Studies on molecular response to heavy metal ions in *Arabidopsis thaliana*

(シロイヌナズナの重金属イオンに対する分子応答の研究)

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博士論文要旨

カドミウム、水銀などの重金属は動植物に深刻な悪影響を与える。近代産業はこれらを多用し、多くの被害を引き起こした。汚染修復には膨大なコストと時間がかかる。有機水銀で汚染された熊本県・水俣湾では、150トンもの水銀ヘドロの処理のため13年の歳月と485億円を費やした。一方で、大地に根ざす植物には様々な元素を吸収する能力がある。効率的、経済的な環境浄化方法の開発が期待される中、これを利用して有害物質を除くアイデアが生まれた。Phytoremediation と呼ばれ、経済的かつ吸収させた元素の再資源化も可能として米国では既に事業化も試みられ、数年後に市場規模は400億円に達するとの試算もある。現実にはまだ効率などに難点があるため、分子育種よって重金属吸収能を高めた実用的な浄化植物の創出が期待されている。だが、重金属に関する植物の分子レベルでの知見は少ない。

本研究では、実用的な環境浄化植物の創出を最終目標に、植物に与える重金属元素の影響を分子レベルで明らかにすることをテーマとした。実験材料にはシロイヌナズナ（アブラナ科）とカドミウムを選んだ。シロイヌナズナは全ゲノム配列が明らかになるなどモデル植物として分子生物学的知見が豊富である。また、アブラナ科植物には重金属耐性や蓄積に関する能力が高い種が多い。カドミウムは毒性が高いうえ、生体に対して全く不要な元素とされ、近年、環境汚染の広がりと人体への影響が懸念される汚染物質のひとつである。

本論文では、まず、シロイヌナズナに0-500μMのカドミウム処理を行い、カドミウムによるシロイヌナズナの生理、形態的影響の観察を行った（第1章）。次にこの結果に基づき、蛍光ディファレンシャルディスプレー（FDD）法による遺伝子mRNAのスクリーニング、カドミウムストレスに応答する遺伝子の大規模単離、遺伝子レベルでカドミウムが引き起すストレスの同定を行った（第2章）。さらに、この中から重金属代謝に関わると予想される遺伝子・蛋白質の機能解析（第3章）へと研究を進めた。

植物は酸化的ストレスを受けると、対処するためにアスコルビン酸ペルオキシターゼ（APX）やスーパーオキシドジスムターゼ（SOD）などの様々な抗酸化酵素を働かす。カドミウム処理をした植物では未処理試料に比較してAPX、SODとも活性が一時的に50%程度にまで低下、また培地中H_2O_2濃度も4倍程度にまで上昇した。一方で、抗酸化能を持つ還元糖「マンニトール」をカドミウムに加えて添加すると、培地中H_2O_2濃度の上昇は2倍程度にまで抑制され、カドミウムにより植物体内で酸化的ストレスが生じていることが示唆された。

このストレスに対処するために、植物は遺伝子レベルで様々な対応をしていることが予想された。FDDによるスクリーニングの結果、カドミウムストレスに応答する31の遺伝子を単離でき、うち1/4は機能未知の遺伝子であったが、残りはリン酸化酵素などのシグナル伝達因子、分子シャペロン、硫黄代謝系遺伝子などであった。

これらの遺伝子の12時間の発現パターンは、早期一過的に発現するものや長時間

発現が継続するものなどバラエティに富んでいた。また、蛋白質リン酸化の阻害剤処理により一部の mRNA 蓄積量は有意に減少。リン酸化の関与するシグナル伝達経路がカドミウムストレスで活性化されることも明らかとなった。これらのうち 15 は H_2O_2 や細胞内で酸化的ストレスを生じさせる銅イオン処理に、また、12 は熱や変性蛋白質を生じさせる代謝阻害剤の処理に応答し、酸化的ストレスと蛋白変性ストレスがカドミウムの与える主要な影響である可能性が示唆された。

FDD で単離された遺伝子は、生体内でのカドミウムストレスの軽減に機能することが期待された。よって、単離した遺伝子の幾つかを酵母内で過剰発現させ、カドミウムに対する耐性を調べた結果、低温や乾燥ストレスに応答する ATMEKK1 遺伝子と、機能未知の遺伝子 CdI19 の形質転換体 2 種が、野生型より 2-5 倍高い Cd 耐性を示した。CdI19 にはモチーフ検索の結果、分子内に金属結合に機能するドメインと蛋白質を膜局在させるアミノ酸配列が見つかった。植物は通常、重金属イオンをキレート化などにより隔離して、悪影響を抑える機能が提唱されている。CdI19 はシロイヌナズナでこのような機能を担う因子である可能性が考えられた。

そこで、CdI19 の機能についてさらに研究を進めた。蛋白質は金属イオンとの結合で多くはその構造を変化させる。このため蛋白質の大まかな構造観測ができる円偏光二色性（CD）分光法により、大腸菌で発現させた CdI19 蛋白のスペクトルを測定した。その結果、蛋白質の二次構造を反映する 200-240nm の領域で、CD スペクトルはカドミウム、銅、水銀の添加により大く変化する一方、コバルト、マンガン、カルシウムの添加ではほとんど変わらなかった。CdI19 がカドミウムなどと結合し、構造を変化させたためと考えられる。また CdI19 をタバコ BY2 細胞中で緑色蛍光蛋白（GFP）との融合蛋白質として発現させると、細胞膜周辺に GFP に由来すると思われる強い蛍光が観測され、CdI19 が細胞膜上に存在する可能性が示唆された。

GUS 遺伝子をレポーターとして CdI19 遺伝子の 5 末端上流部 1Kb のプロモーター配列を組み込んだシロイヌナズナの形質転換体は、根の先端付近や中心柱の導管周辺、胚軸、葉柄、花芽の付け根付近で GUS 遺伝子の強い発現を示した。ノーザン解析から、CdI19 は処理後 4 時間でカドミウムのほか、水銀、鉄、銅、マンガンイオンにも応答し、mRNA 蓄積量を増加させることが分かった。CdI19 はこれらの組織で上述のイオンに応答して発現、機能し、地上部、さらに葉や花などの重要組織に不要な重金属イオンが侵入するのを防いでいる可能性が考えられる。

本研究は、カドミウムが植物体内で酸化的、蛋白変性ストレスを中心として様々の生理的変化を生じさせることを明らかにした。これに対応するため多数のシグナル伝達経路が活性化され、多くの遺伝子、蛋白質が発現、機能していることも示した。また、新たに得られた遺伝子の中で、CdI19 は重金属イオンと直接、相互作用し、植物体内における重金属イオンの隔離や恒常性維持に関与する因子であることが考えられた。これまで報告例の少ない植物と重金属との分子レベルでの関係について多くの新しい知見を得ることができ、今後、これらの結果の応用とさらなる研究により実用的な環境浄化植物の創出が実現できるものと期待している。

Studies on molecular response to heavy metal ions in *Arabidopsis thaliana*

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Contents

Abbreviations

APX	ascorbate peroxidase
Az	azetidine-2-carboxylate
Ca	calcium
CD	circular dichroism
Cd	cadmium
cDNA	complementary deoxyribonucleic acid
Cu	copper
CL	chemiluminscence
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
FDD	fluorescent differential display
GFP	green fluorescent protein
GSH	glutathione S-transferase
GUS	β-glucuronidase
H_2O_2	hydrogen peroxide
MOPS	3-(N-morpholino) propane sulfonic acid
mRNA	messenger ribonucleic acid
MS medium	murashige-skoog medium
RACE	rapid amplification of cDNA ends
ROS	reactive oxygen species
RT	reverse transcription
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
SSC	sodium chloride/sodium citrate

Introduction

Heavy metals are defined as metal group possessing weight higher than 5 g/cm^3. There are about 40 elements that fall into this category. These elements are localized mainly in dispersed form in rock formations. As a consequence of the industrial revolution, there has been an enormous and increasing demand for heavy metals, resulted in high emission of heavy metals into the biosphere. These elements have been largely increased in the aquatic and soil phases, and their toxicity is one of the major environmental health problems in modern society with potentially dangerous bioaccumulation through the food chain (Fig. 1).

Several approaches are currently used to remove these pollutants from our environment but conventional engineering techniques often accompany high cost. For example, to remove soil contamination, landfilling after excavation, transport and deposition of contaminated soil in a permitted hazardous waste landfill are necessary. With a hectare of soil (to a meter depth) weighing approximately 12000 tons (Cunningham and Ow, 1996), this translates to a minimum cost of about a million dollars per hectare by landfilling. The cost-effective technology has been demanded.

One relatively inexpensive and ecologically responsible alternative is phytoremediation. This new approach is to use plants to degrade pollutants in situ. Many plants possess an ability to absorb heavy metals from soil and water (Fig. 2). In many ways, living plants can be compared to solar driven pumps that can extract and concentrate certain element (Table 1). The possibility to remove toxic heavy metals by phytoremediation technology has drawn much attention because of low cost, low impact, visually benign and environmentally sounds (Table 2). Many investigators began to study the possibility to use plants for this purpose.

However naturally occurring plant species are commercially not enough utilizable to detoxify heavy metals due to low heavy metal tolerance, small biomass and insufficient

absorption efficiency. This lead to the molecular breeding, using modern genetic technologies to improve plant traits by modifying the biochemical processes involved in heavy metal uptake, transport accumulation, and resistance. However physiological and molecular mechanisms of plant counteraction or adaptation to heavy metals have not been well known.

In this study, I selected the heavy metal element, cadmium and investigated its effect on *Arabidopsis thaliana*, since Cd in soil and water causes serious problems for organism (Table 3, Fig. 4) and widespread in our environment as the result of contamination by power stations, metalworking industries, and waste incineration. In plants, it reduces absorption of nitrate and its transport from root to shoots by inhibiting nitrate reductase activity (di Toppi and Gabbrielli, 1999). Cd also gives damage to photosystems I and II (Siedlecka and Baszynsky, 1993), and may displace with Ca^{2+}, Zn^{2+} and Fe^{2+} in proteins (Stohs et al., 2000). This results in inactivation of proteins and release of free ions, generating reactive oxygen species. These toxic effects eventually induce growth inhibition and death of the organism (Zenk, 1996).

The response of plant genes to Cd and molecular mechanism of Cd toxicity are not clear yet. In this thesis, I first evaluated physiological changes and occurrence of oxidative stress by Cd (Chapter I). Second, I screened for mRNA populations that change their levels upon Cd-treatment and classified them into functional groups (Chapter II). Third, I analyzed functions of these genes (Chapter III). Overall, this work revealed various new aspects of plant interaction with heavy metals. These knowledge are of great help to create plants with efficient clean-up ability, and I believe phytoremediation is a new frontier of remediation approach and will become a widely accepted technology in future.

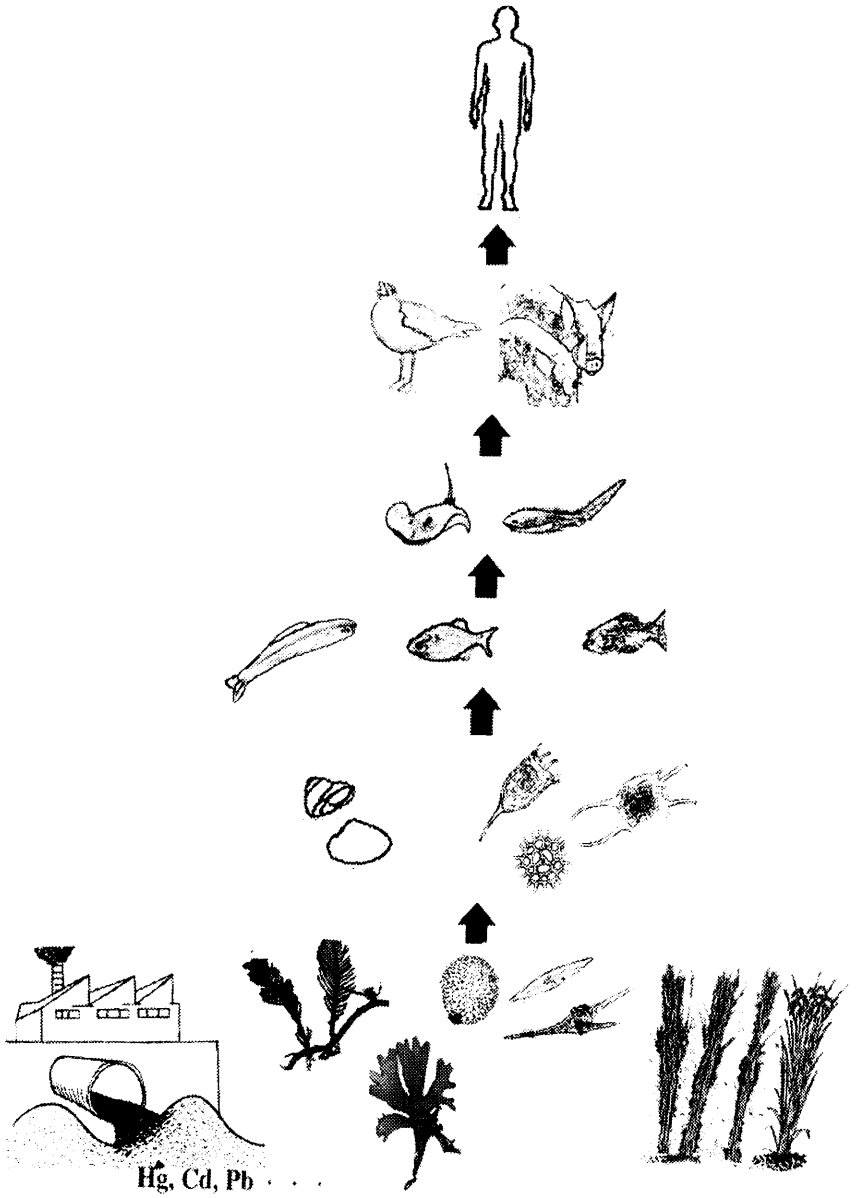


Figure 1. Model illustrating of the heavy metal bioaccumulation. The arrows in this simplified diagram show the pathways in food chain. Cadmium, methylmercury etc are far more serious environmental pollutant and tend to enter the aquatic food chain and biomagnify. It has been estimated that methylmercury bioconcentrates in fish six to seven orders of magnitude above concentrations found in polluted waters. As a result of food chain, harmful effect is found in the upper class living beings. In addition to the food chain, smoking is noticeable Cadmium's pathway from tobacco leaf to man.

