we determined the pH values in our sap

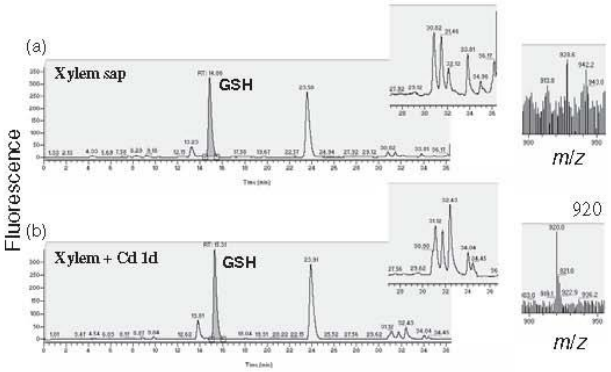


Figure 7. Glutathione (GSH) was the most abundant thiol in the xylem sap of *Brassica napus*. Non-protein thiols in the xylem sap from (a) control and (b) Cd-exposed plants were measured by fluorescence HPLC. No obvious phytochelatin (PC_2) peak was detected, but mass spectrometry revealed a compound with the retention time and mass expected for PC_2 (*m/z* 920). This peak was found in four out of seven xylem sap samples measured (three independent samples for 1 week of treatment and four independent samples for 2 weeks of Cd exposure). The PC_2 peak accounts for <1% of the thiols present from GSH.

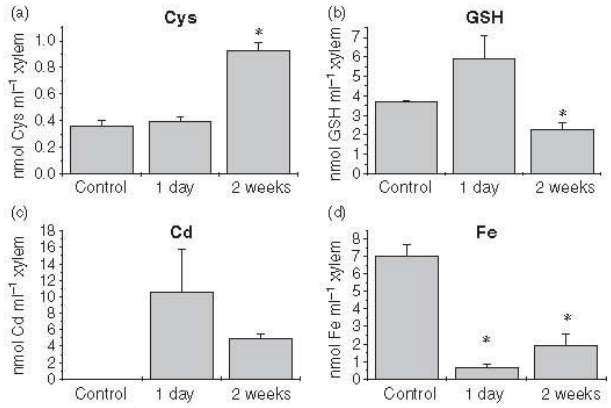


Figure 8. Thiol and heavy metal content in the xylem sap of *Brassica napus*.
(a) The cysteine content in xylem sap increased after 2 weeks of Cd exposure but
(b) glutathione (GSH) was the most abundant thiol at all times measured.
(c) Cadmium was present in the xylem in significant amounts after 1 day of Cd treatment, decreasing to a concentration of 4 μM after 2 weeks of Cd exposure.
(d) Iron content in the xylem sap significantly decreased after 1 day of cadmium exposure. Asterisks denote statistically significant differences from untreated plants. Values shown are $n = 3$–4 experiments; two to three samples were measured per experiment.

samples. Phloem sap had a basic pH (pH 7.7, $n = 2$) compared with the acidic xylem sap (pH 5.8, $n = 2$), suggesting that the environment within the xylem sap is not as favorable as in the phloem sap for stable thiol–Cd complex formation.

Discussion

Phytochelatin accumulation in the phloem sap in response to cadmium

Long-distance transport of PCs among roots and shoots in transgenic Arabidopsis is a phenomenon that was discovered only recently (Chen *et al.*, 2006; Gong *et al.*, 2003). The present study shows that PCs occur naturally and at high levels in the phloem sap of *B. napus* upon Cd exposure. The fact that wild-type plants, rather than transgenic or manipulated plants, were used in the present study strengthens the notion that long-distance transport of PCs is a physiological process and that PCs can function in source-to-sink transport of metals. Analyses of phloem and xylem thiols and Cd concentrations suggest distinct mechanisms of Cd transport in both vascular pathways.

Glutathione and PCs as molecules involved in long-distance transport of cadmium

Long-distance transport of organic thiols through the phloem has previously been reported (Lappartient and Touraine, 1996; Li *et al.*, 2006; Rennenberg *et al.*,1979). However, it remained unknown whether phloem sap thiols are present in sufficient quantities during Cd exposure to account for physiologically relevant Cd chelation and long-distance source-to-sink transport of Cd through the phloem.

Cadmium is a highly reactive heavy metal with high affinity towards the major functional groups of biomolecules (i.e. amino, carboxy, phosphate and thiol groups). These interactions are the biochemical basis of the Cd toxicity. Within biological environments, Cd is expected to be found as complexes rather than existing as a free ion (Mendoza-Cózatl *et al.*, 2005; Sillén and Martell, 1964; Vatamaniuk *et al.*, 2000). It can then be predicted that if Cd is being transported over a long distance within the plant, such transport may take place in the form of complexes. Among the different biochemical moieties, thiols have the highest affinity towards Cd (Sillén and Martell, 1964). Furthermore, among the thiols, PCs are the strongest Cd chelators. The dissociation constant (K_d) for a stable PC–Cd complex is 7.9×10^{-17} M (Dorcák and Krezel, 2003), lower than the dissociation constants for the Cd-chelating proteins metallothioneins (3.44×10^{-11} M) or monothiol molecules such as Cys (1.28×10^{-10} M), GSH (3.16×10^{-11} M) and from other physiological Cd-chelating compounds such as histidine (2.23×10^{-6} M), glutamate (1.25×10^{-4} M) and phosphate-containing compounds such as ATP or pyrophosphate (2.51×10^{-6} M; Erk and Raspor, 1998; Sillén and Martell, 1964). Due to the thiol–Cd affinity constants and because the ratio of ([GSH] + [PCs]) to [Cd] in the phloem sap was >10 at all measured times (Figures 4 and 5), it is likely that GSH and PCs form physiologically relevant levels of stable complexes with Cd in the phloem sap. Other heavy metals such as iron, zinc, or manganese may also form complexes with thiols, but Cd has higher affinity for the thiol moiety (Sillén and Martell, 1964). For example, the K_d of Cys–metal complexes are: cadmium (1.28×10^{-10} M), zinc (1.38×10^{-10} M), iron (6.3×10^{-7} M) and manganese (7.9×10^{-5} M; Sillén and Martell, 1964). The K_d of Cys–copper complexes is lower (6.3×10^{-20} M); however, under our conditions copper was below the detection limit (157 nM) in the phloem sap. In phloem sap samples obtained from plants exposed to Cd for 2 weeks, the ratio of [thiols from PCs]/[Cd] was 5:1 (Figures 4 and 5). This ratio satisfies the 4:1 stoichiometry needed for stable PC–Cd complex formation (Dorcák and Krezel, 2003; Pickering *et al.*, 1999; Salt *et al.*, 1995). On the other hand, the ratio of [PCs]/[Cd] after 1 week of Cd exposure was significantly lower (1.5:1), suggesting that other molecules, most likely GSH, may participate in Cd chelation and transport through the phloem sap.

Glutathione and PC derivatives were detected in the phloem sap after 1 week of Cd exposure (Figure 3). Synthesis of glutathione and PC derivatives seems to be dependent on substrate availability rather than on the expression of specific enzymes (Iturbe-Ormaetxe *et al.*, 2002; Oven *et al.*, 2002). For example, both Arabidopsis phytochelatin synthase and *Glycine max* homo-phytochelatin synthase are able to synthesize PC derivatives, with a C-terminal amino acid other than glycine, with the only apparent requirement that GSH be the donor of the γ-EC moiety and the modified GSH the acceptor (Oven *et al.*, 2002). On the other hand, homo-glutathione synthetase cloned from pea nodules has a higher catalytic efficiency with β-alanine as a substrate than glycine, but the same enzyme is able to synthesize both GSH and γ-glutamyl-cysteinyl-β-alanine (γ-ECA; Iturbe-Ormaetxe *et al.*, 2002).

It has also been documented that Cd exposure induces protein degradation, leading to an increase in the pool of free amino acids (Wu *et al.*, 2004). It is plausible that after several days of Cd exposure the substrates for the synthesis of GSH derivatives are more available, leading to synthesis of the corresponding modified peptides. Interestingly, mass spectrometry also showed a serine-like GSH and PC homolog, 46 Da heavier than the corresponding hydroxymethyl–GSH and hydroxymethyl–PC_2 (Figure 3). These peptides were confirmed to be γ-EC derivatives by tandem mass spectrometry (Figure S2). The tandem mass spectrometry of the *m/z* 574 peptide showed a neutral loss of 64 units, consistent with a methane sulfenic acid loss (CH_3SOH). The only 46 Da modification reported to date corresponds to the addition of methylsulfide (SCH_3) to the β-carbon of an aspartic acid (Kowalak and Walsh, 1996). Further investigation is needed to confirm a similar modification in a serine, which in turn would result in β-methylthioserine.

Cadmium impairs iron homeostasis in xylem and phloem sap

Of the heavy metals measured in the xylem and phloem sap, only iron showed a significant decrease in both the phloem and the xylem in response to Cd exposure (Figures 5b and 8d). This decrease was independent of the NA content in the phloem sap. In Arabidopsis, Cd can enter the root through the iron transporter IRT1 (Rogers *et al.*, 2000; Vert *et al.*, 2002), but iron accumulation in *B. napus* roots was not reduced by Cd (Figure 6). The significant decrease in the iron content of phloem, leaves and xylem (Figures 5b, 6 and 8d) suggests that Cd impairs vascular loading and/or root-to-shoot iron translocation. It is possible that iron loading into the xylem is inhibited by Cd, thus reducing iron accumulation in leaves and iron loading into the phloem. A similar observation was made in maize (*Zea mays*), where it has been suggested that Cd inhibits the phytosiderophore–Fe root-to-shoot translocation system. It should be noted that Cd inhibited the transport system itself and was not attributed to Cd–phytosiderophore complex formation (Meda *et al.*, 2007). No value has been reported for the dissociation constant of the phytosiderophore–Cd complex. However, 2′-deoxymugineic acid is able to form a 1:1 complex with Cd. This complex is weak, and Cd can be easily replaced by adding equimolar concentrations of iron or zinc (Meda *et al.*, 2007). Thus, phytosiderophores appear not to be good candidates for long-distance transport of Cd in plants.

Distinct functions and mechanisms of phloem and xylem Cd transport

Three findings in this study suggest that phloem is an important route for long-distance source-to-sink transport of Cd in *B. napus*: (i) Cd was more concentrated in phloem sap (20 μM) than in xylem sap (4 μM; Figures 5a and 8c). (ii) Thiols were more than 50-fold more abundant in phloem sap than in xylem sap samples (Figures 3 and 7a,b) and PCs were almost exclusively located in the phloem (Figures 2 and 7). (iii) The slightly basic pH of phloem sap would allow for greater stability of metal–ligand complexes compared with the xylem, which exhibited an acidic pH. More stable Cd–ligand complexes would reduce the interaction of Cd with other molecules (i.e. proteins, RNA), preventing further toxicity induced by Cd. The role of the phloem in long-distance source-to-sink mobilization of Cd is also supported by a recent study in Arabidopsis, where significant amounts of Cd where found in the cytoplasm of the phloem and companion cells (Van Belleghem *et al.*, 2007). Several studies have also found that Cd can be redistributed from leaves to other parts of the plant including roots, implicating a role of the phloem in Cd transport (Cakmak *et al.*, 2000; Reid *et al.*, 2003). In contrast, only traces of PCs were found in the xylem, which is in agreement with previous reports showing that Cd in the xylem sap is mainly present in unknown complexes that include oxygen- and nitrogen containing compounds (Salt *et al.*, 1995). Moreover, the fact that the ratio of [thiols]/[Cd] in the xylem sap was below 1 (Figure 8) suggests that molecules other than thiols are responsible for the root to shoot translocation of Cd through the xylem sap.

It should be noted that the relative contribution of xylem sap and phloem sap in the Cd partitioning through the plant will be determined not only by the concentration of metabolites (thiols and Cd) but also by the flux through each vascular system. Several studies have pointed out the role of xylem in the root-to-shoot translocation of heavy metals (Kerkeb and Kramer, 2003; Salt *et al.*, 1995). Further, our data correlate with this model, as we found 4 μM Cd in xylem sap samples and the flux through the xylem can be usually expected to be higher than in the phloem. Further, our findings show that transport of Cd through the xylem is not likely to be in the form of thiol–Cd complexes. Once Cd has been transported in the xylem, the phloem functions in the redistribution of Cd to roots and younger leaves (sink-to-source transport) and this transport is highly likely to be as thiol–Cd complexes.

Characterization of the precise structures that PCs, GSH and Cd form in the phloem sap, as well as the identification of transporters capable of loading and unloading thiols, Cd or thiol–Cd complexes into and from the phloem, represent important challenges in the study of the long-distance transport of Cd. Several transporters of different families have been identified as Cd^{2+} transporters in plants, including CAX, Nramp, P_{1B}-ATPases and ZIP transporters (Mäser *et al.*, 2001; Williams and Mills, 2005), as well as the membrane protein low-affinity cation transporter 1 (LCT1) from wheat (*Triticum aestivum*) and rice (*Oryza sativa*; Clemens *et al.*, 1998; rice LCT1 homolog Os06g38120). However, no PC or PC–Cd transporter genes have been identified in plants to date. A member of the Arabidopsis oligopeptide transporter family (AtOPT6) was shown to have the capacity to transport glutathione–Cd complexes in yeast (*Saccharomyces cerevisiae*; Cagnac *et al.*, 2004). However, this result could not be reproduced using a different yeast expression system or the two-electrode voltage-clamp technique in oocytes (Osawa *et al.*, 2006). Therefore, future work is needed to fully understand the mechanisms by which thiols, cadmium and/or thiol-cadmium complexes are loaded and unloaded from the phloem.

Experimental procedures

Plant growth and sap sampling

Brassica napus (cv. Drakkar, Serasem GIE, la Chapelle d'Armentiers, France) seeds were germinated with tap water and grown in hydroponic culture under controlled conditions, 16-h light/8-h dark, 25.7°C day, 20.7°C night and 55% relative air humidity. The hydroponic solution contained (final concentrations) 0.6 mM NH_4NO_3, 1 mM $Ca(NO_3)_2$, 40 μM Fe-EDTA, 0.5 mM K_2HPO_4, 0.5 mM K_2SO_4, 0.4 mM $Mg(NO_3)_2$, 0.8 μM $ZnSO_4$, 9 μM $MnCl_2$, 0.1 μM Na_2MoO_4, 23 μM H_3BO_3, 0.3 μM $CuSO_4$ and pH was adjusted to 4.7 with H_2SO_4. After the fifth week an air-pump was used for aeration of the hydroponic solution and the hydroponic solution was changed every week. Plants were grown for 9 weeks before being exposed to 75 μM of $CdSO_4$ for the indicated lengths of time. This Cd concentration was chosen since it is high enough to induce strong PC synthesis but still allows the plants to flower for phloem sampling. Phloem sap was obtained as described previously (Giavalisco *et al.*, 2006). Unless otherwise stated, collected phloem was diluted 1:1 with a solution of 1 mM DTT to avoid thiol oxidation. Xylem sap was obtained by decapitation of the stems (Horie *et al.*, 2006) and the sample was also mixed with a 1 mM DTT solution to allow the thiols to remain in a reduced state. Phloem and xylem samples were collected at the Max Plank Institute, Potsdam, Germany and shipped frozen (−78.5°C) to the University of California, San Diego, USA, for fluorescence HPLC, mass spectrometry and ICP-OES analyses. Concentrations of glucose, fructose and sucrose in phloem sap were determined enzymatically as described by Galtier *et al.* (1993).

The HPLC analysis and mass spectrometry

Thiols were derivatized using monobromobimane (mBBr) as described previously (Chen *et al.*, 2006) with slight modifications. Ten to 50 μg of protein (phloem sap) or 50–100 μl (xylem sap) were labeled with 3.5 mM of mBBr (final concentration) for 30 min and the reaction was stopped with the addition of perchloric acid 3% final concentration. Protein was precipitated by centrifugation, 5 min at 20 000 ***g***, in a bench microfuge and the supernatant was filtered through 0.45 μm membranes (Millex, Millipore, http://www.millipore.com/) before HPLC separation. Protein was determined by the Bradford assay (Sigma, http://www.sigmaaldrich.com/

) using bovine serum albumin as a standard. Fifty microliters of mBBr-labeled samples were injected into a 4.6 × 250 mm SunFire C18 column, 5 μm particle size (Waters, http://www.waters.com/) and separated with a gradient from 20% to 95% methanol in 0.05% trifluoroacetic acid (TFA). Excitation and emission wavelengths for the mBBr derivatives were 380 and 470 nm, respectively. After column separation 95% of the flow was analyzed with a fluorometer and the remaining 5% was electrosprayed for mass spectrometry analysis in the positive mode using a ThermoFinnigan LCQ Advantage system (Chen *et al.*, 2006). The bimane label accounts for 190 Da on each peptide per thiol labeled. For tandem mass spectrometry, thiol-peptides were separated by HPLC as described above and each thiol was collected independently. After evaporation of the mobile phase, peptides were resuspended in 50% methanol, 1% acetic acid and analyzed on a QSTAR XL ESI Mass Spectrometer (Applied Biosystems, http://www.appliedbiosystems.com/) in the positive mode. All mass spectrometry and fluorescence HPLC experiments were conducted using the UCSD Superfund Mass Spectrometry Facility. Phytochelatin standards PC_2, PC_3 and PC_4 were purchased from Anaspec (http://www.anaspec.com/).

For quantification of thiols derived from proteins in the phloem sap, 5–10 μg of protein were acidified with an equal volume of TFA 0.2% (v/v, pH < 2) containing 10 mM diethylenetriaminepentaacetic acid (DTPA) and mixed vigorously. The protein sample was divided into two and one tube was centrifuged at 20 000 ***g*** for 10 min in a bench top centrifuge to precipitate proteins. The resulting supernatant was transferred to a new tube. The pH of the non-centrifuged sample and the supernatant of the centrifuged sample were adjusted to pH 8 by adding 950 μl of 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) 100 mM, pH 8. Total thiols were titrated with 5,5′-dithio-*bis*-(2-nitrobenzoic acid; DTNB), 1 mM final concentration and the thiols were quantified by measuring the absorbance at 412 nm and using the $\epsilon^{TNB}_{412\ nm}$ = 13 600 M^{-1} cm^{-1} (Ellman, 1959). The difference of thiols between the non-centrifuged sample and the centrifuged sample was considered as the thiol content from proteins in the phloem sap.

Nicotianamine measurements

For NA determination, 10–50 μg of protein (phloem sap) was heated at 80°C for 20 min and then centrifuged at 20 000 ***g*** in a bench top microfuge for 10 min and filtered through 0.45 μm membranes (Millex, Millipore) before HPLC separation. One to five microliters of sample was diluted 1:1 with an equal volume of a *o*-phthaldialdehyde (OPA, Pierce, http://www.piercenet.com/) incubated in the dark for 1 min and the reaction was terminated by the addition of 1 μl of a sulfosalicylic acid 50% (w/v) before injection. The HPLC separation was carried out on the same column described for thiol separation with the mobile phase conditions described by Le Jean *et al.* (2005). The NA standard was a kind gift from Dr Erin L. Connolly (University of South Carolina, USA).

Heavy metal measurements

For elemental analysis, 10–50 μg of protein (phloem sap) or 50–100 μl (xylem sap) was digested overnight in 70% HNO_3, metal trace grade, and then diluted in Milli-Q water (Millipore) to reach 3.5% HNO_3 final concentration. Leaves and roots tissue from *B. napus* were dried in an oven overnight (60°C) and 10–25 mg of dry weight was used for the acid digestion as described above. Roots were harvested directly from the hydroponic culture, therefore iron levels in roots represent the sum of apoplastic and symplastic iron content. Cadmium, iron, zinc and manganese were determined by ICP-OES at the UCSD/Scripps Institution of Oceanography analytical facility.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Tandem mass spectrometry of the GSH-like peptide γ-ECQ.

Figure S2. Tandem mass spectrometry of the ion that is 46 Da heavier than hydroxymethyl glutathione (γ-ECS).

This material is available as part of the online article from http://www.blackwell-synergy.com.

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1.4. Acknowledgements

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Chapter Two:

Saccharomyces cerevisiae as a heterologous system for the screening and characterization of PC-Cd transporters.

2.1. Abstract

Phytochelatins (PCs) are glutathione-derived peptides involved in heavy metal detoxification in plants and other organisms including nematodes and some yeast. These thiol peptides are synthesized upon exposure to metalloids and heavy metals such as cadmium and have been shown in plants and *Schizosaccharomyces pombe* to function through the chelation and subsequent vacuolar sequestration and stabilization of the metals in the form of high molecular weight (HMW) complexes. In *S. pombe*, the transporter HMT1 has been implicated in the transport of PC-Cd into the vacuole, however in plants the responsible transporter remains unknown. In addition to their intracellular role, PCs have recently been shown to undergo long distance transport between roots and shoots, largely through the phloem. Such movement suggests that there may also be plasma membrane transporters responsible for the vascular loading and unloading of PCs or PC-Cd complexes that have not yet been identified. In an attempt to screen for potential PC-Cd transporters, the wheat PC synthase gene *TaPCS1* was integrated into the genome of the naturally PC deficient *Saccharomyces cerevisiae*. This strain is being used to screen wheat and Arabidopsis cDNA libraries looking for cDNAs capable of increasing the Cd tolerance specifically in the presence of PCs. The limit of Cd tolerance was characterized and *Arabidopsis* and wheat cDNA libraries in yeast expression vectors were screened in this strain above the upper limit of Cd tolerance. In addition, the strain was used to test whether SpHMT1, the only reported PC-Cd transporter, confers tolerance in a PC dependent manner.

2.2. Introduction

Phytochelatins (PCs) are heavy metal binding peptides that are synthesized from glutathione (GSH) by the enzyme phytochelatin synthase (PCS), which is only active in the presence of heavy metal ions and metalloids (Grill et al., 1989; Schmoger et al., 2000) and is activated most strongly in the presence of Cd^{2+} (Grill et al., 1989; Vatamaniuk et al., 2000). PCs are believed to be synthesized and to chelate heavy metal ions within the cytosol, however in both *S. pombe* and tobacco protoplasts PCs and Cd have been found to be primarily located within the vacuole, so it is believed that PC-Cd is being transported into the vacuole (Vogeli-Lange and Wagner, 1990; Ortiz et al., 1992). In *S. pombe*, the vacuolar half-size ABC (ATP-binding cassette) transporter HMT1 has been reported to have PC-Cd transport activity and to be necessary for the formation of HMW complexes of PC-Cd-S (Ortiz et al., 1992; Ortiz et al., 1995). In these HMW complexes, the addition of sulfide has been found to give the complex greater stability, allowing for greater detoxification of Cd than PC-Cd alone (Reese and Winge, 1988). Without the formation of HMW complexes, the *Δhmt1* mutant is highly sensitive to Cd, supporting the idea that their formation is a critical aspect of PC-mediated Cd tolerance (Ortiz et al., 1992; Ortiz et al., 1995). PCs have been found in plant vacuoles (Vogeli-Lange and Wagner 1990) suggesting that the mode of Cd detoxification is similar between yeast and plants, however the plant vacuolar transporter of PC-Cd has yet to be identified.

In plants it has been established that PC-Cd complexes are sequestered in the vacuole (Vogeli-Lange and Wagner, 1990). In addition, it has been recently shown that PCs are capable of undergoing long distance transport between roots and shoots (Gong et al., 2004; Chen et al., 2006). An analysis of the xylem and phloem sap of *Brassica napus*,

a close relative of *Arabidopsis thaliana*, was presented in Chapter I of this thesis and indicates that i) the phloem is a major vascular system responsible for PC and GSH movement through the plant, ii) these thiols have a high enough affinity for Cd and are present in sufficient amounts to predict that they are major chelators of Cd during its movement through the phloem (Mendoza-Cózatl et al., 2008). This long distance transport opens up the possibility for multiple PC-Cd transporters, to serve the need for PC extrusion and import that vascular mobilization may require, in addition to a transporter responsible for the transport of PCs from the cytosol into the vacuole.

Three transporter families have been identified as being involved in the transport of peptides in plants, PTRs (peptide transporters), OPTs (oligopeptide transporters), and ABC transporters. While other transporter families have been found to be involved in the transport of Cd as a free ion, the capacity for peptide transport shared by the PTR, OPT and ABC families makes them more likely candidates for the transport of PC-Cd and glutathione-Cd GS_2Cd complexes.

PTR family transporters are involved in the transport of di- and tripeptides with low selectivity. Although the substrates of these transporters appear to be too small to include PCs, it has been suggested that a member of the PTR family may be able to transport GSH and its precursor, γ-glutamylcysteine (γ-EC) (Tsay et al., 2007).

OPT family transporters are involved in the transport of tetra- and pentapeptides and have been characterized mainly for their uptake of these peptides from outside the cell (Tsay et al., 2007). An OPT family member from *S. cerevisiae*, ScOPT1, shows uptake activity for GSH, GSSG and PC_2 (Osawa et al., 2006). Of the nine OPT transporters in Arabidopsis, only AtOPT6 has been reported to be involved in the uptake

GSH (Cagnac et al., 2004), however other studies have been unable to detect GSH uptake by AtOPT6 (Koh et al., 2002; Osawa et al., 2006; Mendoza-Cózatl, unpublished data). Although no PC uptake activity has yet been reported for any of the Arabidopsis OPT family members, the majority of the AtOPTs show primarily vascular expression (Stacey et al., 2006) and AtOPT3 has been found to increase metal accumulation when expressed in *S. cerevisiae* (Wintz et al., 2003). Taken together with the transport activity of ScOPT1, these factors make it tempting to speculate that some PC or GSH transport within the AtOPT family remains to be found, and that OPT transporters may be involved in the cellular import of PC or GSH following long-distance translocation.

ABC transporters are the largest family of transporters in Arabidopsis, with a total of 103 putative members, and are defined as containing one or two ATP-binding cassettes and two to three transmembrane domains made up of up to six transmembrane α-helices each (Sánchez-Fernández et al., 2001). ABC transporter subfamilies that are of interest in the Cd tolerance mechanism of plants include the ATM (ABC transporter of the mitichondrion), PDR (pleiotropic drug resistance), MDR (mulitdrug resistance) and MRP (multidrug resistance-associated protein) transporters.

Among these selected subfamilies, the ATM subfamily is the only made up of half-sized ATP transporters. At 53-56% similarity, this subfamily consists of the closest Arabidopsis homologues of SpHMT1, the only reported vacuolar PC transporter (Chen et al., 2007). AtATM3, which has highest percent identity and similarity to SpHMT1, has been shown to be involved in tolerance to Cd and Pb (Kim et al., 2006). AtATM3 has been suggested to be involved in the transport of GSH-Fe (Kim et al., 2006) and it has been previously shown to transport FeS from the mitochondria to the cytosol (Kushnir et

al., 2001) but the exact mechanism by which AtATM3 is involved in heavy metal resistance is not yet certain (Chen et al. 2007).

Whatever the mechanism for AtATM3 mediated heavy metal tolerance, however, it is doubtful that an ATM transporter contributes to the extrusion, uptake or vacuolar sequestration of GSH, PCs or Cd, as all ATM subfamily members have been found to localize to the mitochondria (Chen et al., 2007). In contrast, a proteomic analysis of Arabidopsis organelles indicates a plasma membrane localization for all PDR and MDR subfamily transporters detected (PDR 6,7,8,9; MDR 1,4,6,8,11) and a vacuolar localization for all MRP subfamily transporters detected (MRP 1,2,3,4,5,6,10) (Dunkley et al., 2006).

The MRP subfamily is made up of full-size ABC transporters that localize to the vacuole in both yeast and plants (Dunkley et al., 2006; Jungwirth and Kuchler, 2006). In *Saccharomyces cerevisiae*, a yeast which naturally lacks the ability to synthesize PCs, the MRP family member ScYCF1 has been shown to transport bis(glutathionato)cadmium (GS_2Cd) into the vacuole, a mechanism that is important for the Cd tolerance of the organism (Li et al., 1997). In *S. pombe*, the ScYCF1 homologues SpABC2 and SpABC4 were shown to be glutathione-S-conjugate pumps that contribute to Cd tolerance in the absence of HMT1, indicating a possible role for these transporters in the sequestration of GS_2Cd (Iwaki et al., 2006). Arabidopsis homologues of ScYCF1 include the glutathione-conjugate transporters AtMRP1, AtMRP2 and AtMRP3, which have been found to transport GSH and GSSG (Tommasini et al., 1998). Of these, only AtMRP3 was able to partially restore the Cd sensitivity of the *S. cerevisiae Δycf1* mutant, indicating that it may be involved in the transport of GS_2Cd (Tommasini et al., 1998). *AtMRP3* is

constitutively expressed in both roots and leaves, and root transcript levels increase four-fold upon exposure to Cd but not to other oxidative stress (Bovet et al., 2003), suggesting that AtMRP3 has a particular role to play in the response and detoxification of Cd.

The PDR subfamily is made up of full-sized ABC transporters that have been found to be localized to the plasma membrane of Arabidopsis (Dunkley et al., 2006). In yeast, PDR transporters have been found to be involved in the extrusion of peptides, phospholipids, steroids and herbicides (Schüller et al., 2003), making them possible candidates for cellular extrusion of PC-Cd and GS_2Cd. Recently, *AtPDR8* has been identified as being upregulated in the presence of Cd, and has been reported to be involved in the extrusion of Cd from the epidermis and root tips and has been shown to confer resistance to Cd (Kim et al., 2007). However, it remains unknown whether it transports Cd as a free ion or in a chelated form, making AtPDR8 a potential candidate for PC-Cd or GS_2Cd export. *AtPDR7*, the closest homologue to *AtPDR8*, is also upregulated with Cd exposure (Kim et al., 2007) although *AtPDR7* expression is restricted to the roots (van den Brûle and Smart, 2002). Cd responsiveness and a root specific expression makes AtPDR7 an interesting candidate for the mobilization of PC-Cd.

The MDR subfamily, also known as the PGP (p-glycoprotein) subfamily of ABC transporters, was originally characterized in mammalian tissue for their ability to confer resistance to cancer treatment through the export of toxins. ScSTE6, the only MDR/PGP subfamily member in *S. cerevisiae*, is responsible for the export of a-factor, a hydrophobic peptide involved in yeast mating (Kuchler et al., 1989; Jungwirth and Kuchler, 2006). Of the 22 MDR/PGP subfamily members found in Arabidopsis, only

three have been well characterized (Martinoia et al., 2002; reviewed by Sánchez-Fernández et al., 2001). AtPGP1 and AtPGP19 have both been reported to export auxin, an amino acid derived plant growth hormone, while AtPGP4 is involved in the cellular import of auxin. MDR/PGP mediated long distance transport of auxin is important for proper development and gravitropism (Geisler and Murphy, 2006). This capacity for long distance transport, taken together with the potential for peptide transport makes members of MDR/PGP subfamily valid candidates for PC-Cd of GS_2Cd translocation.

In this project, two approaches were developed in an attempt to uncover PC-Cd transporters. *GAL1::TaPCS1* was integrated into the genome of wild-type *S. cerevisiae*, which naturally lacks PCs, which was then transformed with wheat and Arabidopsis cDNA libraries and plated on media containing a high level of Cd to detect potential enhancers of PC-mediated tolerance. Second, *SpHMT1* was cloned into pYES-DEST52 and this construct was tested in the integration strain to determine whether an enhancement of PC-mediated tolerance could be detected, in order to evaluate the screening system developed.

2.3. Materials and Methods

Integration of *GAL1::TaPCS1-CYC1* into the genome of *Saccharomyces cerevisiae*

GAL1::TaPCS1-CYC1 fragment was amplified by PCR of *TaPCS1*-pYES2 plasmid (Clemens et al., 1999) using primers ycfGAL1-F: AACAGTTTGAGAATAAATTAGGGGTATCGTACTACCGTAAAGAACAAGAAAG GATTAGAAGCCGCCGAGCGG and ycfCYC1-R: ATCCTACGTACCAGATTGTGCGGGACAGGTTTTTATTAGTTTCACAGTTGGCC GATTCATTAATGCAGGGC. The resulting *YCF1* 5'-*GAL1::TaPCS1-CYC1-YCF1* 3' fragment was purified using the QIAquick Gel Extraction Kit (Qiagen). The *S. cerevisiae* wild-type haploid strain BY4741 (Invitrogen, Carlsbad, CA) was transformed with the PCR-amplified fragment using the lithium acetate method (Gietz and Schiestl, 1991). Positive recombinants were selected on YP 2%Galactose 1% Raffinose + 250μM $CdCl_2$.