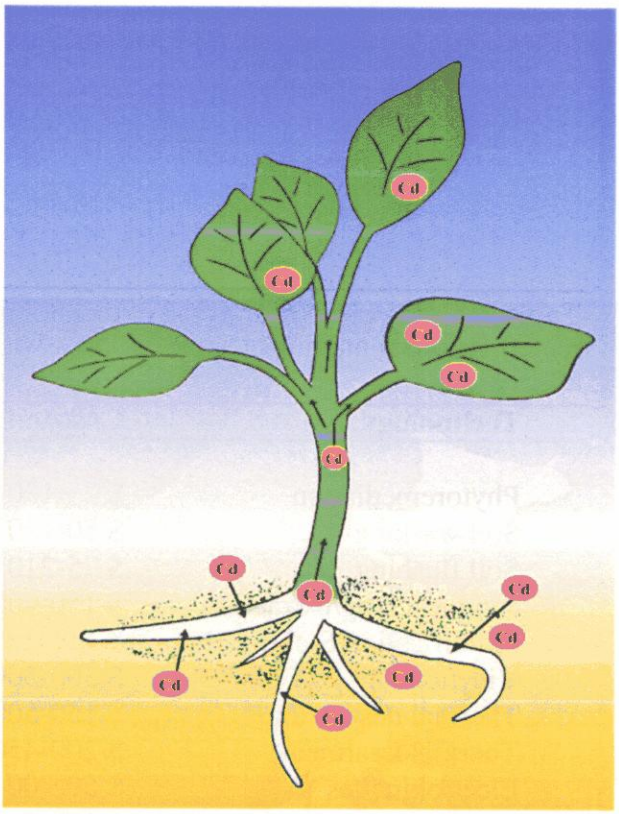


Figure 2. Processes involved in phytoremediation. Plants as solar driven pumps can absorb heavy metals from soil and water

Table 1. Metal concentrations (on dry weight basis) in shoot of known hyperaccumulators (Cunningham and Ow, 1996)

Plant species	Metal	Concentrations in plant
Thalaspi caerulenscens	Cd, Zn	1800 (Cd), 51600 (Zn) mg/kg
Ipomoea alpina	Cu	12300 mg/kg
Haumaniastrum robertii	Co	10200 mg/kg
Thalaspi Rotundifolium	Pb	8200 mg/kg
Macadamia neurophylla	Mn	51800 mg/kg
Psychotria douarrei	Ni	47500 mg/kg

Table 2. Comparison of the cost of technologies to remediate the contaminated soils (Morikawa et al.,1999).

Technology	Cost/ton
Phytoremediation	$ 25-100
Soil washing	$ 50-150
Soil flushing	$ 75-210
Acid leaching/extraction	$ 150-400
Solidification/Stabilization	$ 111-205
Vitrification	$ 300-500
Thermal desorption	$ 150-500
Thermal treatment	$ 200-450
Electrokinetics	$ 50-300
Landfilling	$ 100-500

Table 3. Effects of cadmium to living organism

Human	Renal tubular dysfunction,
	Resorptive inhibition of protein, glucose, and amino acid
Rat	Long-term experimental inhalation of cadmium dust raises the incidence rate of primary lung cancer.
	Long-term intraperitoneal injection, renal tubular dysfunction and osteomalacia occur.
Fish	hypocalcemia, malformation of spine, etc.
	Salmon is easily effected.

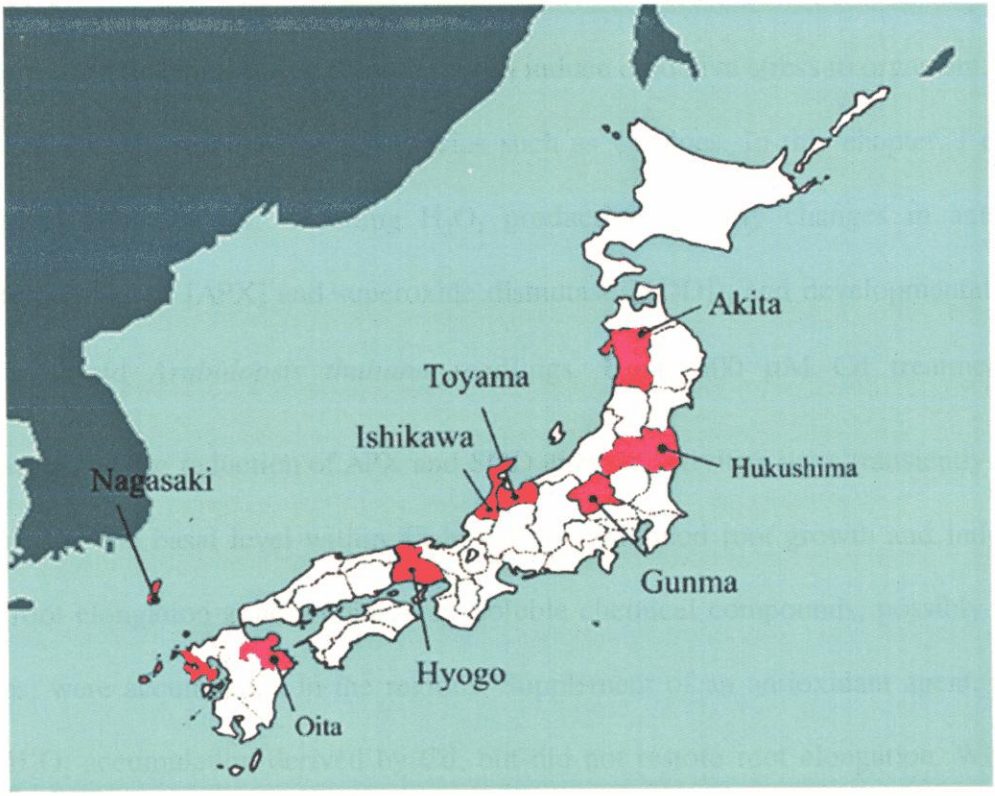


Figure 3. Heavily polluted prefectures by cadmium and occurrence of Itai-Itai Disease. Toyama Jinzu River; the patients certified as victims of Itai-Itai Disease are 181 persons (13 were alive) and certified as requiring follow-up observation are 332 persons (9 were alive)(Dec. 1995). Ishikawa, Kakehashi River; 2 persons, Tetori River; 1 persons, Hyogo Ichi River; 5 persons, Nagasaki Tsushima Island; 9 osteomalacia /11 renal tubular dysfunction (Kasuya, 1999).

Chapter I

Effect of cadmium on root elongation of *Arabidopsis*

ABSTRACT

Cd is a serious toxic element and potentially can induce oxidative stress to organism, but does not produce directly reactive oxygen species such as Cu does. In this chapter, I examined physiological effect of Cd, including H_2O_2 production, activity changes in antioxidants (ascorbate peroxidase [APX] and superoxide dismutase [SOD]), and developmental changes using 2-week-old *Arabidopsis thaliana* seedlings. Upon 500 μM Cd treatment, H_2O_2 accumulation and the reduction of APX and SOD enzyme activities were transiently induced, but returned to the basal level within 48 h. Cd also inhibited root growth and induced cell death of root elongation zone within 24 h. Soluble chemical compounds, possibly phenolic substances, were accumulated in the regions. Supplement of an antioxidant agent, mannitol reduced H_2O_2 accumulation derived by Cd, but did not restore root elongation. When roots were treated with 500 μM Cd in the presence of 30 mM Ca, H_2O_2 accumulation increased, but inhibition of root growth and Cd influx were reduced. These results suggest that Cd temporary induced H_2O_2 accumulation due to an imbalance of redox systems, and that some metabolism changes are also induced, resulting in decrease of root elongation.

INTRODUCTION

Cd is highly toxic but molecular mechanism of Cd toxicity is not unclear yet. Physiologically, it has long been known that Cd inhibits plant growth (Arduini et al., 1996; Arisi et al., 2000). Root elongation is strongly suppressed, which is the most sensitive response to Cd exposure among many other physiological responses (Schutzendubel et al., 2001). At the cellular level, Cd damages to photosystems and severely inhibits mitochondrial oxidative phosphorylation. Cd was proposed to generate reactive oxygen species (ROS) (Stohs and Bagchi, 1995) and to increase lipid peroxidation. However, it is unlikely that Cd directly produce ROS, since Cd does not belong to a group of transition metals such as Cu that obey fenton reactions.

An imbalance in generation and removal of ROS also results in oxidative stress (Finkel and Holbrook, 2000). Plants, like all aerobic organisms, possess an array of hydrophilic and lipophilic antioxidants, glutathione, ascorbic acid (vitamin C), α–tocopherol (vitamin E), phenolic isoflavanoid compounds, and carotenoids (Fryer, 1993; Creissen et al., 1999). Reduced form of these compounds, coordinately scavenges ROS and other products of oxidative reactions with antioxidant enzymes, SOD, APX, catalase (CAT) and glutathione peroxidase (GPX). It is supposed that Cd affects glutathione metabolism (Rauser, 1995; Zenk, 1996; Xiang and Oliver, 1998; Arisi et al., 2000) and activities of antioxidative enzymes, but controversial results have been reported. For example, when plants are exposed to Cd, APX activity increased in *Phaseolus aureus* and *Phaseolus vulgaris*, and decreased in *Helianthus annuus* (di Toppi and Gabbrielli, 1999).

Cd could directly stimulate production of toxic free radicals, resulting in induce of defense response in a similar ways to induce other biotic or abiotic environmental stress response. It has speculated that Cd uptake and toxicity in animals is caused by its interaction with Ca, which acts as a signaling molecule (Hinkle et al., 1987). Intracellular Ca concentration is critical to cope with oxidative stresses (Price et al., 1994). Ca influx is

required for the activation of ROS generation (Schwacke and Hager, 1992; Baker et al., 1993; Harding et al., 1997) and also involved in metabolic control and signal transduction in plant cell (Rivetta et al., 1997). H_2O_2 is the most stable ROS and functions as a signaling molecule. Generation of H_2O_2 is one of the earliest cellular responses to pathogens and elicitor molecules (Lamb and Dixon, 1997). H_2O_2 induces expression of many genes (Desikan et al., 2001) and mediates oxidative cross-linking of cell wall under process that does not require transcription of genes. (Bradley et al., 1992).

Although induction of oxidative stress and alteration of related metabolism are considered to be the major toxicity of Cd in Arabidopsis, few substantial observation has been reported to confirm this. Thus I examined activities of antioxidant enzymes, SOD and APX, H_2O_2 production and developmental changes using 2-week-old Arabidopsis. Hydroponically grown seedlings, which were exposed to 0-500 μM Cd, showed temporal reduction SOD and APX activities and increase in H_2O_2 accumulation. Soluble fluorescent chemicals accumulated in root tissues, indicating change of secondary metabolism.

MATERIALS AND METHODS

Plant materials and Cd treatments

Seeds of *Arabidopsis thaliana* (var Columbia) were surface sterilized by a 10-min incubation in 70 % ethanol and by a 20-min incubation in 5% (w/v) sodium hypochlorite (active chlorine 8.5 - 13.5%, Nacalai Tesque, Kyoto, Japan) containing 0.05 % (v/v) Tween 20. After three washes with distilled water, seeds (10 - 15 per bottle) were cultivated in half strength 10 ml Murashige-Skoog (MS) medium supplemented with 1 % sucrose in a greenhouse at 23 °C under a continuous light.

Chlorophyll, H_2O_2 and Enzyme assay

For these assays, all operations were performed at 4 °C by keeping the samples on an ice bath until assays were completed. 2-week-old plants floated on MS medium were subjected to stress treatment by addition of MS medium containing reagent (0.1 M $CdCl_2$; 1 M $CaCl_2$; 1M mannitol).

Chlorophyll was extracted in 1 ml 80 % acetone (Wako, Osaka, Japan). 0.2 mg of plants were homogenized and vortexed well. Then samples were incubated for 15 min and spun at 15,000 g for 10 min. The supernatant fraction was used for assay. The aliquots were taken to measure the A_{663} in a spectrophotometer (DU640, Beckman, CA, USA). Relative chlorophyll content was calculated according to the absorbance.

The amount of H_2O_2 in medium was determined by a modified Chemiluminscence (CL) procedure (Sasabe et al., 2000). 25 µl of half strength MS medium cultivated plant were mixed with 15 µl luminole (3-aminophthaloylhydrazine, Wako) solution and chemiluminescece was immediately measured for 30 seconds with luminometer (Lumat LB 9507, Berthold Japan, Tokyo, Japan). Luminole solution was containing 2 mM luminole, 1 M Tris/HCl (pH8.0) and 2 mM $CuCl_2$.

Extracts for determination of SOD, APX activities were prepared from 0.5 g of total plants homogenized under ice cold conditions in 1 ml of extraction buffer. Bradford's reagent (BioRad) was used to determine the concentration of solubilized protein with bovine serum albumin as a standard. SOD were extracted with K-PO_4 buffer (50 mM, pH 8.0) containing Na-EDTA (1 mM) and PVP (1 %). Tissue extract was spun at 15,000 g for 10 min and the supernatant fraction was used for assay. For the measurement of SOD activities, 10 µl extracted solution containing 10 µg total protein was mixed in a microplate with 180 µl reaction buffer containing 34 mM K-PO_4 (pH7.8), 28 µM riboflavin (Nacalai Tesque), 28 mM N, N, N9, N9-tetramethylethylenediamine (Wako) and 2 mM nitroblue tetrazolium (Wako) without light. The microplate was illuminated with shaking for 10 min and the changes in A_{595} were measured with a microplate reader (model 3550, BioRad). The relative activities of SOD were calculated by absorbance in A_{595}.

APX activity was determined to measure the decrease of ascorbate because the enzymes can use ascorbate as an electron donor to scavenge H_2O_2. APX was extracted with 100 mM K-PO_4 buffer (pH 7.8) containing 2 mM Na-EDTA, 8 % glycerol, 2 % PVP and 5 mM ascorbate. Samples were centrifuged at 15,000 g for 10 min and 10 µl aliquots of supernatant were used for assay. Extracted solution was measured at the changes in A_{290} at 25 °C in reaction medium containing 50 mM K-PO_4, 5 mM Na-EDTA, 0.5 mM ascorbate and 1 mM H_2O_2 in a final volume of 100 µl. All assays were run six times.