Peptide extraction from *Saccharomyces cerevisiae*

Cells were harvested by centrifugation at 1,000×g for 10 minutes and washed twice with 50mM Tris-HCl pH7.8. Acid-washed glass beads (425-600μm, Sigma) were added in equal volume to the cell pellet. 50mM Tris-HCl pH7.8 was bubbled with N_2 gas for 10 minutes and 500μl was added to each cell pellet. The cells were then vortexed for 2 minutes followed by a 2 minute incubation on ice, and the vortex-ice incubation was repeated 10 times to achieve optimal protein extraction. Cell debris was pelleted by centrifugation at 20,000×g for 10 minutes. Soluble extract was filtered twice through a 0.45μm syringe driven filter unit (Millipore). Protein was quantified using the Bradford

assay (Sigma) and samples were normalized to protein content through the addition of 50mM Tris-HCl pH7.8.

HPLC-MS analysis of thiol peptides from *Saccharomyces cerevisiae*

65µl of cell peptide extraction normalized to protein content were labeled and thiol peptides were detected as described in Part 1 of this thesis (Mendoza-Cózatl et al., 2008).

Cadmium sensitivity

Cells were grown in YNB –ura to OD600 0.35 and 3ml of culture was harvested by centrifugation at 1,000×g for 10 minutes. Cells were resuspended in H_2O to a final OD600 of 1.0. The resulting cell suspension, followed by five 1 in 5 serial dilutions were plated on YNB –ura 2% galactose 1% raffinose with varying concentrations of $CdCl_2$. The resulting dilution optical densities plated were 1×10^{0}, 2×10^{-1}, 4×10^{-2}, 8×10^{-3}, 1.6×10^{-3}, 3.2×10^{-4}.

Size-exclusion chromatography of yeast cell extract

250µl of cell peptide extraction normalized to protein content was injected through a BioLogic DuoFlow System (BioRad) and the sample was run at 1 ml/min over a Superdex-75 column (GE Healthcare) using 30ml of 20mM Tris-HCl pH7.8. 2ml fractions were collected for further analysis by ICP and HPLC. 1.5 ml was allocated for ICP, and diluted HNO_3 was added to 3.5ml final volume and final concentration 3.5% HNO_3. 0.5ml was allocated for analysis by fluorescence HPLC-MS. 500µl was

concentrated by CentiVac Concentrator (Labconco) to approximately 50µl. Volume was adjusted to 65µl with the addition of 20mM Tris-HCl pH 7.8, and samples were labeled with mBBr as described in Part I.

2.4. Results

Design of a PC-Cd transporter screen

Schizosaccharomyces pombe carries a gene encoding phytochelatin synthase, *SpPCS*, and is known to use phytochelatins to chelate cytosolic Cd and be transported into the vacuole where more stable PC-Cd-S high molecular weight complexes are formed. Another yeast, *Saccharomyces cerevisiae*, lacks phytochelatin synthase and instead relies on an analogous system whereby glutathione chelates Cd and the resulting GS_2Cd complex is transported into the vacuole by the ABC transporter ScYCF1. While the mechanisms of cadmium detoxification used by *S. pombe* and *S. cerevisiae* both involve the vacuolar sequestration of thiol-Cd complexes in the vacuole (see Figure 9), PCs have 2×10^6 more affinity than GSH for Cd, leading the level of resistance conferred to be far greater in *S. pombe*. Therefore, it was hypothesized that the disabling of the natural Cd defense mechanism through the deletion of *ScYCF1* would provide a Cd sensitive yeast that would be partially rescued by the expression of phytochelatin synthase and enhanced by plant transporters responsible for the extrusion or vacuolar sequestration of PC-Cd.

While standard methods for gene knockout in *S. cerevisiae* involves homologous recombination between the gene to be knocked out and an antibiotic resistance marker, for this study a *GAL1::TaPCS1-CYC1* cassette was used and homologous recombinants were screened on the basis of Cd tolerance.

Before creating a knockout of *YCF1* with *TaPCS1*, screening conditions were tested to find the optimal Cd concentration for screening the homologous recombinants, using *GAL1::TaPCS1*-pYES2 and empty pYES2 vectors in wild-type and *Δycf1*. The

original aim was to create a strain with only moderate Cd tolerance by disabling the natural *S. cerevisiae* resistance mechanism and simultaneously introducing phytochelatins to the organism. However Figure 10 shows that the *Δycf1* deletion yeast containing the *TaPCS1*-pYES2 vector is more tolerant to Cd than wild-type yeast containing the *TaPCS1*-pYES2 vector. While this result was initially unexpected, knocking out YCF1 would create a larger cyltosolic pool of GS2Cd, giving *Δycf1* expressing *TaPCS1* the ability to synthesize more PCs, which may account for the increased tolerance. These results suggest that a *Δycf – TaPCS1* integration yeast would be more tolerant to Cd and provide a smaller window to detect improved Cd resistance than *TaPCS1* integrated into the genome of yeast with the *YCF1* gene remaining intact. Therefore, it was decided to use a TaPCS1 intregration strain that resulted from a non-specific recombination event, so that the YCF1 gene remains intact.

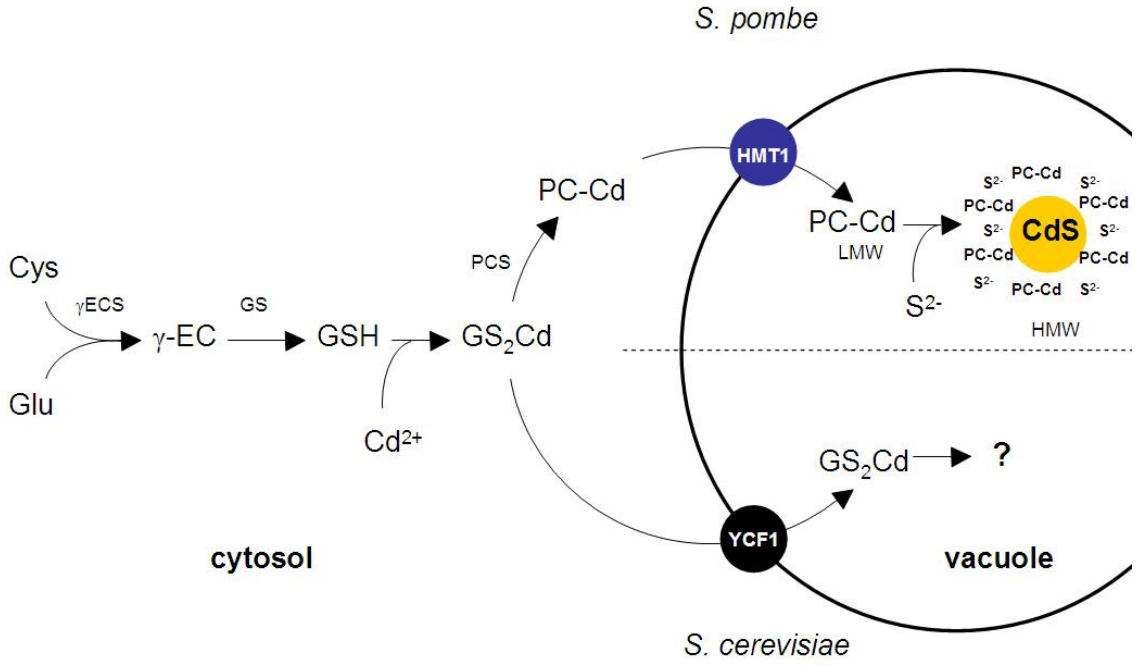


Figure 9. Cadmium detoxification pathways proposed for *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. LMW – low molecular weight, 0.5-2 kDa. HMW – high molecular weight, >10 kDa. γ-ECS - γ-glutamylcysteine synthetase. GS – glutathione synthetase. PCS – phytochelatin synthase.

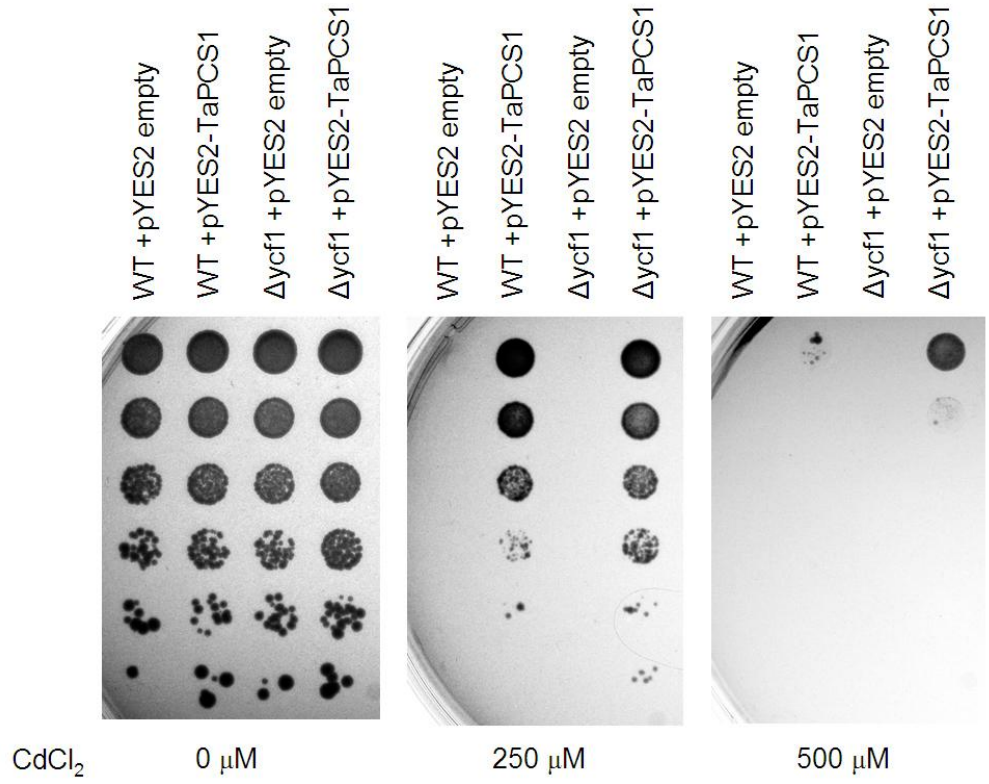


Figure 10. Cadmium tolerance of *Δycf1* – *TaPCS1*. One in five serial dilutions of wild-type *S. cerevisiae* strain BY4741 with empty pYES2 vector, WT with *TaPCS1*-pYES2, *Δycf1* with empty pYES2 vector and *Δycf1* with *TaPCS1*-pYES2 were plated on YNB – ura 2% galactose 1% raffinose with 0μM, 250μM and 500μM $CdCl_2$ and grown for 4 days.

Characterization of the *TaPCS1* genomic integration strain of *Saccharomyces cerevisiae*

Wild-type yeast was transformed with purified PCR-amplified fragment and the successful insertion of *GAL1::TaPCS1-CYC1* was confirmed by PCR of *TaPCS1* among Cd resistant colonies and growth on 5-Fluoroorotic Acid indicates that the *TaPCS1*-pYES2 vector is not present. Positive recombinants were then screened for the retention of the *ScYCF1* gene by colony PCR. Yeast that were found to contain both *TaPCS1* and *ScYCF1* were grown in YP 2% galactose 1% raffinose with 100μM $CdCl_2$ and tested for the ability to synthesize phytochelatins. The yeast with the highest level of PC production was selected for the screening process. Presence of the *TaPCS1* gene in this line was confirmed by PCR (Figure 11a) and the successful expression of *TaPCS1* in the integration strain was confirmed by RT-PCR (Figure 11b, 11c). Figure 13 shows the presence of PCs in the *TaPCS1* integration strain, indicating the production of functional phytochelatin synthase enzyme.

To determine the appropriate concentration of $CdCl_2$ to screen for plant PC transporters, the tolerance of the *TaPCS1* integration strain was assessed (Figure 12). Surprisingly, although wild-type yeast containing *TaPCS1*-pYES2 and the *TaPCS1* integration strain show similar levels of expression (Figure 11), the *TaPCS1* integration strain appears to have higher levels of PC_2 after Cd exposure(Figure 13), but lower tolerance to Cd than yeast containing the *TaPCS1*-pYES2 vector. No growth was detected for the *TaPCS1* integration strain at 400μM $CdCl_2$ after five days.

Upon transport of PCs into the vacuole, wild-type *S. pombe* has been reported to form high molecular weight complexes of PC-Cd-S. To determine whether *S. cerevisiae*,

upon synthesis of PCs, is also able to form analogous high molecular weigh complexes, a reduced PC-Cd standard (Figure 14a) and cellular extracts of wild-type and *TaPCS1* integration *S. cerevisiae* harvested after 20 hours of 50μM $CdCl_2$ exposure and normalized to protein content (Figure 14b), were separated by size-exclusion chromatography using a Superdex-75 column eluting with 20mM Tris-HCl pH 7.8. Non-Cd exposed wild-type cell extract was also injected with 50μM $CdCl_2$ added directly prior to injection, to indicate Cd binding to cellular contents non-specifically (Figure 14b). The peak in fraction 4 appears to be Cd binding cellular proteins, and Cd in fraction 8 represents Cd binding to low molecular weight cellular components. In the reduced PC-Cd standard, PC-Cd was found to elute in fraction 7. The *TaPCS1* integration strain also shows a Cd peak at fraction 7, and no apparent HMW complexes were detected, suggesting that in *S. cerevisiae* cells Cd chelation occurs in the form of low molecular weight PC-Cd complexes.

To test the screening method designed, wild-type and *TaPCS1* integration yeast were transformed with the only reported vacuolar PC transporter, *SpHMT1*. Figure 15 shows one in five serially diluted wildtype and *TaPCS1* integration with empty vector and *SpHMT1*-pYES2, grown on 0 and 300μM $CdCl_2$ for 4 days (Figure 15). Both wild-type and *TaPCS1* integration yeast show enhanced Cd resistance upon expression of the HMT1 transporter, therefore validating the screening strategy. Interstingly, expression of *SpHMT1* conferred a yellow phenotype in both wild-type and *TaPCS1* integration *S. cerevisiae* when grown in the presence of Cd (Figure 16).

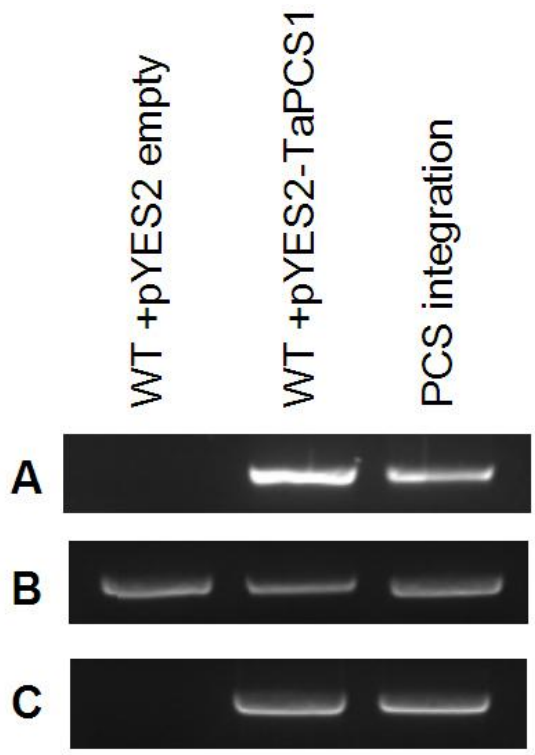


Figure 11. Genomic integration of *GAL1::TaPCS1* and expression of *TaPCS1*. (A) Genomic PCR of wild-type (BY4741) with empty plasmid, WT +TaPCS1-pYES2, and *GAL1::TaPCS1* integration line. RT-PCR of *TaPCS1* expression in WT with empty plasmid, WT + *TaPCS1*-pYES2, and *GAL1::TaPCS1* integration line. (B) *Actin*, 25 cycles, (C) *TaPCS1*, 30 cycles.