Measurement of Cd content by atomic absorption

2-week-old Arabidopsis cultivated in 10 ml half strength MS medium supplemented with 1 % sucrose treated for 24 h with 500 µM $CdCl_2$ or 500 µM $CdCl_2$ plus 30 mM $CaCl_2$. The sample

was washed two times with distilled water and homogenized with sea sand C (Nakalai Tesque) and 3 times of its weight of buffer (50 mM Tris-HCl, pH 7.2). The crude extract was centrifuged at 12500 g for 15 min and the supernatant was diluted with 19 volume of extraction buffer. The concentration of Cd of the diluted supernatant was measured with an atomic absorption/film emission spectrophotometer (AA-6500S, Shimadzu, Kyoto, Japan).

Cd toxicity, mannitol and Ca effect assay

1-week-old Arabidopsis hydroponically cultivated in 10 ml half strength MS medium supplemented with 1 % sucrose in a greenhouse at 23 °C under a continuous light. To determine the effect of Cd toxicity, plants were transferred to 1 % agarose plate containing half strength MS medium and 1 % sucrose with 0-500 μM $CdCl_2$. Mannitol and Ca effect was also tested by the same condition. Plants were cultivated on solid half strength MS medium containing reagents (mannitol effect assay 1 mM mannitol, 150 μM $CdCl_2$; Ca effect assay, 30 mM $CaCl_2$, 200 μM $CdCl_2$). After 1 or 2 weeks, root growth was observed.

Histochemical staining of root

1-week-old Arabidopsis root was used. Plant was cultivated same conditions of Cd toxicity and Ca effect assay. Roots attached to the plants were stained for 10 min in half strength MS medium supplemented with 0.0 5% (w/v) evans blue (Wako) or fluorecein diacetate (Wako). Root tissue for analysis of anatomical changes was fixed in 5 % glutaraldehyde (Wako) and 0.16 M K-PO_4 buffer (pH6.8) for 2 h. Then the root was separated and washed 2 times with 0.16 M K-PO_4 buffer and 3 times with distilled water. The sample was placed in the solution of 25 % ethanol / 75 % H_2O_2 and replaced 4 times every 5 min in new solutions. The percentage of ethanol raises with steps 50, 70, 90 and 100 %. After 5 min incubation of 100 %

ethanol, the solution was changed liquid resin (Technovit 7100, Heraeus Kulzer, Wehrheim, Germany). The tissue was infiltrated with resin over 12 h in room temperature and then polymerized and sectioned at 1.2 μm thickness. These sections were stained with 0.2 % safranin for 5 min and 0.5 % fast green for 25 min. All samples were observed by a microscope (PROVIS AX70, Olympus, Tokyo, Japan) equipped with a fluorescence module. Fluorescent images were captured separately using a CCD camera (monochrome images, CoolSNAP-HQ, Photometrics; color images, DP12, Olympus).

RESULTS

Effects of Cd on root growth

Plants hydroponically cultured for a week were transferred to solid medium containing various Cd^{2+} concentrations. Root growth was strongly suppressed with 150 - 200 μM Cd and samples died upon treatment with 500μM for a week (Fig. 1). When samples were exposed to 200 μM, root elongation was almost retarded, while the shoot was still alive and grew slowly.

Generation of H_2O_2 and activity change of antioxidant enzymes, APX and SOD

Crude extract of Cd-treated plants was used for assay. Initial activities of APX and SOD were decreased to 25 % and 50 % of untreated control, respectively. However this was only transient, beginning to increase there after reaching to and basal level 48 h later (Fig. 2A). In contrast, H_2O_2 concentration in culture media reached a maximum level, being 4 fold higher than the basal level after 8 h and then gradually decreased to the basal level. When an antioxidant, mannitol, was administered together with Cd, the maximum level was 2 fold of control and partial prevention of increase in H_2O_2 accumulation was observed (Fig. 2B).

Inhibition of root growth and effect of Ca

Growth of root tips is inhibited seriously in the presence of Cd (200 μM) and one of the reasons is thought to be oxidative stress. Since mannitol is known to decrease the generation of H_2O_2, 1-week-old seedlings were transferred to solid medium containing both mannitol and Cd, and observed for 2 weeks. Results showed addition of mannitol to be ineffective to improve root elongation (Fig. 3). Other antioxidant reagents, DTT (dithiothreitol), GSH (glutathione) and ascorbate, were also challenged, but no effects were observed (data is not shown). On the other hand, treatment with 30 mM Ca together with 200 μM Cd recovered

root growth completely (Fig. 4). Under this condition, plants were alive on the plate contained over higher Cd of 500 μM (data is not shown). However APX and SOD activities decreased to half level (Fig. 5A) and H_2O_2 concentration increased 12 fold that of control within 24 h (Fig. 5B). Chlorophyll abundance gradually decreased but significant differences were not observed with or without Ca (Fig. 5C).

Effect of Ca to Cd influx

To know reason why Ca in the incubation medium reduced the Cd-induced inhibition of plants, amounts of endogenous Cd were estimated by atomic absorption spectrophotometer. During the first 24 h incubation, the content of Cd per gram fresh weight was reduced from 46.7 μg in the absence of Ca to 17.4 μg in the presence of Ca (Fig. 6). At the same time, the content of Zn was measured but significant difference was not observed (data was not shown).

Effects of Cd on root tissues and metabolism

Since H_2O_2 accumulation and effects of redox system were transient, I examined microscopically root tissues to identify other physiological changes caused by Cd. Cell death was monitored by staining them with dyes, Evans blue or fluorecein diacetate. When plants were exposed to Cd, the root tip color changed brown and root adjacent to brown area was stained with Evans blue (Fig. 7A, B, and C). Addition of Ca inhibited appearance of brown color and restored the fluorescence of fluorecein diacetate (Fig. 7D, E). When plants were incubated for 2 weeks with sublethal level of Cd (200 μM), accumulation of chemical compound which have yellow or blue fluorescence in the endodermis, pericycle, or cambium cells was observed (Fig. 9). The fluorescence was detected weakly in the sample treated for 48 h (Fig. 8G). The fluorescence of control samples was undetectable. Cells, which

accumulated these chemicals, were stained red with safranin and fast green, indicating the existence of phenolic compounds (Fig. 10).

DISCUSSION

Effect of Cd on oxidative stress

The mechanism of Cd suppression of plant growth is not clear yet (Arduini et al., 1996; Arisi et al., 2000). Recent studies have shown that metals such as Fe, Cu, V, Cr, Pb, Hg, Ni and Cd are able to produce reactive oxygen species (ROS), resulting in lipid peroxidation, DNA damage, depletion of sulfhydryls and altered calcium homeostasis in the cell (Stohs and Bagchi, 1995; Rivetta et al., 1997). However, since Cd, does not belong to the group of tradition metals like Cu, which induces oxidative stress via fenton reactions, this idea awaits further confirmation. The alteration possibility is that it influences oxidation-reduction systems in the cell.

In order to address this question, the relationship between enzyme activity of SOD and APX and the abundance of H_2O_2 were investigated. Results clearly showed that temporarily suppressed their activities with concomitant increase of H_2O_2 in growth media. It is known that SOD is the first enzyme involved in detoxifying process of ROS by converting superoxide radicals to H_2O_2 (Asada, 1992). Since APX catalyzes the breakdown of H_2O_2, a quick recovery of SOD activity and low activity of APX may lead to a transient accumulation of H_2O_2. Some other antioxidant enzymes such as CAT, can also scavenge H_2O_2, and it is likely that Cd inactivate most of them (Creissen and Mullineaux, 1995). Simultaneously it may suppress the system for H_2O_2 removal by affecting enzymes and substrates, GSH, GR and CAT (Schutzendubel et al., 2001).

It was proposed that generation of ROS is one of the main Cd toxicity (Stohs et al., 2000) and it is known that Cd especially inhibits root elongation, but the relationship witt ROS is not clear. In this study, I found that an antioxidant reagent, mannitol, induced reduced accumulation of H_2O_2, when administered together with Cd, however it did not restore inhibition of root elongation, these observations indicate that at least root elongation

suppression is not due to ROS. In contrast, Ca administration induces recovery of root growth, although SOD and APX activities and high accumulation of H_2O_2 was the same as control during the experimental period. The possible reason is that both Ca and H_2O_2 are signaling molecules that mediate cross-tolerance to a variety of stresses (Bowler and Fluhr, 2000). For example, it was shown that Ca influx in the cell is required for activation of ROS (Schwacke and Hager, 1992; Baker et al., 1993; Harding et al., 1997) and that the presence of Ca may lead to alteration of redox system.

This is consistent with the present data showing the reduction of Cd-induced inhibition by Ca was accompanied by a reduction of Cd uptake and, suggesting that the recovery of root growth is due to reduction of Cd uptake, instead of decrease of H_2O_2. It was proposed that Cd is transported into plant cells through Ca channels located on plasma membrane (Thuleau et al., 1994; Malho et al., 1995), and our results support this showing excess Ca might interfere unfavorable Cd uptake.

Cell death and metabolic alteration

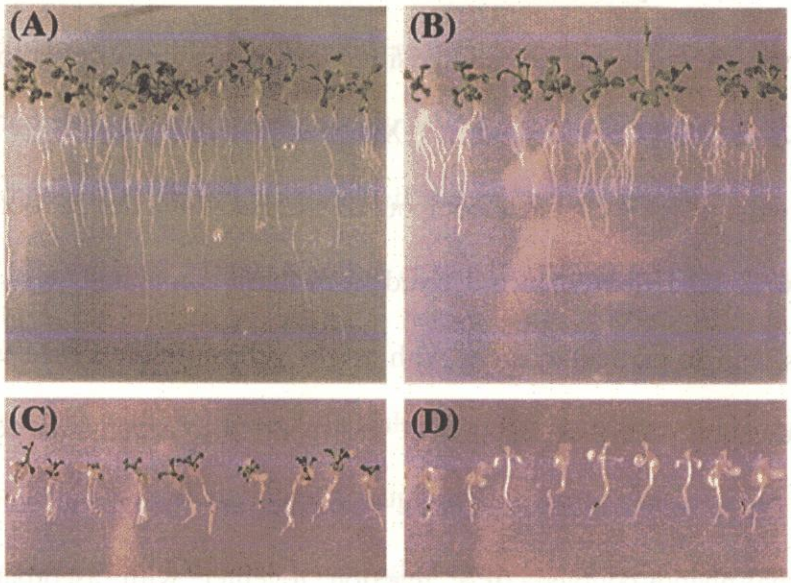
Under our experimental condition, Cd concentration between 150 and 200 μM was critical for inhibition of root elongation. It is known that aluminum (Al) also induces inhibition of root growth due to Al interactions within the root apex (Buchanan et al., 2000). We also observed that cell death appeared first in root meristem or adjacent to meristem, forming probably root elongation zone, where protoxylem exists and cells are actively. Cell death was not observed when seedlings were cultured in the presence of Ca, suggesting again that influx of Cd critical for cell death.

Sublethal concentration of Cd seriously inhibited root elongation without inducing cell death. The affected tissues contain enlarged and unusual formed cells, instead of normal observed small, densely cytoplasmic cells that are dividing and expanding in size

(Meyerowitz and Somerville, 1994). The mechanism by which morphological change occurred is not clear, it is possible that Cd altered some metabolic pathways. For example GSH deficiency was shown to lead to cell division block in plant roots (Vernoux et al., 2000), and Cd was shown to affect GSH metabolism (Xiang and Oliver, 1998).

It is notable that soluble chemical compounds were accumulated in root tissues after Cd treatment. Yellow or blue fluorescence derived from these compounds was detected in Cd-damaged regions, and in particular, areas with bright yellow fluorescence were stained red with safranin, indicating phenolics. Scots pine was reported to accumulate soluble phenolics under Cd stress (Schutzendubel et al., 2001). Phenolics are thought to contribute in H_2O_2 destruction in the phenol-coupled APX reaction together with ascorbate, resulting in protection of tissues from oxidative stress (Schutzendubel et al., 2001). In cultured tobacco cells, phenolics were found to protect cells from aluminum toxicity (Yamamoto et al., 1998). Despite these observations, a direct protective function of phenolics against Cd is not clear yet.

The present results support the idea that Cd induces oxidative stress and abnormal metabolic and morphological changes. However, we found that the former is not causative factor for the latter at least is root elongation. Conceivably, two phenomena may simultaneously occur and affect independently plant growth and differentiation.



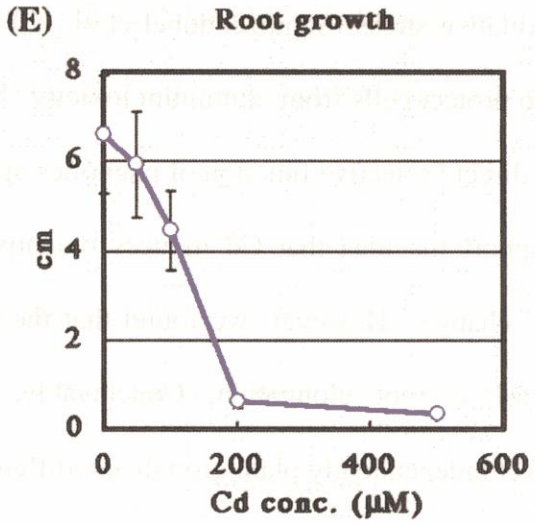


Figure 1. Effects of Cd on root growth of *A. thaliana*. 1-week-old plants were transferred to solid medium containing $CdCl_2$ (A, 0 μM; B, 100 μM; C, 200 μM; D, 500 μM). After 1 week of cultivation, root growth of samples was estimated. The elongated length at each concentration (0, 50, 100, 200, 500 μM) was measured and plotted (E). Values were calculated from 10 samples.

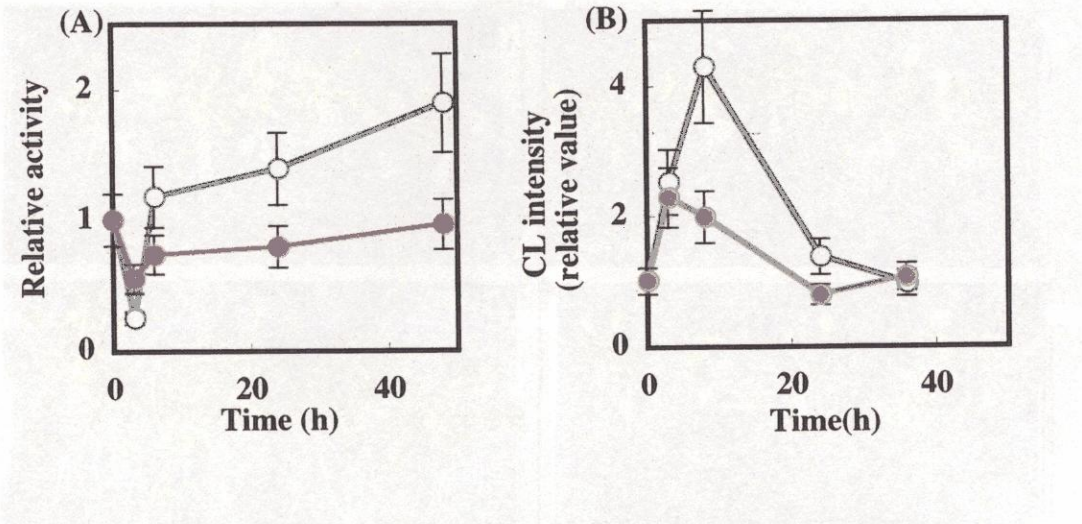


Figure 2. Activity of antioxidative enzymes and accumulation of hydrogen peroxide. The value was expressed relative to the value in control sample (=1). (A) Total plant was treated with 500 μM Cd. Activity changes of SOD (○) and APX (●) were measured at various time points (0, 3, 6, 24, 48 h). (B) Total plants were incubated in the presence of Cd (500 μM, ○) or Cd plus mannitol (1mM, ●).Contents of hydrogen peroxide in the culture medium were measured at various time points (0, 3, 8, 24, 36 h).

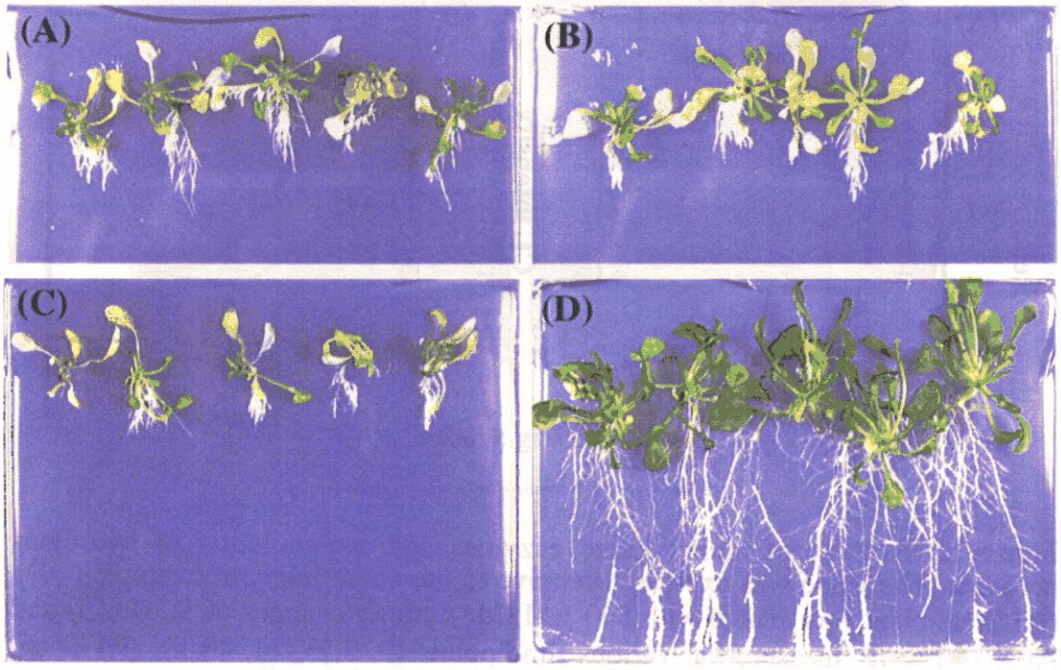


Figure 3. Effects of mannitol on Cd toxicity in *Arabidopsis*. (A) Plants grown on medium with 150 μM Cd. (B) Plants grown on 150 μM Cd medium supplemented with 1 mM mannitol. (C) Plants grown on 150 μM Cd medium supplied with 10 mM mannitol. (D) Plants grown on medium with 10 mM mannitol. After 2 weeks of culture, root growth was observed.

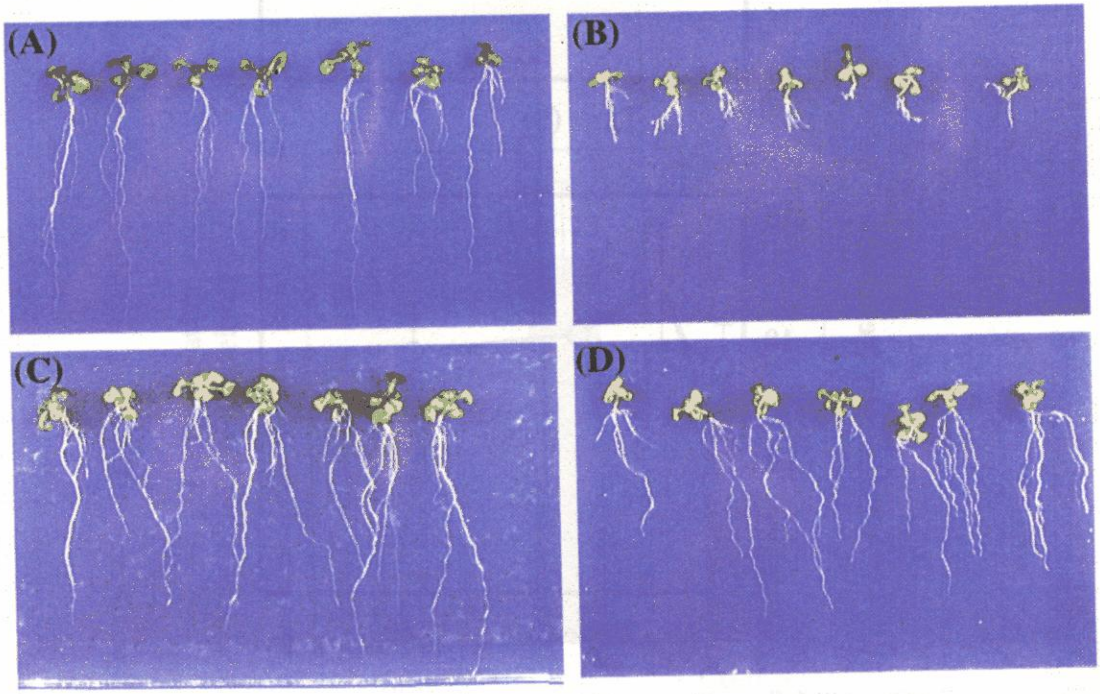


Figure 4. Effects of Ca on Cd toxicity. 1-week-old plants were transferred to solid medium. Plants were grown on 1/2 MS medium (A, control) and 1/2 MS medium containing 200 μM Cd (B), 30 mM Ca (C) and both 200 μM Cd and 30 μM Ca (D). After 1 week, root growth of samples was observed.

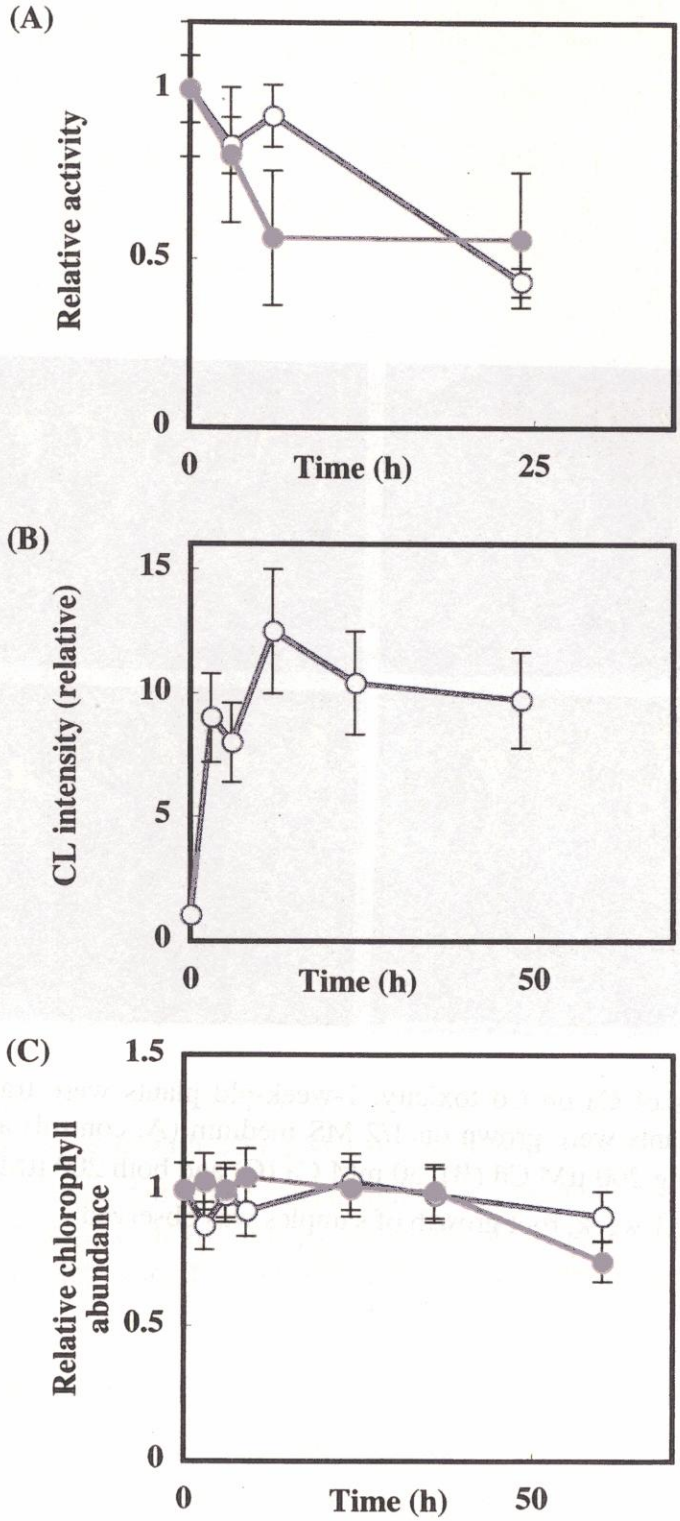


Figure 5. Effects of Ca on enzyme activities, hydrogen peroxide, and total chlorophyll abundance. The value was expressed relative to the value in control plants (=1). (A) The activities of SOD (○) and APX (●) at various time points (0, 3, 6, 24 h). (B) The content of hydrogen peroxide in the culture medium. (C) Chlorophyll abundance in plant treated with Cd (○) or Cd plus Ca (●). Samples were treated with 30 mM Ca (A and B):or with 500 μM Cd or both, 30mM Ca and 500 μM Cd (C).

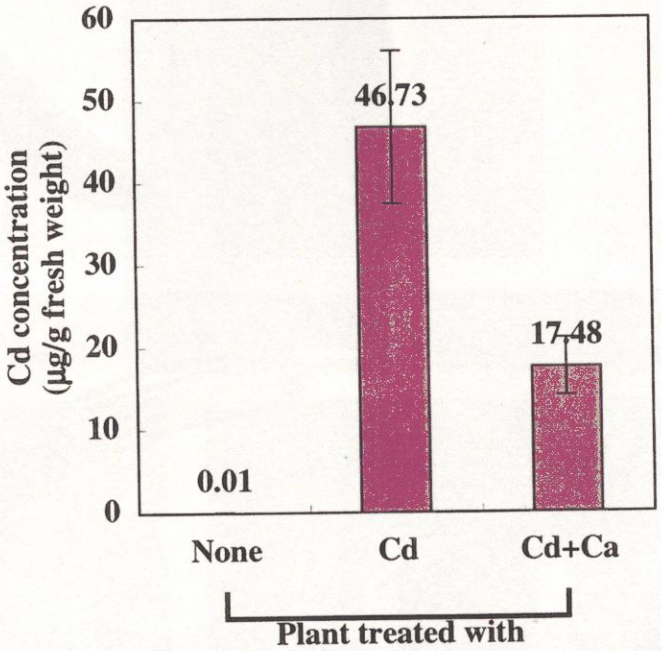


Figure 6. Cd accumulation.Whole plants were treated for 24 h with Cd (500 μM) or Cd plus Ca (30mM), and amount of endogenous Cd were extracted and measured.