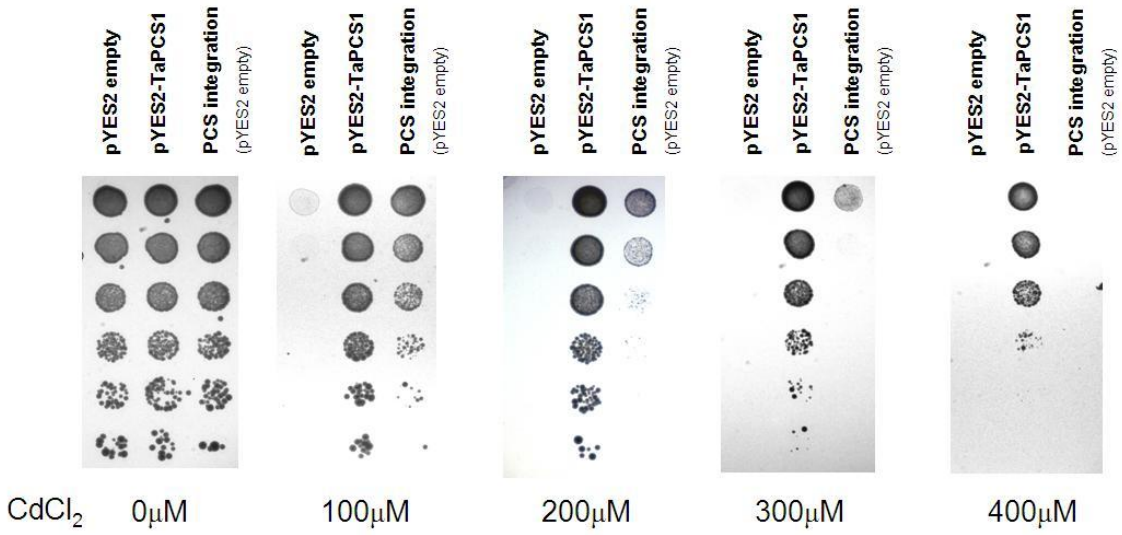


Figure 12. Cadmium tolerance analysis of *TaPCS1* integration line. One in five serial dilutions of WT (BY4741) with empty vector, *TaPCS1*-pYES2 and *TaPCS1* integration with empty vector were plated on YNB –ura 2% galactose 1 % raffinose with 0μM, 100μM, 200μM, 300μM, and 400μM $CdCl_2$ and grown for 5 days.

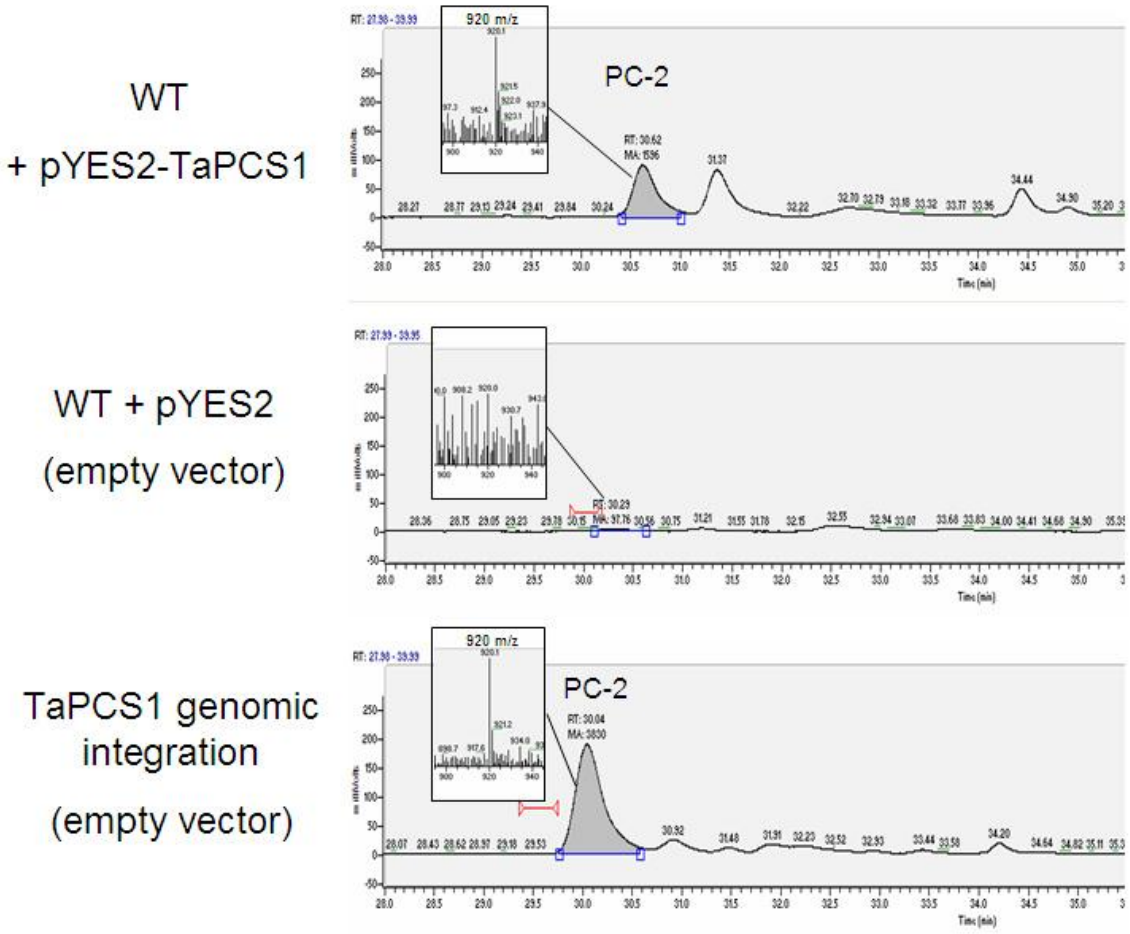
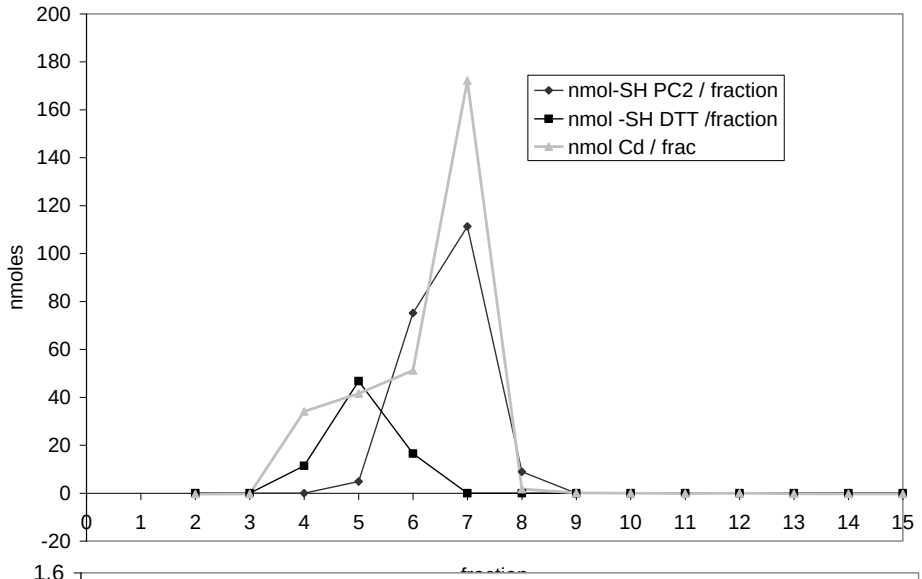


Figure 13. Detection of phytochelatins in *TaPCS1* integration strain. Yeast cultures were grown 2 days in YNB –ura 2% galactose 1% raffinose, then exposed to 50µM $CdCl_2$ for 20 hours. Samples were normalized to protein content prior to labeling. Inlay indicates mass spectrometry of the fluorescence peak. mBBr labeled phytochelatins are identified as m/z 920.1.

A.



B.

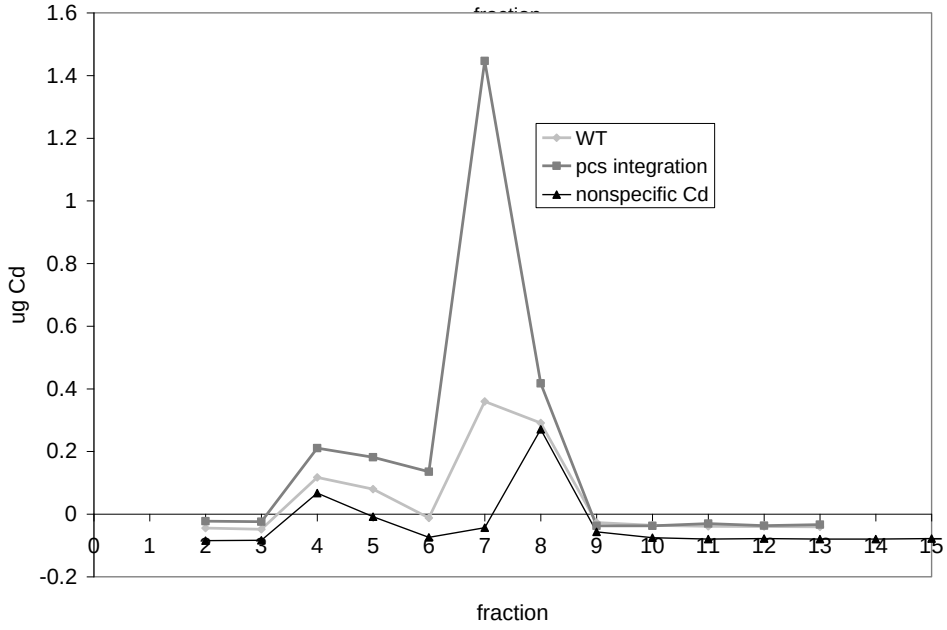


Figure 14. Size-exclusion chromatography to determine Cd complexes. (A) 500µM Cd 1mM PC_2 1mM DTT fractionated over a Superdex-75 column. Thiol content was quantified by fluorescence after HPLC separation. Cd content was quantified by ICP-OES (B) WT and *TaPCS1* integration with pYES2 vector were grown for 2 days, then exposed to 50µMCd for 20 hours. Nonspecific Cd indicates peptide extract from WT with pYES2 grown 3 days, 50µM Cd was added prior to injection.

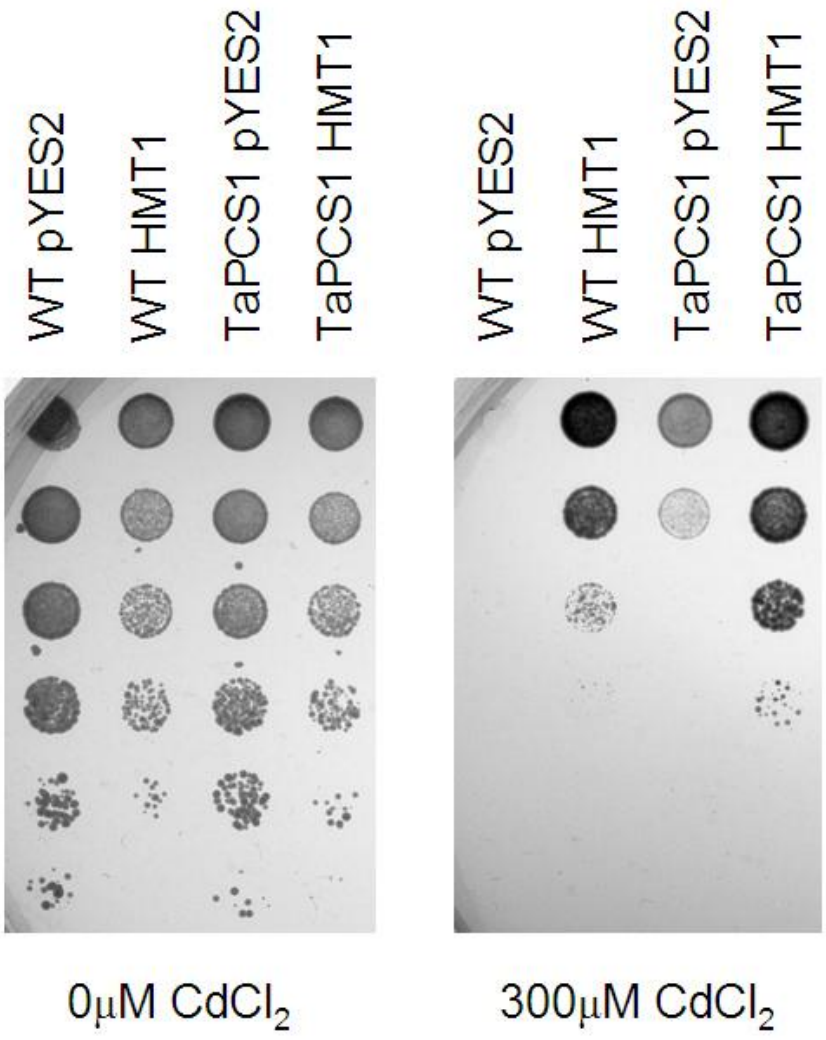


Figure 15. Cadmium tolerance analysis of SpHMT1. 1 in 5 serial dilutions of wild-type and *TaPCS1* integration *S. cerevisiae* with empty vector or *SpHMT1*-pYES2, plated on YNB –ura 2% galactose 1% raffinose with 0 or 300μM $CdCl_2$ and grown for 4 days.

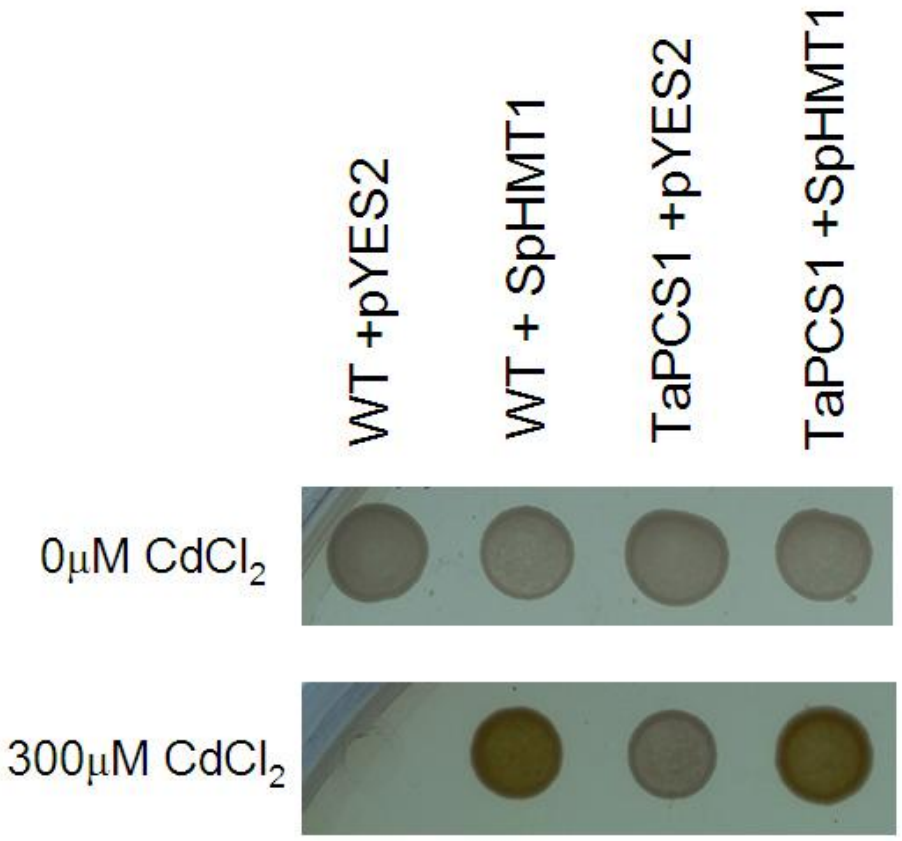


Figure 16. SpHMT1 confers a yellow phenotype upon Cd exposure. Cells were plated at 1.0 OD600 onto YNB – ura 2% galactose 1% raffinose with 0μM or 300μM $CdCl_2$ and grown for 4 days.

Screening plant cDNA libraries in the *TaPCS1* integration strain

Screening for Cd resistant colonies resulting from transformation with size-selected >1.5kb pYES2 wheat root library (Schachtman and Schroeder, 1994) is being carried out at 400μM $CdCl_2$ fourteen days after plating to allow more time for colony enlargement. 41,500 colonies were screened from this library, as determined by plating 5μl of each transformation reaction on YNB –ura without $CdCl_2$. Table 1 describes the cDNAs that were isolated from Cd tolerant colonies. Although nine clones were isolates, none were determined to encode possible transporters, and some of the vectors isolated contained no cDNA.

Screening of Cd resistant colonies resulting from transformation with the pFL61 *Arabidopsis* seedling cDNA library (Minet et al. 1992) is being carried out at 400μM $CdCl_2$ seven days after plating, in an attempt to select cDNAs more specifically involved in Cd tolerance rather than non-specific enhancers of yeast growth. 500,000 colonies were screened so far, and Table 2 describes the cDNAs isolated from the Cd tolerant colonies. Of the five library clones were isolated, three were empty plasmids, one contained a cDNA too short to be a membrane protein and the one other clone isolated contained a partial cDNA.

Genes identified from pYES2 *Triticum aestivum* root cDNA library

clone number	Closest *A. thaliana* homologue	putative function
*Ta*01	At1g79740	hAT dimerization domain containing protein
*Ta*02	At5g26680	Putative endonuclease, DNA repair
*Ta*03	At3g08580, At5g13490	ADP/ATP translocator
*Ta*04	pYES2 empty vector	
*Ta*05	No homologues identified	
*Ta*06	At2g21170	triose-phosphate isomerase
*Ta*07	At1g60200	RNA splicing factor
*Ta*08	No homologues identified	*T. aestivum* 28s ribosomal RNA gene
*Ta*09	pYES2 empty vector	

Table 1. Wheat root cDNAs isolated. Clones are numbered in order of isolation. Arabidopsis homologues are indicated, along with the putative function of the resulting protein.

Genes identified from pFL61 *Arabidopsis* seedling cDNA library

clone number	ATG Number	Function	Protein size	TMD?
*At*01	pFL61 empty vector			
*At*02	pFL61 empty vector			
*At*03	pFL61 empty vector			
*At*04	AT1G51360	unknown	210aa	No
*At*05	AT2g07360*	SH3 domain containing protein	1196aa	No

Table 2. Arabidopsis cDNAs isolated. Clones are numbered in order of isolation. Gene identification number, protein function and size, and predicted transmembrane domains are listed. * indicates the isolation of an incomplete cDNA.

2.5. Discussion

The two yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have similar but distinct modes of cadmium detoxification. In *S. cerevisiae*, Cd is chelated by GSH and the resulting GS_2Cd has been shown to be transported into the vacuole by the transporter YCF1. *S. pombe* instead synthesizes PCs and has been shown to form stable high molecular weight PC-Cd-S HMW complexes within the vacuole. The *S. pombe* transporter HMT1 has been reported to be responsible for the transport of PCs into the vacuole, and in its absence *S. pombe* is unable to form PC-Cd-S complexes and shows a large reduction in Cd tolerance. A large reduction in Cd tolerance is also seen in the *S. cerevisiae Δycf1* mutant, indicating the importance of vacuolar sequestration and leading to the hypothesis that *S. cerevisiae* may also form analogous vacuolar thiol-Cd complexes using GSH rather than PCs.

Because of concerns that the potential formation of GS-Cd-S complexes within the vacuole may confer enough tolerance to partially mask PC-mediated Cd resistance, it was originally decided to design a screening system in which *TaPCS1* was inserted and the *YCF1* gene was deleted. However, Cd tolerance experiments done to validate this design showed that a deletion of *YCF1* actually enhances PC mediated Cd tolerance in *S. cerevisiae* carrying the TaPCS1 gene (Figure 10). PCs are synthesized from glutathione upon Cd exposure, and the increased cytosolic content of GS_2Cd resulting from the inhibition of its transport into the vacuole would allow for increased synthesis of PCs. PCs are known to be more stable chelators of Cd than GSH, and it is likely that PC-Cd confers more tolerance than vacuolar sequestration of GS_2Cd . This idea is supported by a

previous study in which expression of phytochelatin synthase in a vacuole deficient strain of *S. cerevisiae* was found to increase Cd resistance (Clemens et al., 1999).

In order to facilitate the screening procedure, the objective was to maximize the window in which an increased Cd tolerance could be observed. A condition with less background Cd tolerance was favored, in order to more clearly detect enhancers of PC-mediated Cd tolerance. It was therefore determined that a strain in which *TaPCS1* has been integrated into the genome without the disruption of *YCF1* would be more favorable as a screening tool.

The method used to create the *TaPCS1* integration stain follows PCR-mediated gene disruption, a method that has been reported to produce 5-10% site specific recombination events (Lorenz et al., 1995). It had been determined that an integration of *TaPCS1* with the *YCF1* gene remaining intact would be better for the screening process. While it is not common practice in the *S. cerevisiae* organism for a study to be done on a gene integrated into an unknown location, in plants the insertion of a gene through T-DNA without knowledge of the integration site is a well established and commonly used practice. PCR of *TaPCS1* and *YCF1* was used to select colonies that had undergone non-specific recombination. The resulting strain contains the *TaPCS1* gene (Figure 11a) and is able to grow on 5-Fluoroorotic Acid, indicating the integration of the *GAL::TaPCS1-CYC1* cassette and confirming the absence of the pYES2 plasmid. The *TaPCS1* integration strain shows successful expression of the *TaPCS1* gene (Figure 11b and 11c), presence of PCs (Figure 13), and increased Cd tolerance (Figure 12). The upper limit of growth for the *TaPCS1* integration strain was determined to be 300μM $CdCl_2$ (Figure 12). No growth was seen at 400μM $CdCl_2$, and it was determined that the screening should be

conducted at this level of Cd. By screening just above the upper limit of Cd tolerance, the aim was to detect anything that would increase tolerance of Cd in the presence of PCs, to enable the potential identification of PC-Cd transporters.

A wheat cDNA library (Schachtman and Schroeder, 1994) was originally selected for the screening. This cDNA library was size selected to contain inserts greater than 1.5kb, and has been used successfully in the cloning of the wheat phytochelatin synthase gene, *TaPCS1* (Clemens et al., 1999). A size selected library presents a particular advantage for this screen, since some potential transporters are likely to be quite large (2.5 to 5kb), and will be present in greater proportion in a library selected for larger inserts. From this library, 41,500 transformants were screened for growth after 14 days and nine resistant colonies were identified.

While no peptide transporters were identified, a number of genes identified can be logically explained as enhancers of Cd tolerance by helping the yeast to overcome toxic effects of Cd rather than facilitating Cd chelation and removal. Cadmium causes cellular damage through many mechanisms. It binds thiol groups on proteins with high affinity, resulting in the inhibition of protein function, such as those involved in electron and proton transport and of the activity of enzymes involved in the Krebs cycle, glycolysis, pentose phosphate pathway, and the Calvin cycle in plants (reviewed by Seregin and Ivanov, 2001). In addition, cadmium can displace zinc, iron and copper from proteins, again leading to the inhibition of protein function and also leading to an increase of reactive oxygen species (ROS). Cd also promotes the formation of ROS in the mitochondria through an increased buildup of semi-ubiquinone, resulting in an increase production of superoxide anions (Bolduc et al., 2004). *In vitro* experiments have shown

that Cd and hydrogen peroxide together can induce DNA damage, through the creation of free hydroxyl radicals (Badisa et al., 2007). Oxidative stress has also been reported to cause lipid peroxidation, and a product of this reaction, 4-hydroxynonenal, has been shown to be mutagenic (Singh et al., 2005). In addition to promoting DNA mutations, Cd has also been shown to inhibit DNA repair through a specific inhibition of mismatch repair (Jin et al., 2003; Clark and Kunkel, 2004).

Among the cDNAs identified from the wheat root library were a protein putatively involved in DNA repair, triosephosphate isomerase, and an ADP/ATP translocator (Table 1). Because cadmium is known to induce processes that are mutagenic and at the same time inhibits DNA repair, it is possible that an increase in DNA repair proteins may enhance the ability of the yeast to grow in the presence of Cd. Triosephosphate isomerase is an enzyme involved in glycolysis and the calvin cycle, processes that are both inhibited by cadmium. While the enzyme itself has not been reported to be affected by Cd exposure, expression of triosephosphate isomerase has been shown to be upregulated after Cd exposure (Shin et al., 2003), although the reason for its upregulation is not yet understood. While the relationship between Cd exposure and behind triosephosphate isomerase is not so clear, the reasoning behind the detection of an ADP/ATP translocator can be better understood. By inhibiting glycolysis, the pentose phosphate pathway, electron transport and the Krebs cycle, cadmium limits the availability of ATP within the cell. It is possible that an increased translocation of ADP for ATP can help to maximize the availability of ATP for necessary cellular processes, enabling the yeast to grow more vigorously in the presence of Cd.

While some of the cDNAs identified as increasing Cd resistance of the *TaPCS1* integration yeast appear to be related to the toxic effects caused by Cd and are potentially helping the cell to resist aspects of Cd toxicity, some of the cDNAs identified have no apparent connection to Cd to support the resistance observed. In fact, two out of the nine cDNAs selected from the screen were identified as empty pYES2 vector, suggesting that 400μM Cd is not entirely inhibitive of *TaPCS1* integration strain growth after 14 days. Also, one cDNA isolated was not able to be identified as it bears no homology to any known plant proteins.

The genome of *Triticum aestivum* has not been sequenced, and this makes it more difficult to identify cDNAs that increase Cd tolerance. Because of the difficulty of identifying selected genes, and the concern over colonies appearing on 400μM Cd plates with empty plasmid after 14 days of incubation, two modifications were made to the screening procedure. First, the incubation time was shortened from 14 days to 7 days, with the hope that this would allow the identification of cDNAs that were specifically enhancing Cd tolerance. Second, it was decided to switch from screening a wheat library to an Arabidopsis cDNA library in order to be able to identify the exact genes detected during the screen.

An Arabidopsis seedling cDNA library (Minet et al., 1992) was selected for the modified screening procedure. Approximately 500,000 transformants were screened, revealing 5 resistant colonies after 7 days of incubation. Unfortunately, shortening the incubation time did not lower the amount of nonspecific growth, as 3 of the 5 colonies were found to contain only empty vector. Another cDNA that was identified, At2g07360, was found to contain only the final 600bp of the 3.6kb coding sequence indicating that

this was probably another false positive. One vector was isolated from a resistant colony that contained the complete cDNA of the gene At1g51360, a gene with unknown function that contains a stress-responsive alpha-beta barrel. While the protein has no predicted transmembrane domains and is too small to be a transporter (only 21 kDa), it seems logical that a stress responsive protein would be revealed when screening based on tolerance to Cd toxicity. While many or the cDNAs isolated appear to make sense in the context of the cellular toxicity of Cd, retransformation of *S. cerevisiae* with these plasmids has not yet been done and would be necessary to determine whether the cDNAs actually enhance Cd tolerance.

Shortening the selection time appears to have not made the screen any more specific for enancers of Cd tolerance, and other modifications to the screening procedure may be necessary. The colonies of *TaPCS1* integration strain are visible after 4 days on 200μM Cd, indicating that genes involved in the stabilization or removal of Cd from the cytosol may allow for growth within a similar time period. Screening at a lower concentration of Cd after a shorter period of time may improve the chance of catching a cDNA that detoxifies Cd directly. Additionally, plating cells in lower density would ensure that all of the library transformed cells have equal contact with the Cd containing media, which could potentially reduce the appearance of false-positive colonies. Another way to allow all cells to be equally exposed to Cd would be to screen library transformants in liquid culture containing Cd. This method has been successfully applied to Cd tolerance screening before, allowing for the isolation of the wheat phytochelatin synthase gene (Clemens et al., 1999). The cDNA library screening with the *TaPCS1* integration line is still in progress, and these modifications will be tested.

Another concern over this screening method has been the large size of some of the candidate transporters. The wheat root pYES2 cDNA library is size selected to contain fragments over 1.5kb in length and the Arabidopsis pFL61 cDNA library was determined to have an average insert size of 1.6kb. These libraries both contain relatively large cDNAs, full sized ABC transporters range from 3.5-5 kb, making their presence in either of cDNA library less frequent, although larger cDNAs have been successfully isolated from the pFL61 library. No PC transporters have yet been identified in plants, although some transporters such as MRP3, PDR7 and PDR8 have been identified as promising candidates. In addition to continuing the screening of library cDNAs, a more direct approach would be cloning candidate transporters and testing their effect on the Cd tolerance of WT and the *TaPCS1* integration yeast strain. In addition, the only reported transporter of PCs, SpHMT1, was identified as being necessary for the formation of HMW complexes. In order to test whether any of these transporters result in the transport of PCs into *S. cerevisiae* vacuoles, it first needs to be established whether the yeast is able to form HMW complexes without any additional transporter. In order to test this, a reduced PC-Cd standard was separated over the column and it was identified that low molecular weight PC-Cd elutes in fraction 7 (Figure 14a). When whole cell extract of the Cd exposed TaPCS1 integration strain was separated in the same manner, the majority of cellular Cd was detected in fraction 7, indicating *S. cerevisiae* is not capable of forming HMW complexes (Figure 14b). However, recent data from *Δycf1 S. cerevisiae* vacuole preparatrions suggests that *S. cerevisiae* is capable of PC transport in an ATP-dependent manner (Mendoza-Cózatl, DG, Song WY, Martinoia E and Schroeder JI, unpublished data). If there is a transporter in *S. cerevisiae* capable of PC transport, it is possible that

the vacuoles are not forming HMW complexes due to a lack of vacuolar sulfide, rather than a lack of vacuolar PCs.

To evaluate the approach of testing cloned candidate transporters in the *TaPCS1* integration strain, *SpHMT1* was cloned and Cd tolerance was analyzed in wild-type and the *TaPCS1* integration strain with and without the transporter. Surprisingly, HMT1 conferred Cd resistance in *S. cerevisiae* regardless of whether the strain could synthesize PCs. HMT1 has been previously reported as a PC-Cd transporter because it is required for the formation of HMW PC-Cd-S complexes (Ortiz et al., 1992; Ortiz et al., 1995). However, the closest *Arabidopsis* homologues of SpHMT1 are mitochondrial FeS transporters (Kushnir et al., 2001; Chen et al., 2007). Homology to FeS transporters, taken together with the Cd tolerance conferred in the absence of PCs suggests that HMT1 may have other substrates able to establish vacuolar HMW complexes, such as the vacuolar transport of sulfide or CdS. Indeed, during the analysis of Cd tolerance it was observed that both wild-type and *TaPCS1* integration yeast expressing *SpHMT1* acquired a yellow phenotype (Figure 16), which may be indicative of a metal-sulfide precipitate (Merck Index, 1989; Di Toro et al., 1990).

More experiments are necessary to explore the possibility that *S. cerevisiae* may possess an endogenous PC transporter, the potential that it may lack sufficient vacuolar sulfide for the formation of high molecular weight complexes, and the possibility that HMT1 may confer Cd tolerance through the transport of sulfide. The next step will be to determine whether HMW PC-Cd-S complexes are formed in the integration strain expressing *SpHMT1*, and whether analogous GS-Cd-S complexes are present in wild-type yeast expressing *SpHMT1*.

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Chapter Three:

Increased synthesis of thiol-peptides together with the shoot expression of a vacuolar thiol-Cd transporter as a strategy for Cd phytoextraction.

3.1. Abstract

Heavy metals are released into the environment as byproducts of many industrial processes, and they are detrimental to human health in trace amounts. Plants naturally accumulate metals that exist at trace levels in the soil, and thus phytoextraction has been proposed as a viable solution for the growing problem of heavy metal contamination of soils. Cadmium (Cd) and other heavy metals are chelated by the thiol-peptides glutathione (GSH) and phytochelatins (PCs) intracellularly and sequestered in the vacuole. Aims to improve Cd retention in plants have focused on the overexpression of enzymes involved in the biosynthesis of thiol-peptides, however many of these approaches have led to the development of plants that are unexpectedly more sensitive to Cd. Another approach has been the expression of the *Saccharomyces cerevisiae* vacuolar glutathione-Cd (GS_2Cd) transporter YCF1 in the whole plant. Although this approach was successful, it would be limited by the level of available GSH. This study uses a combined approach to increase GSH levels through the increased expression of the limiting enzyme in GSH synthesis (γ-glutamylcysteine sythetase - γECS), while also increasing GS_2Cd shoot retention through the shoot specific expression of the YCF1 transporter. Moderate expression of bacterial γECS increased GSH levels by 2-fold and resulted in only a mild sensitivity to Cd. The promoter *CAB2p* was found to have primarily shoot expression, and a *CAB2p::YCF1* plant expression construct was created and transformed into wild-type and plants already expressing bacterial *γECS*. Surprisingly, all *YCF1* expressing T2 lines (in wild-type and *EcγECS* lines) were found to accumulate more Cd in root, and less in shoot tissue as compared to Columbia wild-type. Possible explanations for this phenotype are discussed.