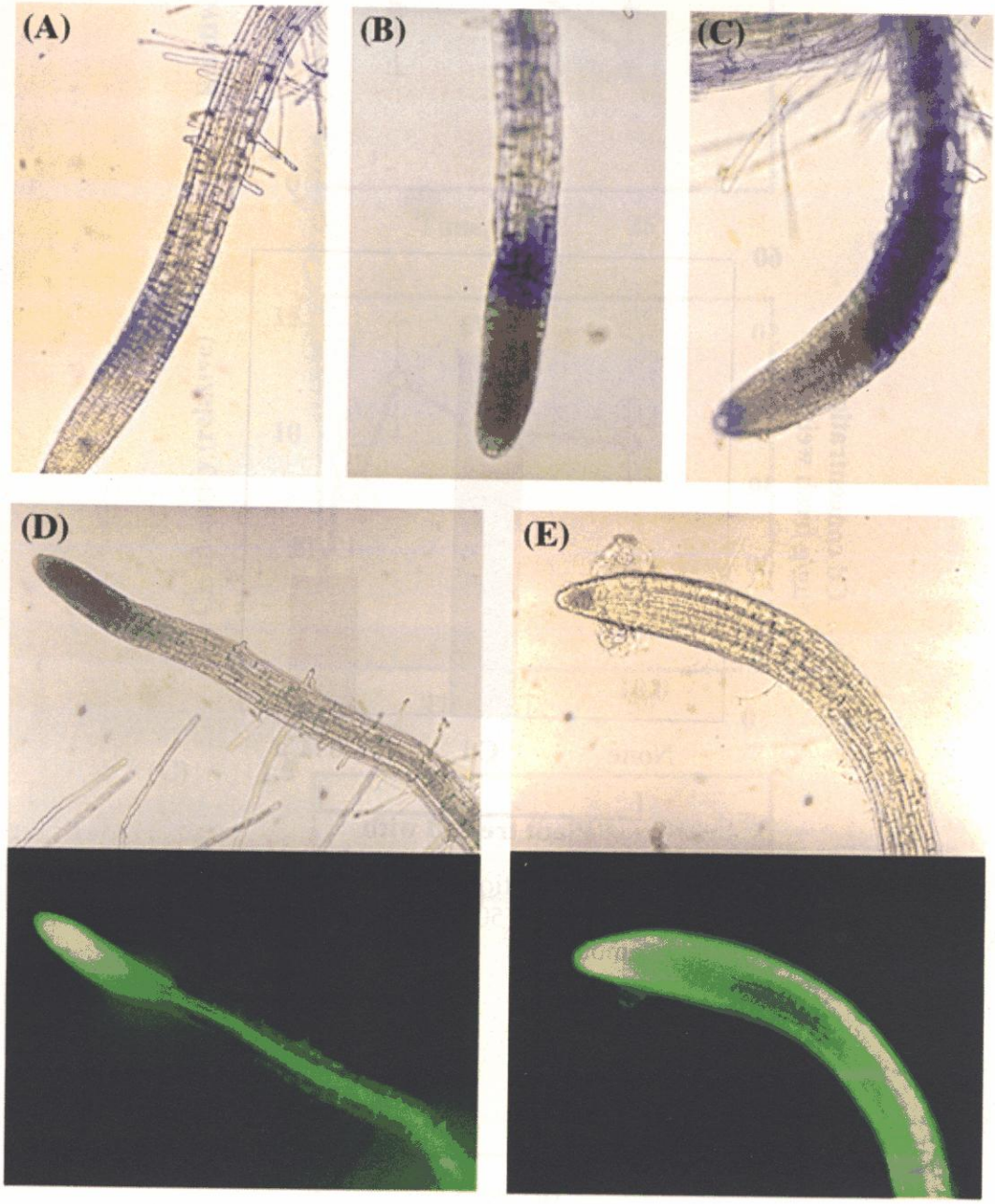


Figure 7. Cell death in roots of 1-week-old seedlings of *Arabidopsis* grown in liquid culture. Plants were treated with 500 µM Cd (A, 0 h; B, 24 h; C and D, 48 h) or 500 µM Cd plus 30 mM Ca (E, 48 h). Samples were stained with evans blue (A, B and C) or fluorecein diacetate (D and E). The excitation wavelength for fluorescence images (D and E) is from 470 to 490 nm (UV and IB-excitation filters).

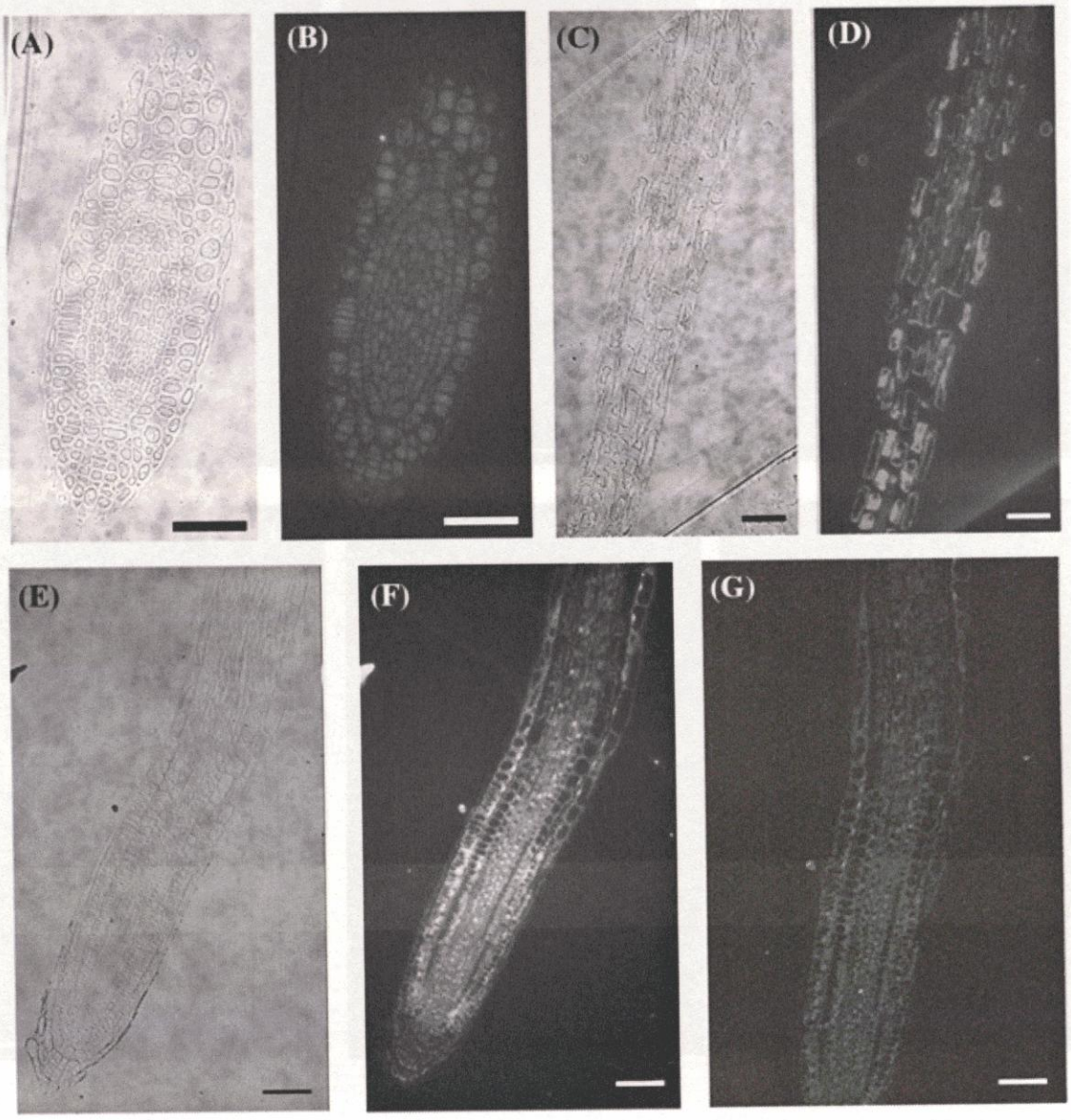


Figure 8. Visualization of fluorescent chemicals in root of *Arabidopsis*. Plants were grown in medium with (E, F and G) or without (A, B, C and D) Cd for 48 h. 2 sections (root tip; A and B, mature root; C and D) were prepared for control. Fluorescence images (B, D, F and G) and corresponding differential interference contrast images (A, C and E) were captured by a CCD camera. The excitation wavelength is from 330 to 385 nm (UV and U-excitation filters). [Bar = 50 μm]

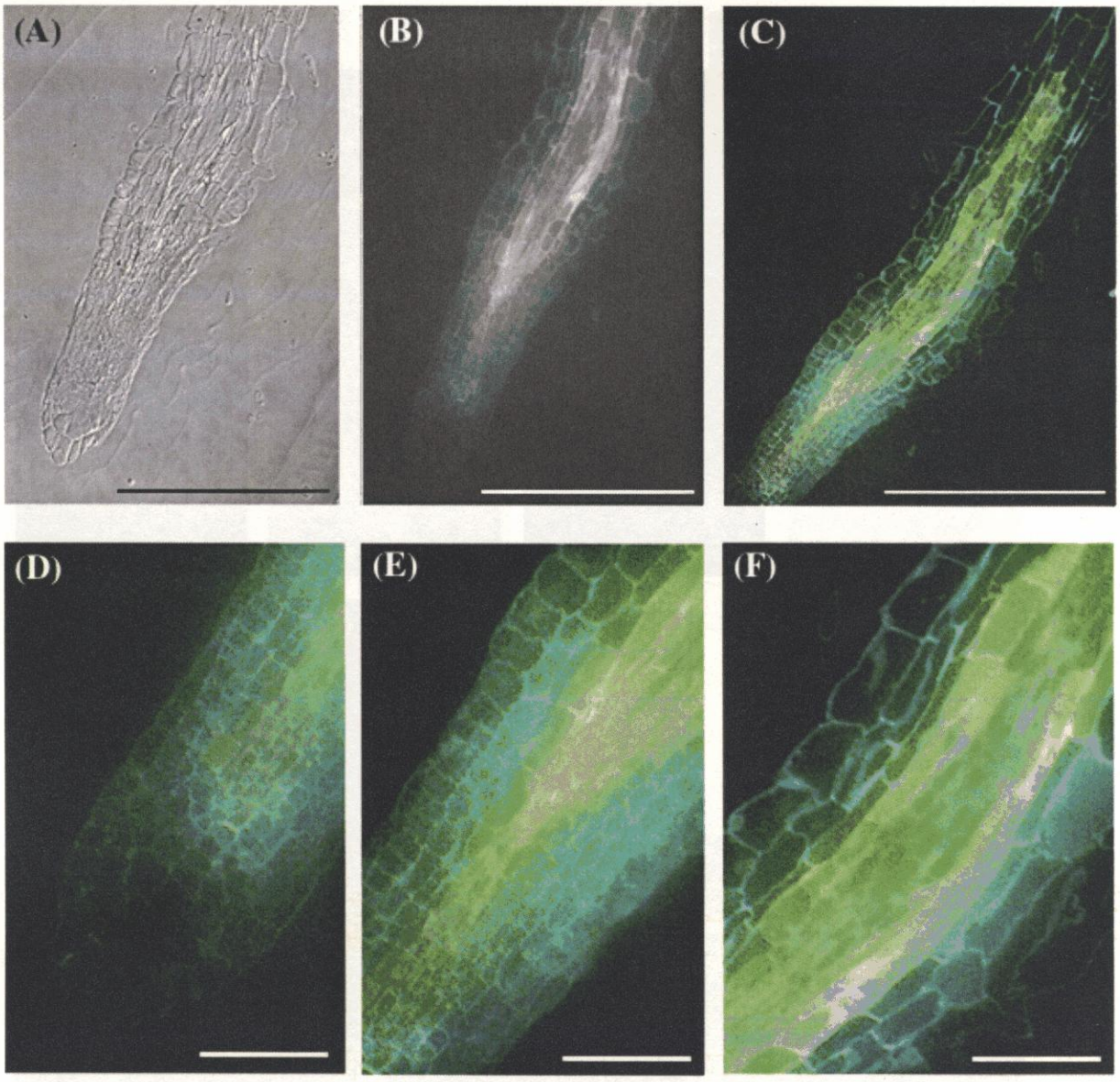


Figure 9. Fluorescent chemicals accumulated in root of *Arabidopsis*. Plants were incubated for 2 weeks in medium with sublethal level of Cd. Fluorescence images (B - F) and corresponding differential interference contrast image (A) were captured by a CCD camera. The excitation wavelength is from 330 to 385 nm (UV and U-excitation filters). [Bar = 250 µm (A - C) and 50 µm (D - F)]

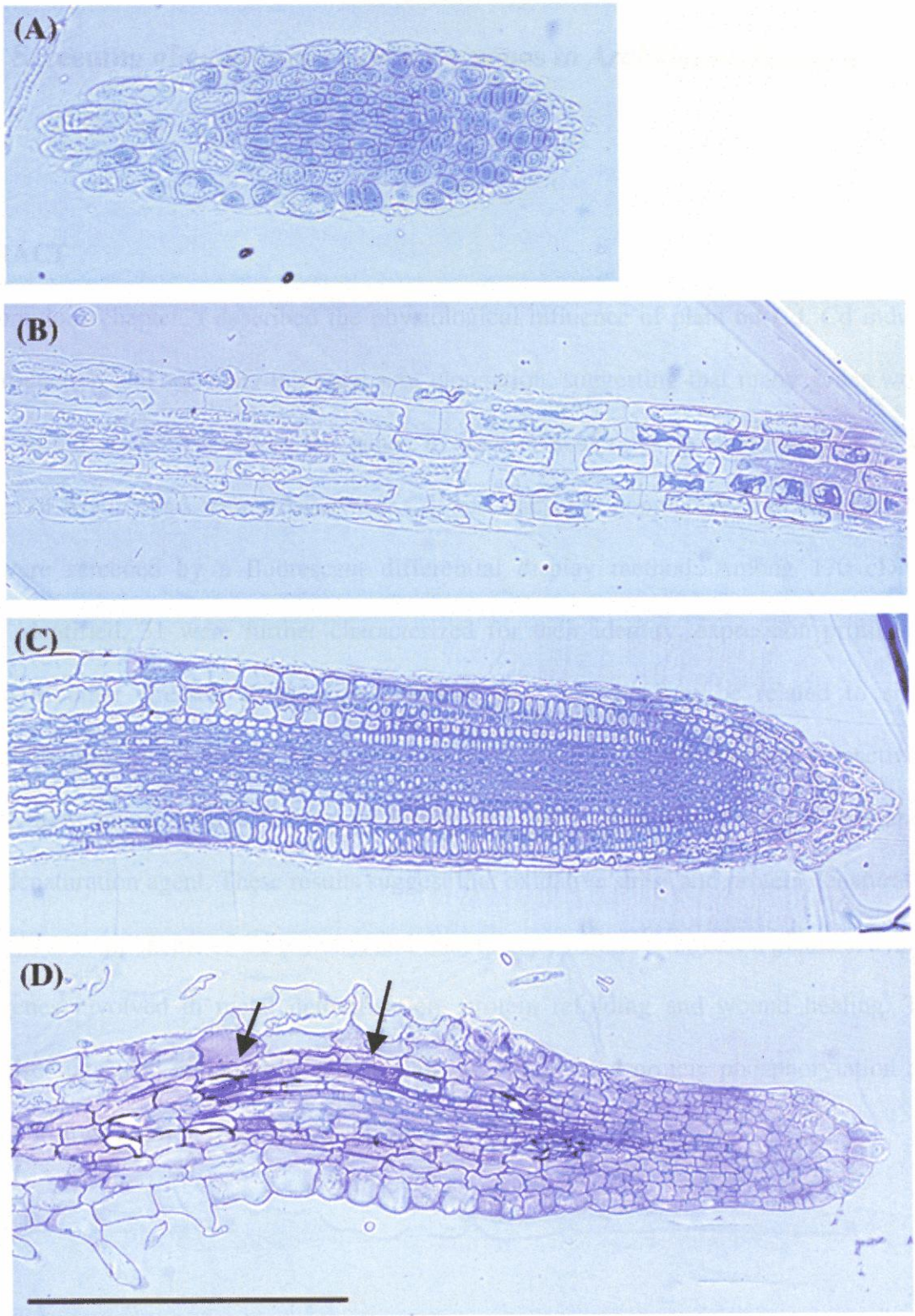


Figure 10. Localization of phenolic compounds in root of *Arabidopsis*. Plants were exposed to 200 µM Cd for 0 h (A, B), 48 h (C), and 2 weeks (D). 2 sections (A, root tip; B, mature root) were prepared for control. All samples were stained with 0.2 % safranin for 5 min and 0.5 % fast green for 25 min. Arrows indicate the localization of phenolic compounds. [Bar = 250 µm]

Chapter II

Screening of cadmium-responsive genes in *Arabidopsis thaliana*

ABSTRACT

In the previous chapter, I described the physiological influence of plant on Cd. Cd induced H_2O_2 production and seriously inhibited root elongation, suggesting that many genes would respond to Cd stress. To reveal the genes to play critical roles in detoxification of Cd, seedlings of Arabidopsis were treated with Cd, and transcript populations that changed their levels were screened by a fluorescent differential display method. Among 170 cDNAs initially identified, 31 were further characterized for their identity, expression profile and response to other stresses. Sequencing revealed 10, 12 and 15 to be related to signal transduction, protein denaturing stress, and responses to active oxygen species, respectively. Many of these genes responded not only to Cd, but also to oxidative stress, Cu ions and a protein denaturation agent. These results suggest that oxidative stress and protein denaturation are important components of Cd toxicity, and that, to cope with such stresses, plants activate a set of genes involved in metal detoxification, protein refolding and wound healing. The results also suggested temporarily and spatially well regulated protein phosphorylation and activation of transcription factors, accompanied by their transcription.

INTRODUCTION

Cd toxicity to living cells is caused at very low concentrations, and it is a suspected carcinogen in humans (Clemens et al., 1999). In plants, Cd damages the light harvesting complex II (Krupa, 1998). Total chlorophyll content is decreased and non-photochemical quenching is increased in Brassica napus (Larsson et al., 1998). Probably Cd also interferes with movement of K^+, Ca^{2+} and abscisic acid in guard cells, while inhibiting stomatal opening (Barcelo and Poschenrieder, 1990). Cd alters the synthesis of RNA, inhibits ribonuclease activity (Shah and Dubey, 1995). It may displace some metal ions in proteins (Stohs et al., 2000). Consequently it is highly probable that Cd interferes with transcription or signal transduction mechanisms.

Despite this knowledge regarding toxicity, information about the overall Cd effects on plant cells and their defense response on gene level is limited. In this study, we therefore screened for mRNA populations that change their levels upon Cd-treatment, and classified them into functional groups. For this purpose, we adopted fluorescent differential display (FDD), proven to be a powerful method to investigate changes in gene expression (Ito et al., 1994, Hara et al., 2000). Although there are drawbacks of low reproducibility of fingerprints and high frequency of false positive clones, it is simple and highly sensitive. The most advantageous feature of FDD is that it provides simultaneous comparison of multiple RNA samples which are both up-regulated and down-regulated.

Using this technique, we isolated 31 genes whose transcripts were induced upon Cd treatment of Arabidopsis. Elucidation of their identities suggested that Cd stress causes activation of genes involved in signal transduction pathways, oxidative and protein denaturing stress responses and sulfur metabolism. Many genes with unknown function were also found to be regulated by Cd. The results suggest that plants rapidly and simultaneously change expression of a set of genes to cope with heavy metal stresses.

MATERIALS AND METHODS

Plant materials and stress treatments

Seeds of *Arabidopsis thaliana* (var Columbia) were surface sterilized by a 10 min incubation in 70 % ethanol and by a 20 min incubation in 5 % (w/v) sodium hypochlorite (active chlorine 8.5 - 13.5 %, Nacalai Tesque, Kyoto, Japan) containing 0.05 % (v/v) Tween 20. After three washes with distilled water, seeds (10 - 15 per bottle) were cultivated in half strength 10 ml Murashige-Skoog (MS) medium supplemented with 1 % sucrose in a greenhouse at 23 °C under a 14/10 h light/dark photocycle. Two-week-old plants floated on MS medium were subjected to stress treatments by addition of 10 - 50 µl MS medium containing chemical compounds in H_2O or in DMSO (Sigma). Cd treatment was performed by addition of a final concentration of 500 µM $CdCl_2$ and other heavy metal treatments were performed by addition of a final concentration of 100 µM $CuSO_4$ or $ZnCl_2$. Heat shock stress was performed by incubation at 37 °C. Samples were harvested at an appropriate time point, immediately frozen in liquid nitrogen and stored at –80 °C until use.

RNA extraction

Total RNA was isolated by the acid guanidinium-phenol-chloroform method (AGTC). After stress treatments, frozen tissues (0.5 - 1.0 g) were ground in liquid nitrogen to a fine powder and immediately transferred into 10 ml of extraction buffer (4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5 % sodium N-lauroylsarcosine, 0.1 M 2-mercaptoethanol). After the solution was mixed well, 1 ml of 2 M sodium acetate (pH 4.0), 10 ml of acid phenol saturated with distilled water, and 2 ml of chloroform/isoamyl alcohol (49:1) were added, followed by incubation for 15 min on ice. The solution was subjected to centrifugation at 10,300 g for 15 min at 4 °C, and total RNA was precipitated from the aqueous phase by the addition of 10 ml

of isopropyl alcohol. After incubation at room temperature for 10 min and centrifugation at 10,300 g for 15 min at 4 °C, pellets were dissolved in 600 µl of DEPC-treated water and extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). RNA was left to precipitate with addition of 8 M LiCl (final concentration of 2 M) for 4 h at 4 °C, pelleted by centrifugation at 10,300 g for 20 min (4 °C), and resuspended in 200 µl of DEPC-treated water.

Differential display

The differential display (DD) was performed as described earlier (Hara et al., 2000). Total RNA was treated with DNase I, and cDNA was synthesized using Super Script II (Gibco-BRL), according to the manufacturer's manual. Upon completion, a 20 µl reaction mixture containing 2.5 µg of total RNA and 2.5 µM oligo-dT_{15} as the primer was diluted to 200 µl with H_2O. PCR was performed in a 10 µl reaction mixture containing 1 µl diluted cDNA, rhodamine-labeled 3-anchored primers (Takara) and 12-mer arbitrary primers. The reaction was carried out with 25 cycles of 94 °C for 30 sec, 40 °C for 1 min, 72 °C for 1 min, and 72 °C for 5 min for a final extension. After PCR amplification, samples were denatured at 85 °C for 3 min, and 6 µl aliquots were loaded on a 5 % denaturing polyacrylamide gel. Electrophoresispatterns were analyzed with an image analyzer (FM-BIO, Takara). cDNAs differentially amplified were eluted by boiling the gel pieces in TE buffer for 30 min at 95 °C and precipitation with ethanol. The fragments were reamplified by PCR with the same pair of primers as used for the first amplification and extracted with phenol/chloroform, and concentrated by ethanol precipitation. The resulting product was subcloned into the pT7 blue vector (Novagene).

RNA hybridization analysis

Total RNA (20 μg) was size-fractionated by electrophoresis on a 1.2 % agarose gel containing 2.2 M formaldehyde and 1x MOPS (3-[N-morpholino]propanesulfonic acid) buffer, and transferred to nylon membranes (Hybond-N, Amersham) in 20x SSC by the capillary blotting method. After crosslinking by irradiation with UV, hybridization was carried out at 42 °C for 16 h with gentle shaking in a solution containing 1 mM EDTA, 0.5 % SDS, 50 mM Tris-HCl (pH 7.5), 1x Denhardt's solution, 3x SSC, 50 % formamide, 10 % dextran sulfate. The cDNA probes were prepared from pT7 blue vectors containing FDD fragments by PCR amplification with the pair of M13 primers. After phenol/chloroform extraction and ethanol precipitation, they were labeled with [α-32P]dCTP with a random labeling kit (BcaBEST Labeling Kit, Takara). Membranes were washed with a solution containing 0.1x SSC and 0.1 % SDS at 62 °C, and subjected first to a bioimage analyzer (BAS-2500, Fuji, Tokyo) and then to autoradiography with x-ray film.

RT-PCR analysis

cDNAs were synthesized by the same protocol as for FDD, and RT-PCR with gene specific primers was performed by 24 - 26 cycles of 96 °C for 20 sec, 60 °C for 30 sec and 72 °C for 1 min. Samples were loaded on 1 % agarose gels, separated by electrophoresis and detected by ethidium bromide staining. The PCR products were confirmed by direct sequencing. Actin was used as the internal standard. The reaction was confirmed to be linear by comparison with preliminary calibration curves.

Sequencing analysis

Nucleotide sequences were determined by the dideoxy chain-termination method (ABI PRISM Big Dye Terminator) with a sequencer (model 310, ABI), analyzed using Gene Works

software (Intelli Genetics) and compared with the databases used the BLAST algorithm (Altschul et al., 1990).

RESULTS

Identification of Cd-responsive Genes

In order to identify mRNA populations with altered levels in response to Cd, FDD RT-PCR was performed. Equal amounts of cDNA were synthesized from total mRNAs extracted from 2-week-old seedlings that had been treated with 500 μM Cd^{2+} for 0 - 2 hours. Using 3 independent samples, and 4 kinds of fluorescence labeled anchor primers in combination with 20 arbitrary primers, 80 independent PCRs were performed (Table 1).

A total of 170 cDNA fragments were found to exhibit differential expression (Fig. 1). Most were up-regulated by Cd treatment, but 12 were down-regulated (data not shown). Fragments were eluted from gel, re-amplified by PCR and subcloned. Approximately 600 plasmids were constructed, and 200 were selected for further analysis. Finally, 55 clones were shown by northern analysis to be up- or down-regulated upon Cd-stress (data not shown). Single-sequence runs assigned the 55 cDNAs to 24 contigs, several cDNAs thus being multiply primed. Full-length cDNA information was obtained by 5' -race amplification and a BLAST data search. A summary of information for these clones is provided in Table 2.

Sequence analysis

The database search assigned putative functions to 22 genes. The clones of unknown function were designated as CdI (Cd Induced) with appropriate numbers. Assigned genes were referred to by name (Table 3). The first group contains 10 genes probably involved in signaling pathways, three encoding protein kinases. ATMEKK1 is reported to respond to touch, cold, and salinity stress (Mizoguchi et al., 1996). CdI12 and CdI17 are putative serine threonine protein kinases. Four were found to be genes for transcription factors. ATAF2 belongs to the NAC family of transcription factors functioning during plant development (Kikuchi et al., 2000). DREB2A drives the rd29A gene, which responds to cold and draught stress (Liu et al.,

1998). Both CdI2 and CdI11 are also putative transcription factors. CdI2 has a bZIP motif and CdI11 has a WRKY domain. One gene (CdI1) shows high homology to Hs1-pro gene that has a leucine rich repeat domain, and is possibly involved in signal transduction pathways for defense against pathogen infections (Cai et al., 1997). Two are for calmodulin-related proteins; CdI6 being similar to calmodulin with 4 EF hand motifs and CdI7 being predicted to bind calmodulin. They may participate in signal transduction pathways that involve Ca^{2+} as the second messenger.

The second group contains 3 genes, which encode proteins participating in protein folding. HSC-G8 and HSP81-3 are molecular chaperons and CdI16 resembles cis-trans isomerase that helps protein folding. The third group contains 3 genes, containing blue copper binding protein (BCB), CdI9 and CdI19. CdI9 with a 12 transmembrane domain shows similarity to multidrug efflux proteins in bacteria, with a structure generally found in transporters. It may directly function in metal ion binding. CdI19 has a similar region at the N-terminus with an yeast ATX1 protein, which contains a metal binding motif of CXXC and is proposed to deliver copper ions (Lin and Culotta, 1995). ATX1 shows a similarity to an Arabidopsis ATFP3, which contains a metal binding motif at the N-terminus and a farnesylated motif at the C-terminus (Dykema et al., 1999). CdI19 has two putative metal binding domains similar to those of ATX1 and ATFP3 at the N-terminus, and a farnesylated motif similar to that of ATFP3 at the C-terminus. Although the physiological function of ATFP3 is not determined, the similarities among these three proteins suggest that CdI19 is a metal binding protein.

APS reductase and a sulfur transporter constitute the fourth group. Both of them are involved in sulfur metabolism, and APS reductase has already been reported to be induced by Cd stress in *Brassica Juncea* (Lee and Leustek, 1999). Other genes, cytochrome p450 (CYP83A1), GST6, and CdI3 of group five are probably abiotic stress responsive genes. CAO

is recognition particle protein for chlorophyll (Klimyuk et al., 1999). CTF2b may contribute to ABA synthesis (Bilodeau et al., 1999). The remaining 8 genes encode putative proteins which show no significant similarity to any proteins with known functions.

Profile of transcript accumulation

Transcript accumulation of each clone was estimated during the first 12 h treatment with Cd (Fig. 2). The hybridization signals were densitometrically evaluated and normalized based on the rRNA signal as the internal control. The highest expression point for each sample was given an arbitrary value of 1, to which the comparative values were plotted. The induction profile was grouped into 4 phases; immediate early (1 - 3 h), early (2 - 4 h), late (4 - 12 h) and constitutive (1 h -). Transcripts of 10 genes were immediately induced in the early phase after Cd treatment. Transcripts of this group, including 3 protein kinases, were rapidly increased, being detected within 1h and reaching maximal levels at - 3 h (Fig. 2A). Transcripts of 9 genes were induced in the middle phase, increasing 3 h after treatment and declining by 6 h later (Fig. 2B). This group contained genes encoding heat shock proteins and metal binding proteins. Transcripts of 2 genes encoding transcription factors (CdI2, CdI11) were induced in the late phase (Fig. 2C). Transcripts of 8 genes showed prolonged accumulation, beginning to increase after 1h and maintaining high levels for up to 8 - 12 h (Fig. 2D). The transcripts of 2 genes (CAO and CYP83A1) were decreased during Cd treatment, showing slow and rapid down-regulation, respectively (Fig. 2E).

Effects of Cd on transcription factors

Among 31 genes identified, there were several transcription factors. DREB2A (DRE binding protein) is proposed to be a transfactor of rd29A (Liu et al., 1998), and OBP1 and OBF5 are proposed to be putative transcription factors of GST6 (Chen et al., 1996; Zang et al., 1993).