3.2. Introduction

Cadmium (Cd) is present in the environment largely as a byproduct of industrial processes and through the dumping of Cd containing waste such as cadmium containing pigments and nickel-cadmium batteries. Major routes of Cd exposure in humans include inhalation, ingestion and absorption through the skin, and exposure can cause serious health problems such as kidney damage and bone deterioration (reviewed by Godt et al., 2006). Cadmium is also a carcinogen, causing DNA damage and inhibiting the mismatch repair mechanism, as well as potentially disrupting cell-cell adhesion through a toxic interaction with cell surface proteins (Jin et al., 2003; Clark and Kunkel 2004; Prozialeck and Lamar, 1999). The toxicity of Cd and its increasing accumulation in soil has made the remediation of cadmium-containing waste a concern of public health. In 2007, cadmium ranked 7th on the Agency for Toxic Substances and Disease Registry's CERCLA National Priority List of Hazardous Substances (U.S. Department of Health and Human Services, 2007).

Currently, physical and chemical remediation techniques are applied for the clean up of cadmium contaminated soil. This is comprised of the physical removal of contaminated soil from the site, followed by chemical processing and filtration of the sediment. On whole this process is costly and labor intensive, and consequently there is interest in developing a different approach to cadmium cleanup (Raskin et al., 1997).

Phytoremediation, which uses plants to clean up contaminated soil, is one promising alternative. There are multiple ways in which plants can be used to decontaminate the hazardous substances within soil, including phytostabilization and phytoextraction. Plants are not able to chemically stabilize cadmium in a way that would

make it either permanently non-toxic or non-bioavailable. However, the efficiency by which they accumulate nutrients present at low concentration in soil and the capacity for mobilization of metals throughout the plant have led to the idea that plants can be useful tools for heavy metal phytoextraction. In order for plants to be efficient extractors of Cd, they must be able to both accumulate the metal and direct storage to the aerial, harvestable tissue.

Cadmium enters the roots nonspecifically through transporters responsible for the uptake of essential heavy metals such as iron and zinc (Williams et al., 2000; Thomine et al., 2000; Pence et al., 2000). Upon entering a plant cell, cadmium binds cellular thiol groups, in particular to metallothioneins and to glutathione, with which it forms GS_2Cd complexes. GS_2Cd and GSH are the substrates of phytochelatin synthase (PCS), which upon Cd exposure produces phytochelatins: peptides of the sequence γ-$(gluatmylcysteine)_n$-glycine where n=2-11. After synthesis in the cytosol PC-Cd complexes are believed to be transported into the vacuole, where after sulfide (S^{2-}) incorporation they have been shown to form high molecular weight (HMW) complexes surrounding a CdS core (Reese et al., 1992). The formation of such HMW complexes is believed to be important for the full detoxification of Cd, as they have greater stability than low molecular weight PC-Cd complexes (Reese and Winge, 1988). In addition to their intracellular role in the vacuolar sequestration of Cd, thiol peptides such as PCs and GSH have been reported to be involved in long-distance movement of cadmium between roots and shoots (Gong et al., 2004; Chen et al., 2006; Mendoza-Cózatl et al., 2008). Because of their combined role in the accumulation and movement of Cd throughout the

plant, many attempts have been made at manipulating the thiol peptide mediated Cd detoxification pathway for phytoremediation purposes.

One approach for improving heavy metal tolerance has involved the expression of heavy metal ion transporters. The expression of a bacterial heavy metal extruder *EcZNTA* increased the tolerance and decreased the accumulation of heavy metals (Lee et al., 2003). The overexpression of an endogenous Arabidopsis heavy metal exporter *AtHMA4* improved Cd tolerance and resulted in a slight increase in translocation of Cd to the shoots (Verret et al., 2004).

After phytochelatin synthase was identified, attempts at improving the phytoremediation capability of plants shifted towards the manipulation of the thiol peptide mediated heavy metal detoxification mechanism. An attempt was made to overexpress the PCS enzyme with the aim of creating a plant that would be more tolerant and accumulate more Cd. While the overexpressors did show increased levels of PCs upon exposure to Cd, they were found to be Cd hypersensitive (Li et al., 2004). The reason for this hypersensitivity is not yet fully understood. Multiple studies have attempted to increase the entire thiol peptide biosynthetic pathway through the overexpression of the limiting enzyme, γ-glutamylcysteine synthetase (γ-ECS), with varying success. In a study involving the expression of the enzyme in *Brassica juncea*, bacterial γ-ECS was found to improve Cd tolerance and increase Cd accumulation (Zhu et al., 1999). In another study, the *Actin2* promoter was used to high overexpress bacterial γ-ECS in *Arabidopsis thaliana*. This approach led to significantly enhanced levels of GSH and PCs, however once again the plants were found to be cadmium hypersensitive (Li et al., 2005). The toxicity seen is possibly a result of oxidative stress induced by the

accumulation of oxidized γ-EC (Creissen et al., 1999; reviewed by Mendoza-Cózatl et al, 2005). In addition, the activity of γ-ECS is feedback-inhibited by the level of GSH, preventing the overexpression of γ-ECS from yielding large increases in glutathione level (Mendoza-Cózatl and Moreno-Sánchez, 2006). These difficulties, in conjunction with the finding that the flux through the GSH biosynthetic pathway is controlled mainly by the demand for GSH, have led to the proposal that to increase the production of thiol peptides, both γ-ECS and another protein involved in the consumption of glutathione should be expressed at moderate levels simultaneously (Mendoza-Cózatl and Moreno-Sánchez, 2006).

Another approach to enhance the phytoremediation capacity of plants through manipulation of thiol-peptides has been the expression of the *S. cerevisiae* vacuolar GS_2Cd transporter *YCF1* in *Arabidopsis thaliana*. Expression of this transporter was found to increase the Cd tolerance and accumulation of the transgenic plants, however its impact on the root-to-shoot accumulation ratio was not examined (Song et al., 2003). While no plant PC-Cd transporters have yet been identified, the promising results from the expression of *YCF1* suggest that once one is identified, overexpression of a vacuolar PC-Cd transporter would also enhance Cd accumulation and tolerance. Also, because of the 2×10^6 fold higher affinity that PCs have for Cd, this may be an even more effective method for increasing plant Cd levels.

The expression of GS_2Cd or PC-Cd transporters represents a promising direction for the development of an efficient phytoextraction technique, however two issues must first be addressed. First, if either type of transporter is expressed too highly, it may result in a toxic depletion of available GSH. This could be ameliorated through a system where

GSH demand and GSH biosynthesis are increased in tandem, such as moderate enhancement of *γ-ECS* expression simultaneous to the expression of a thiol-Cd transporter. Second, the efficiency of phytoextraction requires that the toxin be accumulated largely in aerial, harvestable tissue. This requirement may be met by directing the expression of GS_2Cd of PC-Cd transporters specifically to the aerial tissue.

In this study, the *Escherichia coli γ-ECS* (*EcγECS*) under control of the *35S* promoter was transformed into *Arabidopsis thaliana* plants. Four homozygous lines with moderate levels of *γ-ECS* expression were isolated and analyzed to confirm that the expression of *γ-ECS* would not lead to Cd hypersensitivity. To increase the accumulation of Cd in the shoot, the yeast vacuolar GS_2Cd transporter *ScYCF1* was expressed specifically in shoots through the use of the chlorophyll a/b binding protein promoter, *CAB2p*. Promoter-GUS analysis was used to confirm that *CAB2p* directs shoot-specific expression, and *CAB2p::ScYCF1* was transformed into the three independent *35S::Ecγ-ECS* homozygous lines. Cd accumulation of the roots and shoots were analyzed to determine the efficiency of this approach.

3.3. Methods

Creation of homozygous 35S::EcγECS lines.

Plants were transformed with Agrobacterium containing the *35S::EcγECS* construct in the pBIN19 plant expression vector (Noctor et al., 1996) by the floral dip method. T1 seeds were selected on ¼ MS + 50μg/ml kanamycin, and were transferred to soil after 10 days. RNA was extracted from 4 week old T1 plants using the RNeasy Plant Mini Kit (Qiagen) and lines were selected based on expression level. T2 seeds were germinated on guarter-strength Murashige and Skoop medium with 50μg/ml kanamycin and 15 plants from each line were transferred to soil and grown to maturity. T3 seeds were collected from each T2 line and 30 seeds from each T2 line were germinated on guarter-strength Murashige and Skoog with 50μg/ml kanamycin. Lines in which all seedlings germinated were determined to be homozygous.

Root elongation

Seedlings were germinated on quarter-strength Murashige and Skoog agar plates and grown vertically for 7 days, and then seedlings were transferred to ¼ MS with 0μM $CdCl_2$ or 75μM $CdCl_2$. The roots were covered with 60μm Spectra/Mesh Nylon filters (Spectrum Labs) and the position of the root tips were marked. The seedlings were grown for an additional 8 days, and then the length of elongation was measured.

GSH quantification

Seeds were germinated on quarter-strength Murashige and Skoog plates and grown for 10 days. Seedlings were transferred to the hydroponic culture system described

in Chen et al., 2006, with minor modifications. Hydroponic solution (Arteca and Arteca, 2000) was changed every 2 days, and the region that contained hydroponic medium was kept in darkness through the application of a black cardboard mask to prevent the growth of photosynthetic microorganisms such as algae, which are able to synthesize thiol-peptides. 1 week post-bolting, plants were exposed to hydroponic solution containing 20μM $CdCl_2$ for 72 hours. Roots and rosette leaves were harvested and rinsed in Milli-Q water (Millipore). After rinsing, the tissue was blotted to remove excess water, and tissue was frozen in liquid N_2. Frozen tissue was homogenized and massed while frozen. Approximately 1.5ul extraction buffer (6.3mM diethylenetriamine-pentaacetic acid 0.1% trifluoroacetic acid) were added per mg fresh weight. Samples were vortexed for 1 minute and centrifuged at 20,000×g for 10 minutes. 65μl of cell extract supernatant was used for mBBr labeling, and the labeling procedure and following fluorescence quantification were followed as described in Part I of this thesis (Mendoza-Cózatl et al., 2008).

Cadmium quantification

Seedlings were germinated and grown on quarter strength Murashige and Skoog plates for 10 days, and transferred to the hydroponic culture system described above. 1 week post-bolting, plants were exposed to hydroponic solution containing 20μM $CdCl_2$ for 72 hours. Plants were then washed 5 minutes with Milli-Q water (Millipore), 5 minutes with 100mM $CaCl_2$, and then 5 minutes with Milli-Q water. Root tissue and rosette leaves were separated, transferred to 15ml polypropylene Falcon tubes (Becton Dickson) and dried at 60°C overnight. Dry weight was measured, tissue was digested in

1ml trace metal grade 70% HNO_3 (Fisher Scientific) overnight and boiled 30 minutes the following day. Digested plant material was centrifuged at 1200×g for 10 minutes. 0.5 ml of supernatant was transferred to a new polypropylene tube and diluted to 3.5% HNO_3 final concentration with Milli-Q water (Millipore). Samples were then analyzed for metal content by ICP-OES (Inductively Coupled Plasma – Optical Emissions Spectroscopy) at the Scripps Institute of Oceanography's analytical facility.

Construction of *CAB2p::YCF1-Nos*

The *CAB2p* promoter was amplified from Columbia wild-type Arabidopsis genomic DNA using the primers CABF-Kpn: ggggtaccgtgttaacagcttttatagttcaaag and CABR-HindIII: cccaagcttgaaacttttttgtgttttttttttttttatgactaac and was cloned into pGreenII 0179 using the restriction sites KpnI and HindIII. YCF1 was amplified from the *35S::YCF1* plasmid (Song et al., 2003) using the primers YCFF-BamHI: cgggatccatggctggtaatcttgtttcatggg and YCFR-NotI: atagtttagcggccgcttaattttcattgaccaaaccagcc and cloned into *CAB2p*::pGreenII 0179 using the restriction sites BamHI and NotI. The Nos terminator was amplified from pBI121 (Gong et al., 2004) using the primers NOSF-NotI: ataagaatgcggccgcgatcgttcaaacatttggcaataaag and NOSR-SacII: tccccgcggatctagtaacatagatgacaccgc and was cloned into *CAB2p::YCF1*-pGreenII 0179 using the restriction sites NotI and SacII, yielding the construct *CAB2p::YCF1*-Nos in pGreenII 0179. Plants were transformed with the construct by the floral-dip method and positive transformants were selected on 25μg/ml hygromycin plates.

Construction of *CAB2p::GUS*

The *CAB2p* promoter was amplified from the *CAB2p::YCF1-Nos* construct using the high-fidelity Phusion enzyme (Finnzymes) and the primers caccgtgttaacagcttttatagttcaaag and cccaagcttgaaacttttttgtgtttttttttttttttatgactaac. PCR amplifications were run on 1.5% agarose and the corresponding DNA fragment was cut from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen). The purified amplification product was cloned into pENTR using the pENTR/D-TOPO cloning kit (Invitrogen). The sequence was verified (Retrogen, Inc) and the LR clonase enzyme (Invitrogen) was used to transfer the promoter from CAB2p-pENTR into the Gateway compatible pBGGUS destination vector (Riken).

Analysis of *CAB2p*-driven expression

CAB2p-pBGGUS was transformed into Columbia wild-type Arabidopsis plants by the floral-dip method. Resulting seeds were screened on quarter-strength Murashige and Skoog plates with Basta. Positive transformants were identified after 7 days of growth and were transferred to quarter-strength Musashige and Skoog for at least 3 days prior to staining. At the desired stage of growth, seedlings were removed from agar plates and submerged in freshly made staining solution (10% methanol 0.1% Triton X-100 100mM $NaHPO_4$ pH7 0.5mM $K_3Fe(CN)_6$ 0.5mM $K_4Fe(CN)_6$ 10mM EDTA 1mM X-gluc). Plants in staining solution were placed at 37°C overnight, then washed 3 times for 1 hour with 70% ethanol.

3.4. Results.

Overexpression of bacterial *γECS* in *Arabidopsis thaliana*

Expression of *EcγECS* in shoot and root tissue was analyzed by RT-PCR of homozygous T3 plants (Figure 18). γECS2 was found to be have high expression in shoots, while γECS4 was found to have high expression in roots. γECS5 and γECS6 were found to have moderate expression throughout the plants.

Glutathione levels in shoots and roots of Cd exposed plants were determined through fluorescence HPLC-MS of mBBr labeled thiols (Figure 19). γECS4, γECS5 and γECS6 were found to have a statistically significant increase of GSH in the shoot tissue after Cd exposure. All lines, including Columbia wild-type plants were found to have more GSH in shoots than in root tissue.

Overexpression of *γECS* has been previously reported to cause Cd hypersensitivity when the enzyme accounted for 0.1% of total cellular protein content (Li et al., 2005). To determine whether the moderate expression of *γECS* is sufficient to also result in Cd hypersensitivity, root elongation experiments were performed. *γECS* lines were grown on quarter-strength Murashige and Skoog plates for 7 days, then were transferred to quarter-strength Murashige and Skoog with 0 or 75μM $CdCl_2$ and grown for 8 additional days (Figure 20). γECS4, γECS5 and γECS6 were found to have slightly reduced root growth on 75μM $CdCl_2$ as compared to wild-type seedlings, however γECS4 also showed less root elongation than wild-type seedlings at 0μM $CdCl_2$. Shoot tissue of all *EcγECS* expression lines were of comparable size to wild-type, and showed no additional chlorosis or any other indication of being more sensitive to Cd.

While growing *EcγECS* expression lines, γECS2 was noted to have an abnormal developmental phenotype. Some seedlings were found to have a delayed and unbalanced emergence of the first true leaves, in which one true leaf would emerge normally and the second, opposing leaf would emerge later and with an abnormal, twisted morphology. When γECS2 T2 seedlings were germinated on kanamycin, 26% displayed this phenotype, 50% showed normal development and 24% were found to be sensitive to the antibiotic, indicating that the phenotype observed has the expected segregation ratio for a trait related to the T-DNA insertion. Due to this unexpected abnormality in the γECS2 line, it was decided to proceed with an analysis of γECS4, γECS5, and γECS6 only, and to use only these three healthy lines for further transformation with *CAB2p::YCF1*.

Cadmium accumulation in roots and rosette leaves of hydroponically grown wild-type and homozygous *35S::EcγECS* lines was analyzed by ICP-OES. Wild-type shoots and roots showed no statistical difference, and none of the roots or shoots of the *EcγECS* expression lines showed Cd accumulation that was statistically different from wild-type plants.

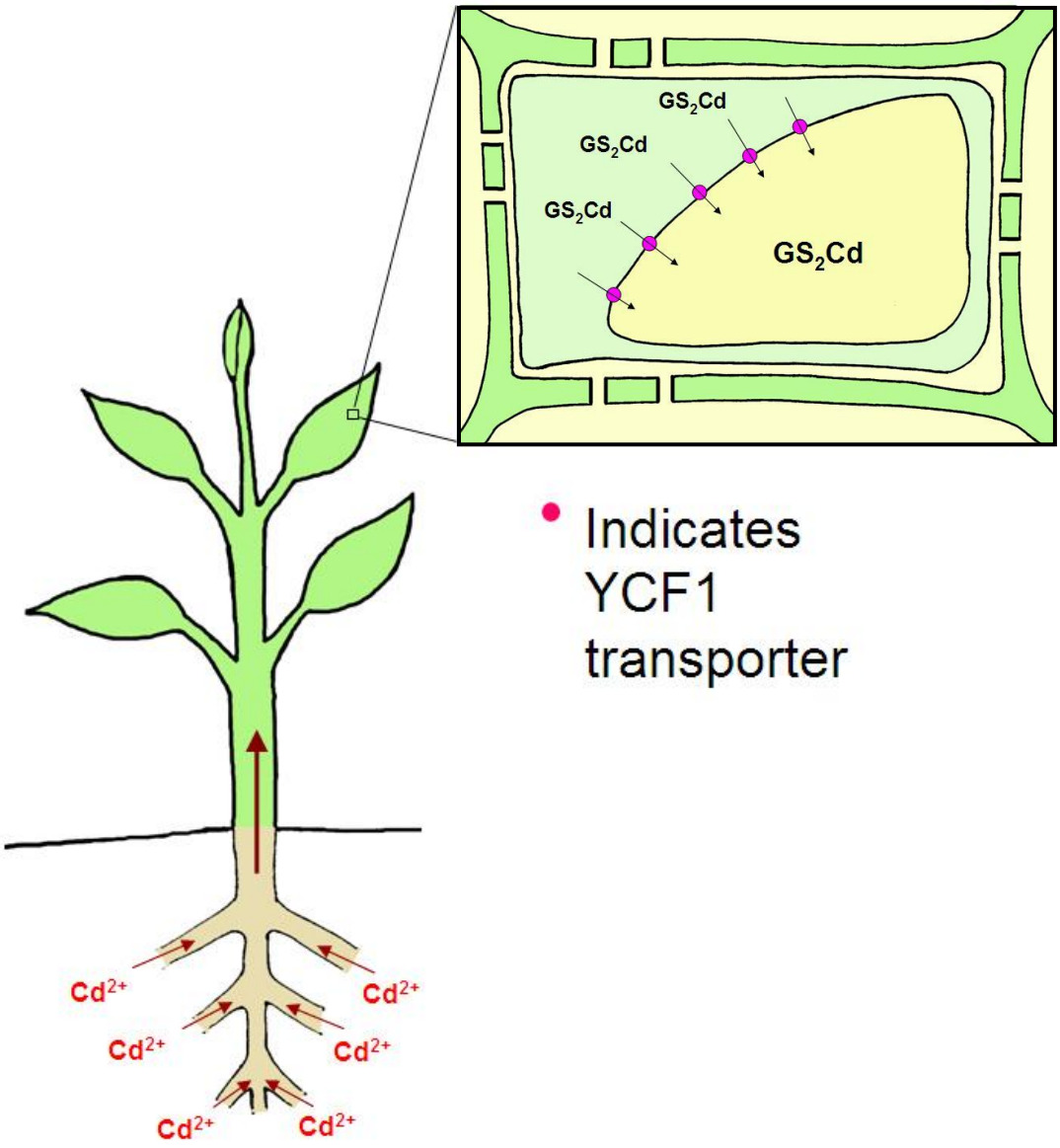

Figure 17. Schematic representation of the phytoextraction technique designed in this study. Cadmium enters through the roots and undergoes long-distance translocation through the xylem and the phloem. GS_2Cd is sequestered in the vacuoles of green tissues by the transporter ScYCF1.

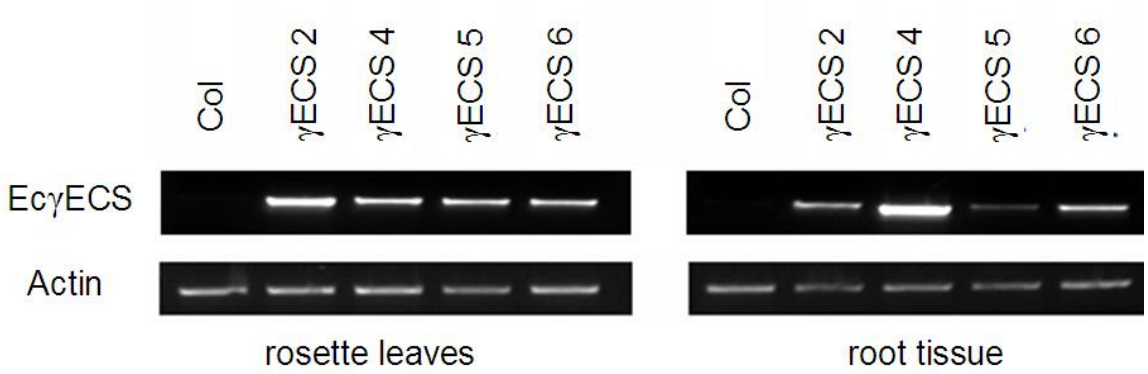

Figure 18. Expression analysis of *EcγECS* lines. Shoot and root expression of *EcγECS*, 35 cycles and *Actin*, 22cycles.

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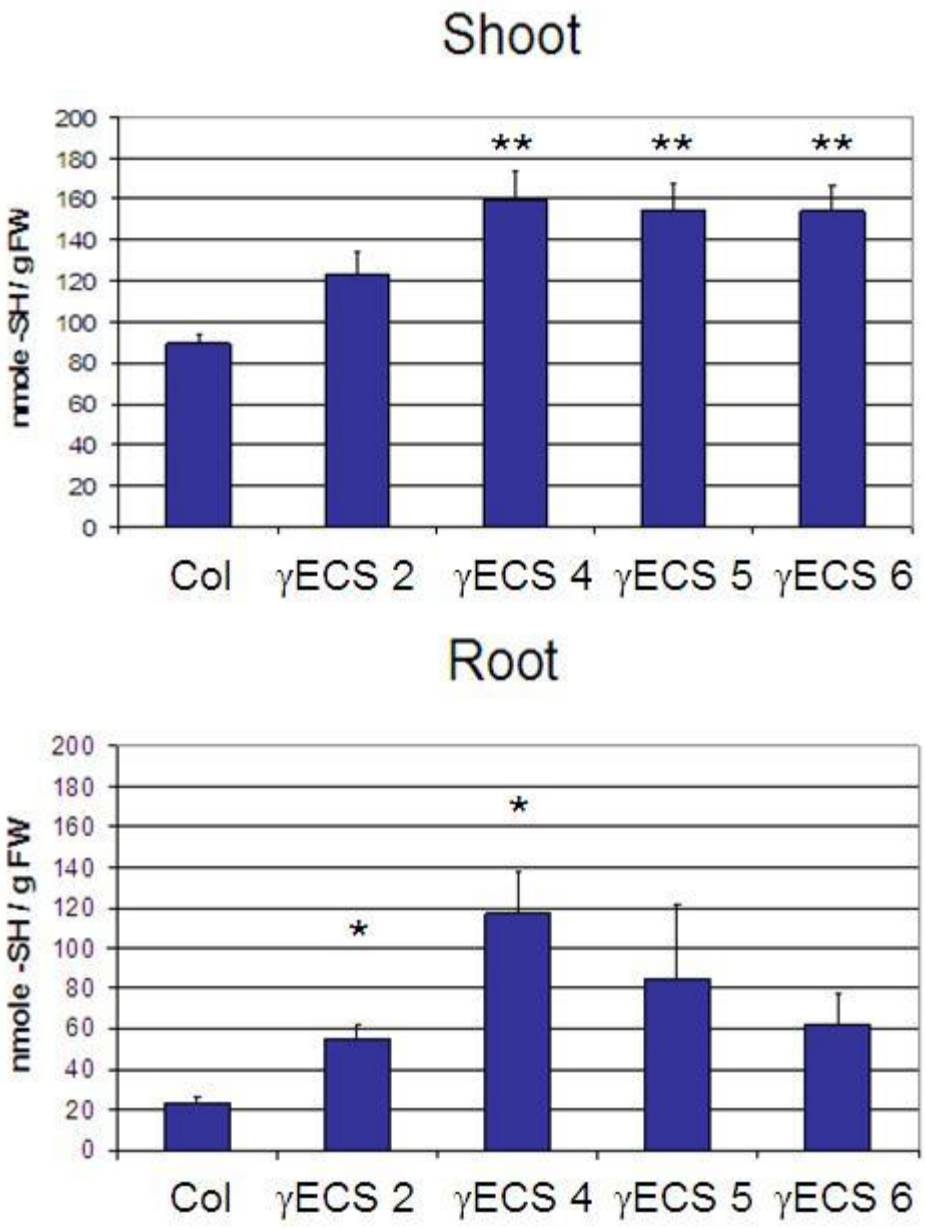

Figure 19. GSH level of *EcγECS* expression lines. Plants were grown hydroponically and exposed to 20μM $CdCl_2$ for 72 hours prior to thiol extraction. n=3, error bars represent standard error. * indicates $p < 0.05$, ** indicates $p < 0.01$ compared to Columbia wildtype.

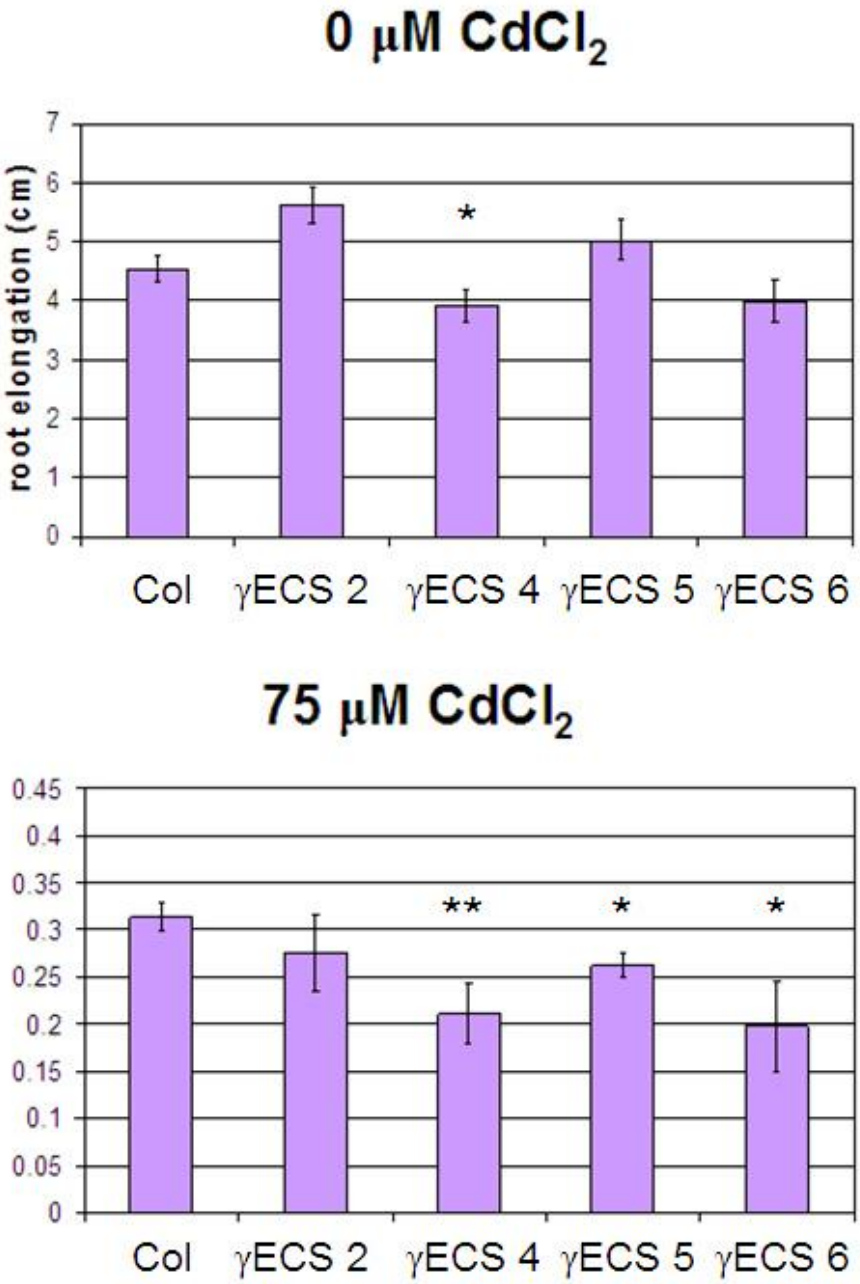

Figure 20. Root elongation of *EcγECS* expression lines. Seedlings were grown on ¼ MS 7 days and transferred to ¼ MS containing 0μM $CdCl_2$ or 75μM $CdCl_2$ for 8 days. n=10, error bars represent standard error. * indicates $p < 0.05$, ** indicates $p < 0.01$ compared to Columbia wildtype.

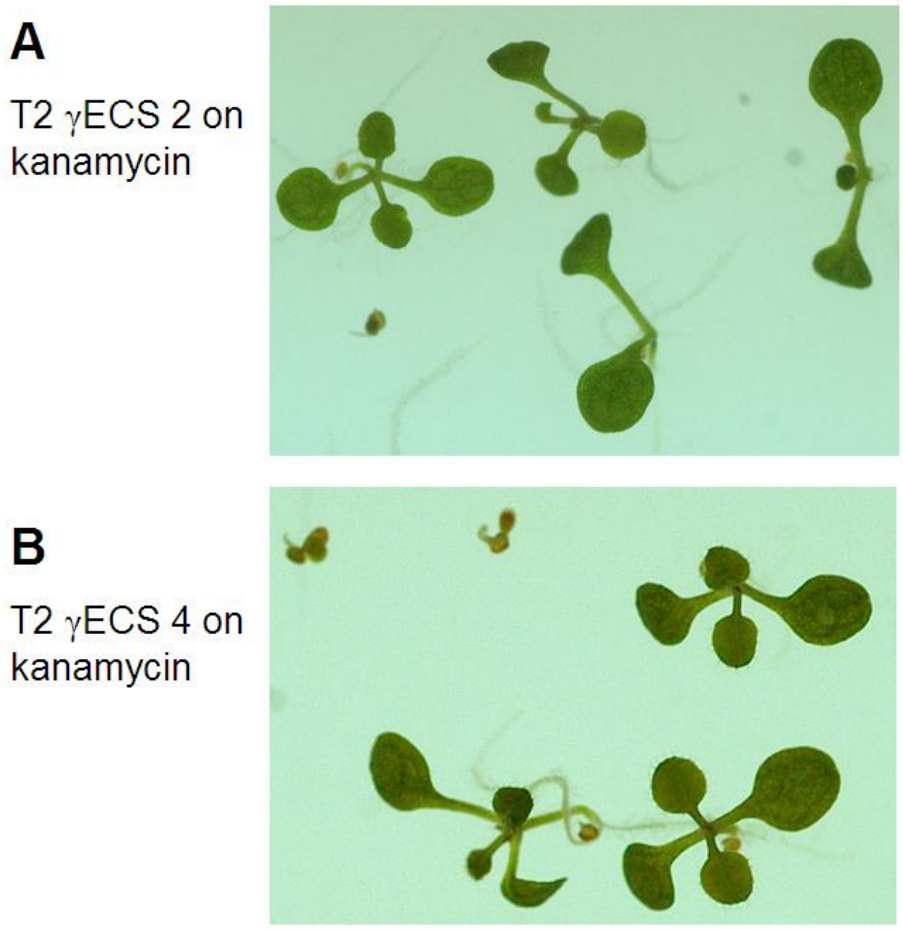

Figure 21. Abnormal growth phenotype of γECS 2. Seedlings representative of γECS2 and γECS4 T2 seedlings grown on kanamycin. An abnormal growth phenotype was observed for γECS2, in which 26% of seedlings showed delayed and unsymmetrical emergence of true leaves, 24% of seeds showed kanamycin sensitivity, and 50% showed normal growth n=58 seedlings (A). Normal growth was seen in the other γECS expression lines, represented here by γECS4, with 31% kanamycin sensitive and 69% showing normal growth, n=81 (B).