Consequently, we examined by RT-PCR the response profile of their related genes upon Cd treatment. The result showed DREB2A, rd29A and OBF5, but not DREB1A and OBP1, to be induced by Cd (Fig. 3). rd29A was shown to be induced by cold, salt and dehydration stresses, and DREB2A was reported to specifically interact with the DRE sequence in the promoter region of the rd29A gene. OBP1 and OBF5 are also DNA binding proteins and can recognize the upstream region of GST6 which is known to be induced by auxin, salicylic acid and H_2O_2 (Chen et al., 1996).

Effects of H_2O_2, Cu, Zn, heat shock and chemicals

Cd is proposed to displace Ca^{2+}, Zn^{2+} and Fe^{2+} in proteins, resulting in generation of active hydroxyl radicals (Stohs et al., 2000). Since BCB and GST6, identified in this study, were reported to respond to ozone (Richards et al., 1998) and H_2O_2 (Chen et al., 1996), respectively, we examined whether Cd-responsive genes are activated by H_2O_2 stress. The results showed transcript levels of 3 genes, sultr4, ATAF2 and CdI13, to be changed by 1 mM H_2O_2 treatment (Fig. 4). Sultr4 and ATAF2 transcripts began to accumulate after 2 h and remained high for up to 8 h. CdI13 transcripts were detectable as early as 1 h after treatment (Fig. 4A). The accumulation profile was similar to that with Cd treatment.

At least 3 new genes identified are activated by Cd and H_2O_2, Consequently, we examined the reverse case, that is, whether or not genes known to be activated by oxidative stress are also activated by Cd. As a model case, Cu/Zn SOD was selected since it responds to oxidative, ozone and H_2O_2 stresses (Sharma and Davis, 1994). A cDNA encoding a cytosolic Cu/Zn SOD (protein ID CAA43270) was prepared by RT-PCR, and used for northern analysis. The results clearly showed SOD transcripts to be induced by Cd treatment, although decline to the basal level was evident 6 h after Cd treatment (Fig. 4B). The fact that free or excess Cu ions catalyze the formation of reactive oxygen species through fenton reactions

(Stohs and Bagchi, 1995) led us to examine effects of Cu ions on the transcript level of isolated genes. Two-week-old seedlings were treated for 2 h either with 100 μM $CuSO_4$ or with 100 μM $ZnCl_2$ as control. Transcripts of 12 genes listed in Table 2 increased in response to Cu (Fig. 5), and only one (CdI12) responded to both Cu and Zn (Fig. 5). The remaining 19 genes responded to neither Cu nor Zn (data not shown).

Since a gene encoding a heat shock protein was identified, we examined effects of heat shock and azetidine-2-carboxylate (Az), which increases unfolded proteins, on the transcript levels of other genes (Koizumi et al., 1999). Those of 2 genes were increased by heat shock, of 4 genes were increased by Az, and of 6 genes by both heat shock and Az (Fig. 6).

Identification of several protein kinases suggested that signaling in response to Cd stress is mediated through a protein phosphorylation cascade. To confirm this, inhibition experiments were performed. Seedlings were pretreated with staurosporine (STU) prior to Cd treatment, and assayed for transcript profiles of isolated genes by northern hybridization. Transcripts of 10 genes declined strongly, and 3 declined weakly in comparison with those from control samples (Fig. 7A, B). Among the affected genes, 3 encode transcription factors (CdI2, DREB2A, ATAF2) and 2 a protein kinase (CdI17, ATMEKK1). This evidences that signaling pathways of these genes are mediated by phosphorylation.

DISCUSSION

This chapter describes identification and properties of a number of genes, products of which are possibly involved in resistance to Cd toxicity. We screened approximately 8,000 cDNA bands by FDD using four different anchored oligo (dT) primers, each in combination with 20 arbitrary 12-mer primers. We initially identified 170 transcripts that were up- or down-regulated by Cd, among which 31 were confirmed to be Cd-responsive, and further characterized. Assuming that total population of transcripts in a given tissue to be 15,000, it was predicted that 450 combination of primers for FDD is theoretically necessary in order to screen all the available transcripts with 95 % probability (Ito and Sakaki, 1996; Hara et al., 2000). Since we obtained 31 individual transcripts by 80 combination of primers, and taking account of transcript redundancy, we calculated that approximately 80 - 100 genes are Cd-responsive under our experimental condition.

Sequencing analyses revealed one third of identified genes (10) to encode signal transduction factors including 3 protein kinases, 4 transcription factors, and 2 calmodulin-related proteins. This suggests that plants respond rapidly to Cd by activating signal transduction pathways, which may include a protein phosphorylation cascade. Indeed, this assumption is partly rationalized by the finding that a protein kinase inhibitor, staurosporine, blocked or attenuated transcriptional induction of 13 genes by Cd treatment.

Activation of genes for transcription factors appears to be critical for the Cd response. DREB1A and DREB2A are proposed to regulate the rd29A gene, although DREB1A differs from DREB2A in the signal transduction pathway (Liu et al., 1998). This is consistent with our results showing rd29A and DREB2A, but not DREB1A, to be responsive to Cd and indicates that signaling to activate rd29A is preferentially mediated through DREB2A. Since rd29A is thought to play an important role under cold, high-salt and dehydration (Liu et al., 1998), Cd may cause damage to plants in common with those stresses. OBF5 is a bZIP-type

DNA binding protein which binds to the promoter region of GST6 (Zhang et al., 1993), the latter being induced by auxin, salicylic acid and H_2O_2 and responding to oxidative stress (Chen et al., 1996). GSTs are primarily involved in detoxification of cytosolic products produced during xenobiotic metabolism and in protection of tissues against oxidative damage. Since our results showed transcript induction of GST6 and OBF5, it is highly probable that Cd disturbs plant metabolism by producing various xenobiotics. Another transcription factor of interest is the WRKY-type, which is generally induced upon pathogen attack (Eulgem et al., 2000), suggesting a common feature between Cd and disease responses. Overall, the majority of transcription factors identified are involved in stress responses, indicating that, to cope with multiple environmental stresses including heavy metals, plants organize a fine network featuring cross-talk of apparently independent signaling pathways.

The other one third of genes that respond to Cd appear to encode proteins which function in actual protection against Cd stress. Those include chaperones, metal binding proteins and abiotic stress response proteins. Heat shock proteins (HSP) are typical. For example, upon exposure to Cd, a significant amount of HSP70 was found to be bound to plasmalemma in cell cultures of *Lycopersicon peruvianum* (Neumann et al., 1994). Since HSP70 is a chaperon that helps misfolded proteins to reattain the correct conformation, Cd may induce denaturation of proteins. To test this assumption, effects of heat shock and azetidine-2-carboxylate, which causes unfolding proteins in plants, were examined. Results showed clearly that both treatments commonly induced transcripts of up to 12 genes, including HSP81-3, HSC-G8, DREB2A, ATAF2, CdI6 and CdI17. It can thus be concluded that protein denaturation is one of the major effects of Cd-toxicity. In order to detoxify Cd, plants produce phytochelatins, which are synthesized from glutathione, a sulfur-containing compound (Cobbett, 2000). The induction by Cd of transcripts for APS reductase and Sultr4, which are key proteins in the sulfur reduction pathway, indicates an urgent necessity of

sulfurs, and supports the idea that plants are equipped with a mechanism to actively remove Cd (Harada et al., 2001).

The other group consisted of 12 genes encoding Cu-responsive proteins. Although Cu is a common co-factor for many enzymes including oxidases, free or excess Cu ion acts as a catalyst in the formation of reactive oxygen species and catalyzes peroxidation of membrane lipids (Stohs and Bagchi, 1995). Activation of genes encoding Cu-responsive proteins may suggest that Cd also induces active oxygen species. This is consistent with induction of CdI19, which shows 40% similarity to the metal binding region (spanning approximately 80 amino acids) of CCH, a functional homolog of yeast ATX1 which can rescue ATX1-deficient yeast mutants (Himelblau et al., 1998). Since yeast protein ATX1 is proposed to deliver copper ions and to defend against oxidative stress produced by SOD deficiency (Lin and Culotta, 1995), CdI19 also functions to metal ion deliver.

In summary, the present data suggest that the toxicity caused by Cd is partly due to protein denaturation, probably through generation of active oxygen species. To cope with such stress, plants activate a set of defense genes encoding metal binding proteins, chaperons and other stress-inducible proteins. In order to initiate their transcription, stress signals appear to be transduced through a protein phosphorylation cascade, and transcription factors are induced (Fig. 8). Although the target genes of these transcription factors are currently not known, it is clear that overall transcription of Cd-responsive genes is temporally and spatially well concerted. Further functional analyses of identified genes will reveal the molecular mechanisms of heavy metal responses of higher plants.

Table 1. PCR primers used in this study (Arbitrary 5' primers (1-20), and 3' poly-T primers (A, C, G, T); V is a mixture of A, C and G

5' primer			
1) 5'-ATGGCGAGGAAG-3'	2) 5'-GACGGCCAGATC-3'	5) 5'-AAGGAGCCGATG-3'	6) 5'-TGCTGCAGACCA-3'
7) 5'-AGGTACGACGCC-3'	9) 5'-CCTTGCTGGGTC-3'	10) 5'-AGTGAGGAGCCG-3'	11) 5'-GATCCGTGAGGC-3'
13) 5'-GCCTCCTTGAGC-3'	14) 5'-AAGGGACGCATC-3'	15) 5'-GCCACGGAGTTC-3'	16) 5'-AGCCATCGTGGT-3'
17) 5'-ACCCAAAGGCAC-3'	18) 5'-AAAGGCACCGTC-3'	19) 5'-GAACTCCGTGGC-3'	20) 5'-TGCCTTGCTGTG-3'
3' primer			
A) 5'-GT_{15}VA-3'	C) 5'-GT_{15}VC-3'	G) 5'-GT_{15}VG-3'	T) 5'-GT_{15}VT-3'

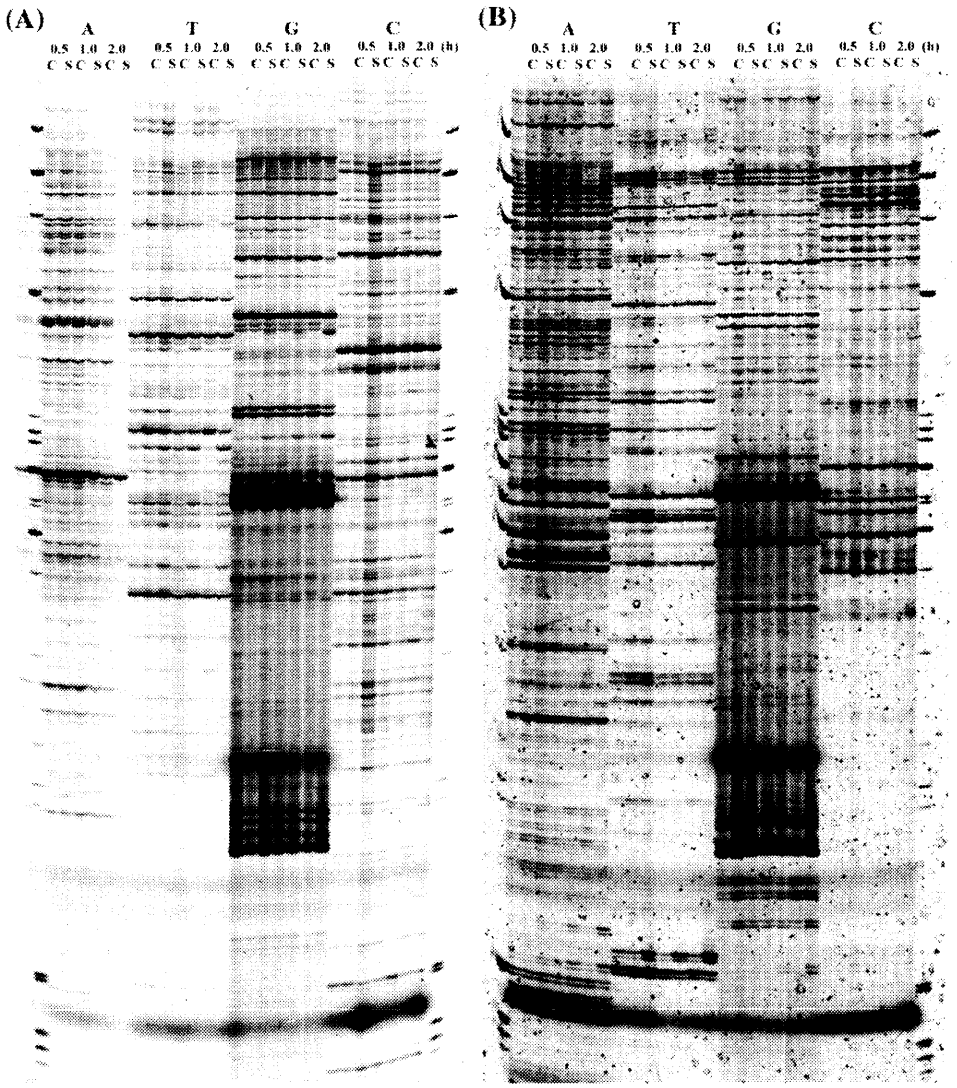


Figure 1. Image of typical FDD finger printings. cDNA were reverse transcripted from mRNAs isolated from seedlings of *Arabidopsis*. (S), Stress treatment (500 μM Cd); (C), control. Numbers (0.5, 1.0, 2.0) indicate the period of stress treatment. 4 kinds of 3' poly T primers (A, T, G, C) and were used in combination with 20 kinds of arbitary primers. This finger printing gel was obtaind by poly T primers and 5' arbitary primer No. 5 (A) and No. 6 (B).