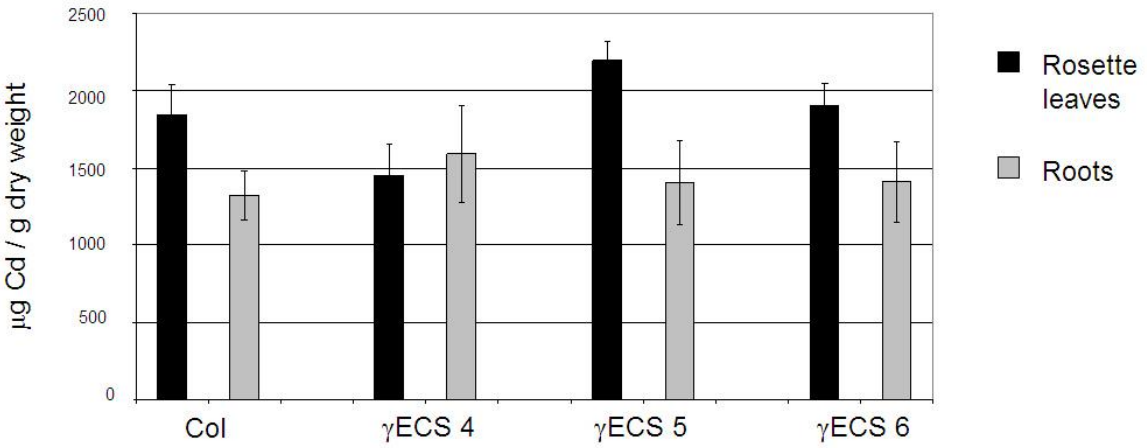

Figure 22. Cadmium accumulation of *EcγECS* expression lines. Plants were grown hydroponically and exposed to 20µM $CdCl_2$ for 72 hours before harvesting. Roots (grey) n = 4, rosette leaves (black) n = 6-8. Error bars represent standard error, none of the *EcγECS* expression lines were statistically different from Columbia wild-type ($p > 0.05$).

Shoot-targeted expression of *ScYCF1* using the CAB2 promoter

In order to analyze the tissue specificity of the chlorophyll a/b binding promoter *CAB2p*, a *CAB2p::GUS* construct was made and transformed into Columbia wild-type Arabidopsis plants. Seedlings stained for GUS showed *CAB2p* promoter activity in the cotyledons and true leaves, but very little expression in root tissue (Figure 23a-c). Likewise, staining of bolting stage adult plants showed high levels of staining of all leaves and very little *CAB2p* promoter activity was detected in the roots (Figure 23d-e). Little GUS staining was detected in the stems, indicating the *CAB2p* directs expression mainly to leaf tissue (Figure 23d).

CAB2p::YCF1 was transformed into Columbia wild-type Arabidopsis and into three homozygous *35S::EcγECS* expression lines. T1 plants were analyzed for expression of *YCF1* and one line from the Columbia and each of the three independent *35S::EcγECS* backgrounds was selected for further analysis. *YCF1* rosette leaf expression levels of the selected lines can be seen in Figure 24. T2 plants hetero- or homozygous for the *CAB2p::YCF1* T-DNA were selected based on hygromycin resistance, and transferred to hydroponic culture. Plants were exposed to cadmium for 72 hours and the cadmium content of shoots and roots was analyzed (Figure 25). Unexpectedly, *CAB2p::YCF1/35S::EcγECS* plants showed a decrease in Cd in the shoots and an increase in Cd of the roots. Also, *CAB2p::YCF1*/Columbia showed lower Cd accumulation in shoots than wild-type Arabidopsis.

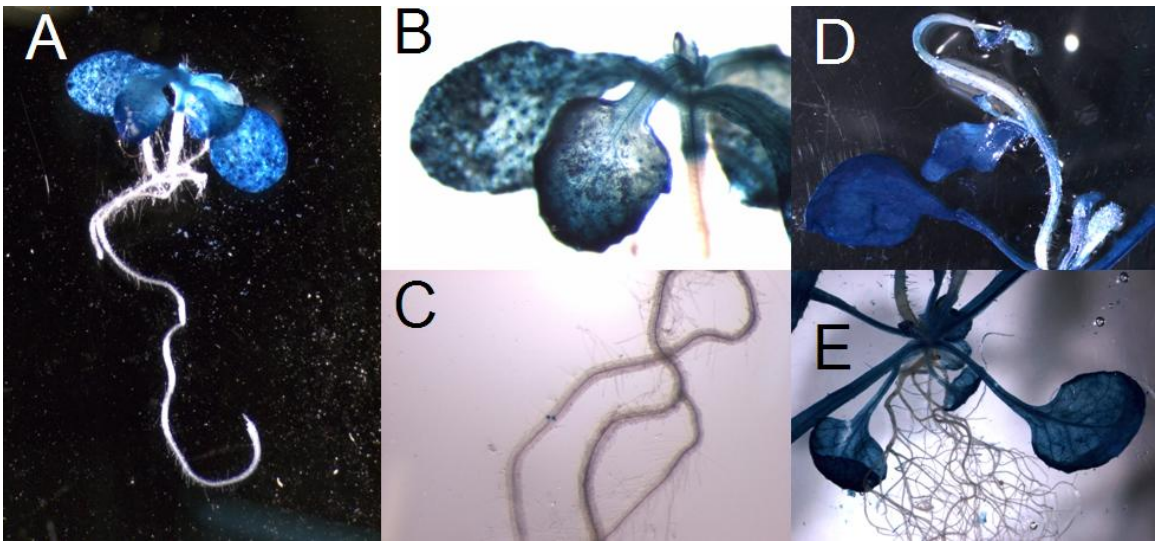

Figure 23. *CAB2p::GUS* promoter analysis. *CAB2p::GUS* transformed T1 seedlings were selected by Basta resistance. Seedlings and bolting stage adult plants were stained for GUS activity. Whole seedling (A), shoots of seedling (B), roots of seedling (C), shoots of adult plant (D), rosette leaves and roots of adult plant (E).

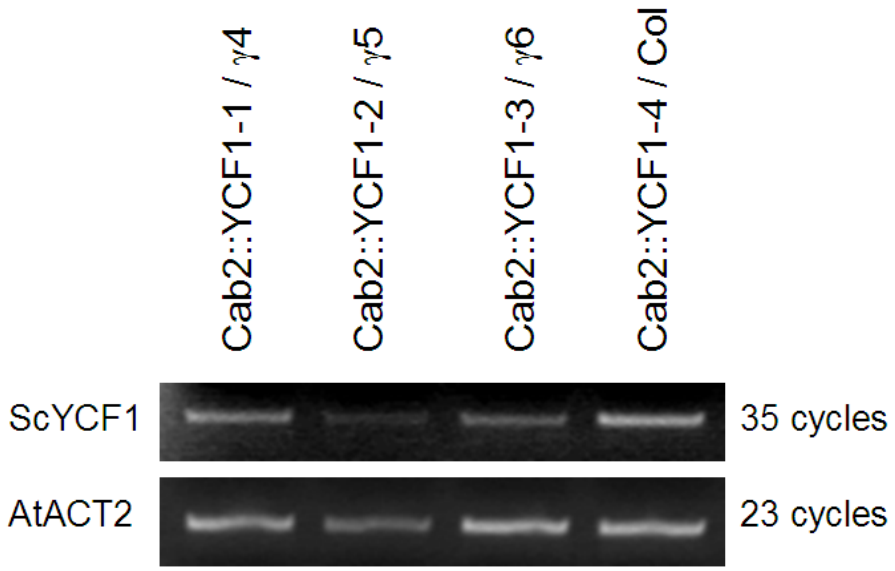

Figure 24. Expression of *YCF1*. RT-PCR of *ScYCF1* in *CAB2p::YCF1* in γECS4, γECS5, and γECS6 and Columbia wild-type backgrounds. YCF1 35 cycles, Actin 23 cycles.

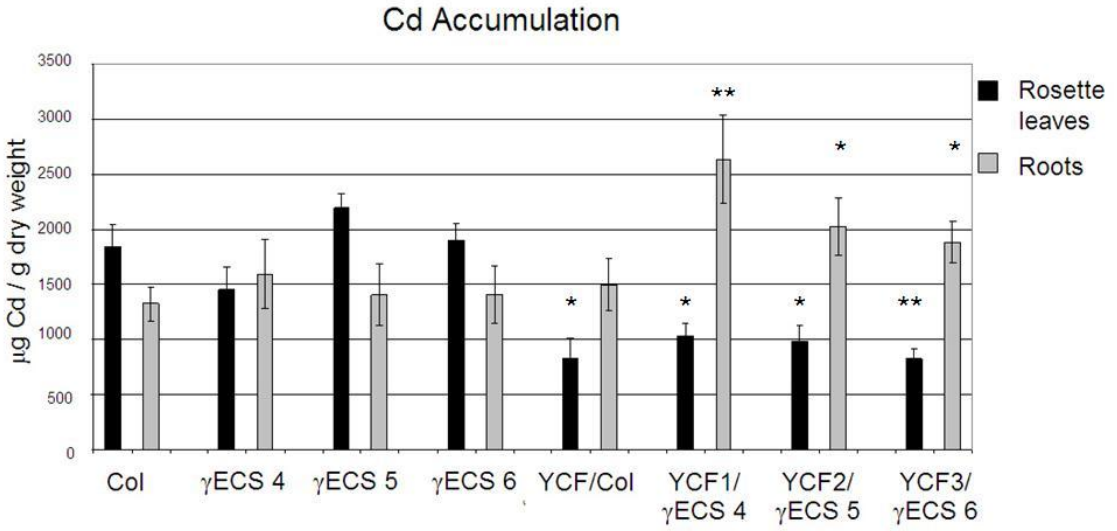

Figure 25. Cadmium accumulation of Columbia wildtype, 35S::EcγECS expression lines, and CAB2::YCF1 in Col and each 35S::EcγECS background. Rosette leaves (black) and root tissue (grey). * indicates $p < 0.05$, ** $p < 0.01$ statistically greater accumulation in roots than shoots.

3.5. Discussion

The main objective of this project is to simultaneously increase GSH production through the expression of bacterial *γECS* and GS_2Cd sequestration in the leaves through the expression of the *YCF1* transporter using a shoot specific promoter. For the first half of this study, *35S::EcγECS* was transformed into Columbia wild-type Arabidopsis, and homozygous lines were isolated and characterized.

In a previous study attempting to increase Cd tolerance and accumulation, *EcγECS* was expressed under the control of the Act2 promoter, yielding γECS enzyme that accounted for 0.1% of cellular protein. Surprisingly, this high overexpression of *γECS* makes plants hypersensitive to Cd (Li et al., 2005). This hypersensitivity can be explained by the accumulation of γEC and its oxidized form, which cause oxidative stress.

During the wild-type Arabidopsis glutathione biosynthesis, the AtγECS enzyme is regulated by feedback inhibition by the level of available GSH, which binds the enzyme reducing its activity. However, the bacterial γECS enzyme has a lower affinity for GSH than the AtγECS enzyme does, meaning that the amount of GSH required for feedback inhibition of the *E. coli* enzyme is much greater and expression of *EcγECS* leads to an increase in the cellular GSH level. By increasing the amount of GSH available within the cytosol, *EcγECS* expression also increases reactions that require GSH as a substrate, such as phytochelatin synthesis. In the study in which *EcγECS* was expressed under the actin promoter, a five-fold increase in PC_2 was observed. While PC_2 has been demonstrated to increase Cd tolerance, a byproduct of the reaction is γEC which in its oxidized form can induce oxidative stress (Creissen et al., 1999). Under physiological conditions, where the bacterial γECS is not present and the AtγECS is limiting GSH

synthesis due to feedback inhibition by GSH, more of the γEC that is produced during the synthesis of PCs is used by the glutathione synthetase enzyme to form GSH, preventing the toxic buildup of oxidized γEC.

The goal of this study was to increase the shoot accumulation of Cd, and in order for it to be successful it is important that the plants are not Cd hypersensitive. Because it is inhibited less by GSH, EcγECS acts as a potent enhancer of the glutathione biosynthesis pathway. Therefore, in order to increase the production of thiol peptides with less of a toxic effect, *35S::EcγECS* transformed plants with moderate levels of expression were selected.

In a previous study overexpressing *EcγECS*, the level of GSH was increased roughly four-fold, and significant sensitivity was seen at 75μM Cd (Li et al., 2005). The moderate levels of *EcγECS* expression in Arabidopsis were found to almost double the level of glutathione in the shoot tissue (Figure 19). While the GSH level was increased, only a slight Cd sensitivity was detected by root elongation assay at 75μM Cd (Figure 20). In addition, chlorosis has been reported as an indicator of the toxic accumulation of oxidized γEC (Creissen et al., 1999). When the *EcγECS* expression lines were grown on 75μM Cd (Figure 20) no chlorosis was observed, suggesting that a moderate level of bacterial *γECS* expression is sufficient to elevate GSH biosynthesis while preventing the oxidative stress associated with the buildup of oxidized γEC. These results appear to indicate that *EcγECS* expressed at lower levels is more advantageous for Cd phytoremediation purposes, where a plant must be both more tolerant and have the capacity to detoxify more Cd.

While there was an increase GSH (Figure 19), Cd accumulation of moderate *EcγECS* expression lines was not significantly altered. This finding is in agreement with a previous study where *EcγECS* was highly overexpressed but no difference in Cd content was detected (Li et al., 2005). This finding is also consistent with the idea that in order to for an increase in thiol peptides to lead to an effective phytoremediation strategy, the additional expression of a GSH consuming, Cd detoxifying element such as the vacuolar GS_2Cd transporter YCF1 is needed.

The chlorophyll a/b binding protein promoter *CAB2p* has been previously used to express proteins in shoot tissues, and in a previous analysis of the promoter indicated that transcript produced with the promoter was present in RNA from whole shoots but none was detected from whole root RNA (Chen et al., 2006). In order to determine in what parts of the shoot the promoter was active, and to confirm that the promoter does not drive expression in roots, a *CAB2p::GUS* construct was created and transformed into plants. In seedlings, GUS staining indicated promoter activity in both cotyledons and true leaves. In bolting stage adult plants heavy staining revealed high *CAB2p* driven expression in leaves, however less staining of the stems indicates the promoter is less active in that part of shoot tissue. In both seedlings and adult plants, some GUS staining was found, however in very low levels. When the amount of staining in the roots is compared to the amount seen in aerial tissues, it appears to be unlikely that such low expression of *CAB2p::YCF1* would interfere with the experimental objective.

The *S. cerevisiae* GS_2Cd transporter has been previously expressed in Arabidopsis and was found to localize to the vacuolar and plasma membranes (Song et al., 2003). YCF1 functions in yeast in the vacuolar sequestration of Cd, and plants expressing the

transporter showed greater accumulation of Cd and higher tolerance, indicating the transporter is also functioning in the sequestration of Cd when expressed in plants (Song et al., 2003). In this study, *YCF1* was expressed in the shoots, in an attempt to increase shoot retention of Cd. However, shoot expression of this transporter was found to decrease in the Cd content of the shoot tissue. While this is contrary to what was expected, it is possible that the decrease in Cd shoot content is a sign of Cd extrusion from the cell. While it was not originally considered in the design of this project, localization of YCF1 to plant plasma and vacuolar membranes as described in Song et al., 2003 does make GS_2Cd extrusion a possible outcome.

In light of the unexpected results received from the expression of *YCF1*, some adjustments will need to be made in order to achieve shoot accumulation of Cd. Another transporter that may be used to increase shoot accumulation of Cd is the *Schizosaccharomyces pombe* vacuolar transporter HMT1. HMT1 has been shown in *S. pombe* to be responsible for the formation of high molecular weight PC-Cd complexes, conferring significant Cd tolerance to the yeast (Ortiz et al., 1992; Ortiz et al., 1995). If the localization of the transporter is also to the vacuolar membrane, its expression may increase vacuolar sequestration and stabilization of Cd, and may allow for increased Cd accumulation. Ultimately endogenous Arabidopsis PC transporters could also be manipulated for the purposes of improving phytoextraction, however these transporters have not yet been identified.

Additionally, shoot expression is not the only way to direct Cd accumulation to the shoots. Overexpression of the heavy metal xylem loader *AtHMA4* led to increased translocation to the shoots (Verret et al., 2004). While increased translocation to the

shoots does slightly increase shoot accumulation of Cd, it is most likely the shoot to root translocation of Cd that limits the effect of AtHMA4. *AtHMA4* could be used more efficiently for phytoextraction purposes if it were expressed at the same time as a transporter involved in Cd sequestration, in the vacuoles of green tissue.

3.6. References

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