Table 2. Properties of cloned cDNAs identified by FDD. Clones with putative functions are designated as CdI with sequential numbers. Genes with known function are referred to by their names. Protein ID was assigned for the highest alignment score in BLAST search. Asterisk indicates cDNA samples that were assigned to multiple genes (> score 80). Status of transcript accumulation in response to denaturation (1), H2O2 or Cu treatment (2), and phosphorylation inhibition (3) was examined. Induction and no effects are indicated by the plus (+) and minus (-) symbols, respectively in columns 1 and 2. Inhibition by staurosporine is indicated by the down-ward arrow (↓) in column 3. BCB is Blue copper binding protein.

cDNA clone	Insert Size	ORF	Best Homology	Protein ID	1	2	3
CdI1	400	1,304	Hs1pro-1	AAB95285.1	-	+	-
CdI2	350	888	bZIP	AAG26018.1	-	-	↓
CdI3	250	1,344	glucosyl transferase	AAG50970.1	-	+	-
CdI4	250	666	unknown protein	AAD23890.1	-	+	↓
CdI5	320	303	unknown protein	CAB61953.1	-	-	-
CdI6	350	486	calmodulin	BAB00596.1	+	-	-
CdI7*	600	1,944	Ca-M binding protein	BAB08793.1	+	-	-
CdI8	480	276	unknown protein	AAD11996.1	+	-	↓
CdI9*	700	1,506	transporter	AAC28507.1	-	-	-
CdI10*	600	792	unknown protein	AAD21443.1	-	-	↓
CdI11	350	971	putative WRKY	CAB79873.1	-	-	-
CdI12*	700	1,482	protein kinase	AAC18814.1	-	+	-
CdI13	310	1,839	unknown protein	AAF79287.1	+	+	-
CdI14	300	918	unknown protein	AAD32777.1	-	+	-
CdI15	500	573	unknown protein	AAC73017.1	+	-	-
CdI16	900	1,533	cis-trans isomerase	CAB80023.1	-	-	-
CdI17*	430	1,270	APK1A	AAF23252.1	-	+	↓
CdI18	420	762	unknown protein	AAF82229.1	+	-	↓
CdI19	370	1,179	ATFP3	CAB83295.1	-	+	↓
APS reductase	500	1,377		AAC49562.1	-	+	-
DREB2A	700	1,004		BAA33794.1	+	-	↓
HSC-G8	500	1,962		CAA70105.1	+	-	-
CAO	740	1,131		AAD01509.1	-	-	-
Sultr4	320	2,058		BAA23424.1	-	+	-
GST6	750	792		AAC63629.1	+	+	↓
BCB*	1,000	519		CAB89345.1	+	+	↓
ATAF2	500	838		CAA52777.1	+	+	↓
CYP83A1(P450)	250	1,509		CAB78419.1	-	+	-
HSP81-3*	300	2,100		BAB09285.1	+	-	-
CTF2b*	500	1377 1287		AAC35227.1 or AAD09952.1	-	+	↓
ATMEKK1	350	1,827		BAA09057.1 or CAB77975.1	-	-	↓

Table 3. Functional classification of Cd-responsive gene products

Function	Number of clones	Identification of clones
Protein kinase	3	CdI12, CdI17, ATMEKK1
Transcription factor	4	CdI2, CdI11, DREB2A, ATAF2
Calcium binding	2	CdI6, CdI7
Protein folding	3	CdI16, HSC-G8, HSP81-3
Sulfur metabolic pathway	2	APS reductase, Sultr4
Metal binding	3	CdI9, CdI19, BCB
Abiotic stress responding	4	CdI3, GST6, CYP83A1, CTF2b
Miscellaneous	2	CdI1,CAO
Unidentified	8	CdI4, CdI5, CdI8, CdI10, CdI13, CdI14, CdI15, CdI18

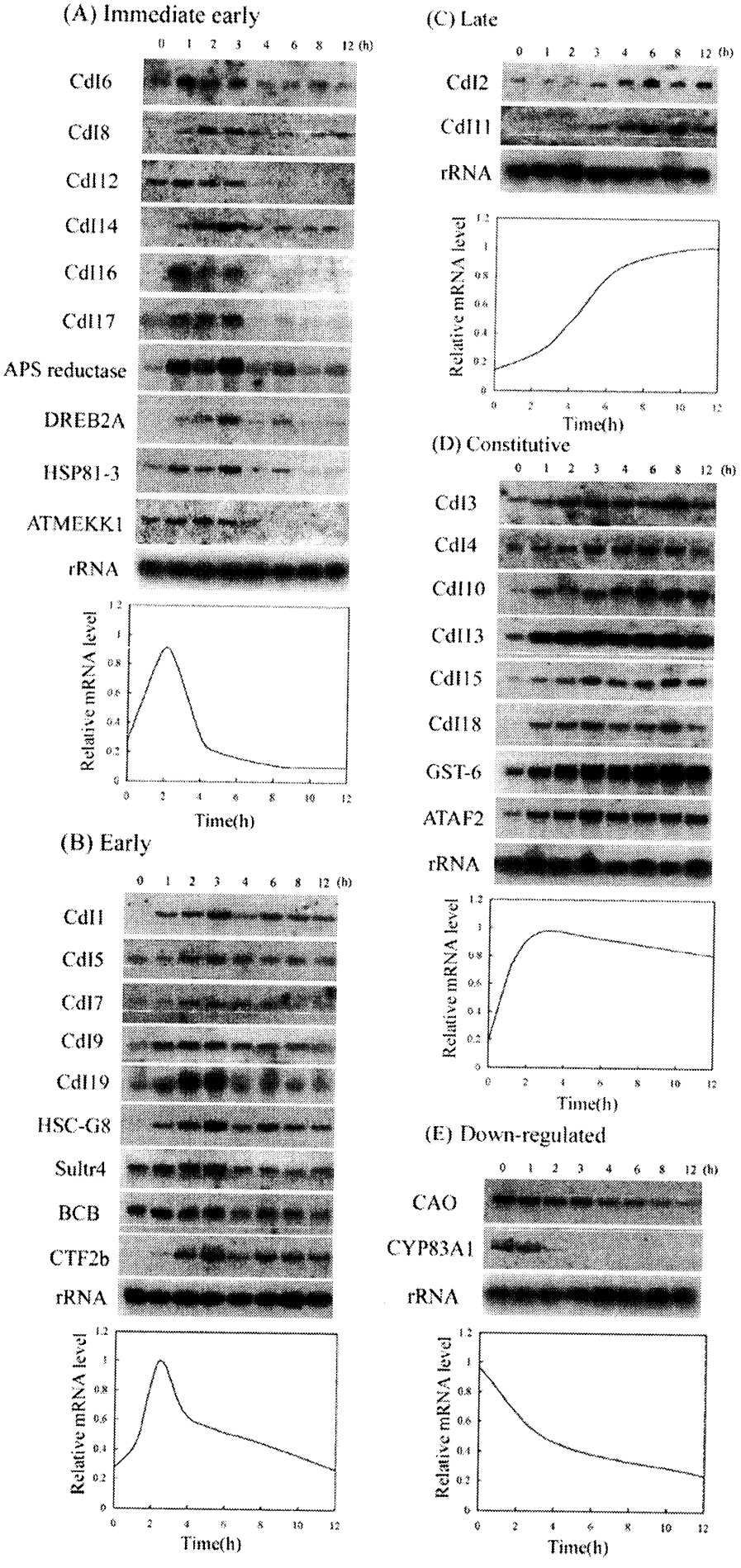


Figure 2. Time course of transcript accumulation. Hyponically cultured Arabidopsis seedlings were treated with 500 μM $CdCl_2$ for 12 h. Total RNA was extracted, fractionated by agarose gel electrophoresis, transferred onto nylon membrane and subjected to northern hybridization assay using probes containing sequences listed in Table 2. Eight identical sheets were prepared, each of which were successively subjected to hybridization with at least 4 different probes. After each hybridization, previous probes were confirmed to completely stripped off with a bioimage analyzer BAS-2500. rDNA was used as the internal standard for RNA loading. The transcripts were classified based on the accumulation pattern into immediate early (A), early (B), late (C), constitutive (D) and down-regulated (E) groups. The relative transcript abundance was densitometrically evaluated, normalized to rRNA levels and an average pattern of the group was estimated, which is depicted as the representative accumulation pattern under each panel.

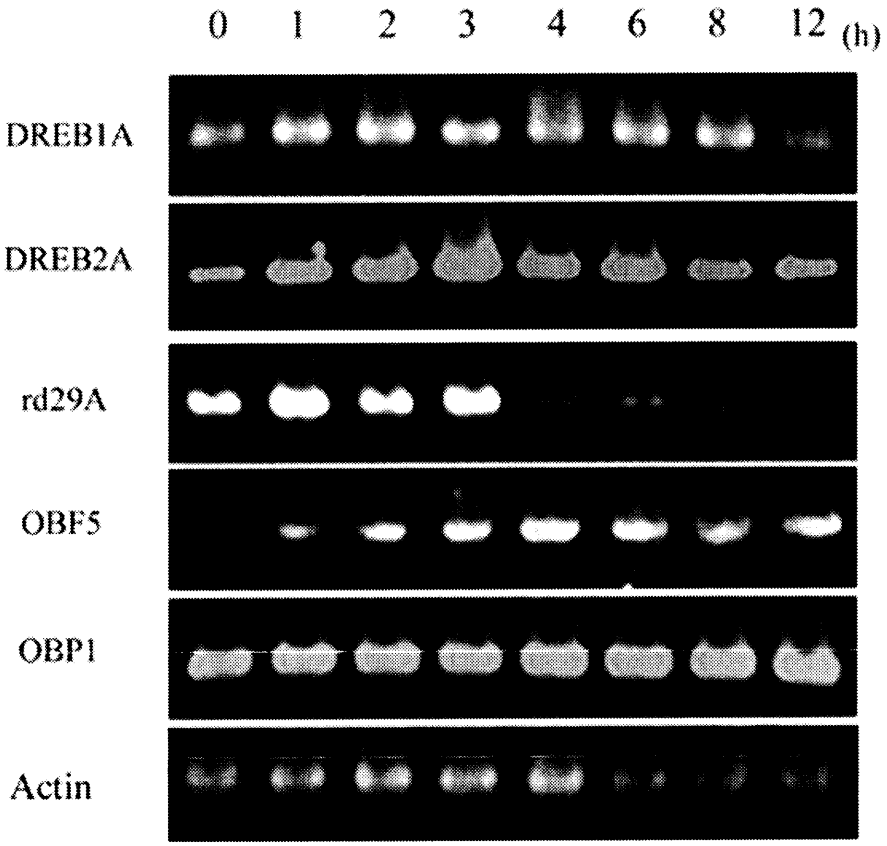


Figure 3. Effects of Cd on accumulation of transcripts for transcription factors. Seedlings were treated with 500 μM $CdCl_2$ and levels of transcripts for the indicated genes were examined by RT-PCR at appropriate time points. The primers used for RT-PCR were: DREB1A; 5'-TGGCCGATCAGCCTGTCTC-3' 5'-CGTCGTCATCATCGCCGTC'-3', DREB2A; 5'-GGAGATGGCAGTTTATGATC-3' 5'-TTGTTTAGTTCTCCAGATCC-3', rd29A; 5'-ATGGATCAAACAGAGGAACCAC-3' 5'-ACAACTCCTGATTCACCTGG-3', OBF5; 5'-ATGGGAGATACTAGTCCAAG-3' 5'-TCACTCTCTTGGTCTGGC-3', OBP1; 5'-GTGTTCCGTTACAAACGACGC-3' 5'-GATGGAAAGATCCGGCCAC-3'. PCR cycles were 24 for DREB1A and rd29A and 26 for DREB2A, OBF5 and OBP, with 96 °C for 20 sec, 60 °C for 30 sec, and 72 °C for 1 min. Samples were fractionated by 1% agarose gel electrophoresis and visualized with ethidium bromide staining. Experiments were repeated at least 3 times independently and similar results were obtained. The signals were not due to genomic DNA contamination as no signal was detected upon PCR using RNA samples prior to cDNA synthesis (data is not shown).

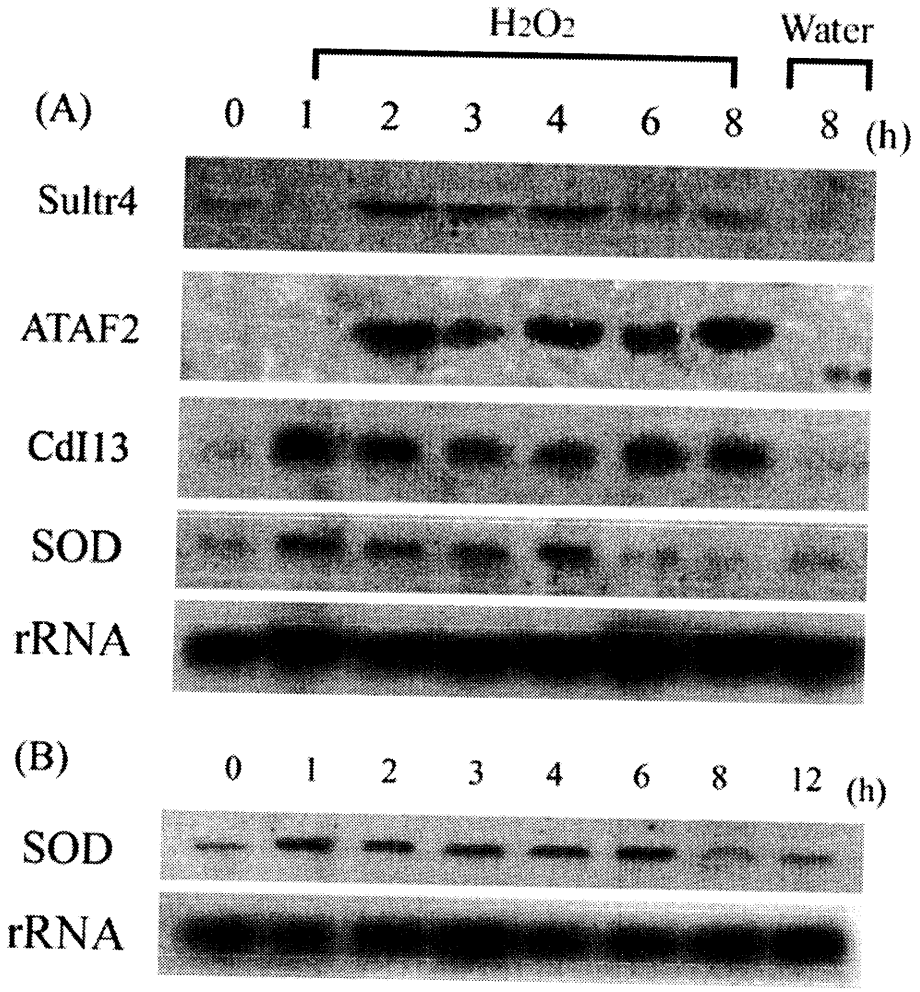


Figure 4. Effects of Cd and H_2O_2 stresses. Seedlings were treated with 1mM H_2O_2 (A) or 500 μM Cd Cl_2 (B) and levels of transcripts for the indicated genes were examined by northern hybridization at the indicated time points. Control was sampled just on stress treatment and 8 h after treatment with water (A). Aliquots of 20 μg of total RNA per lane were subjected to electrophoresis and hybridization was performed at 42 °C for 16 h.

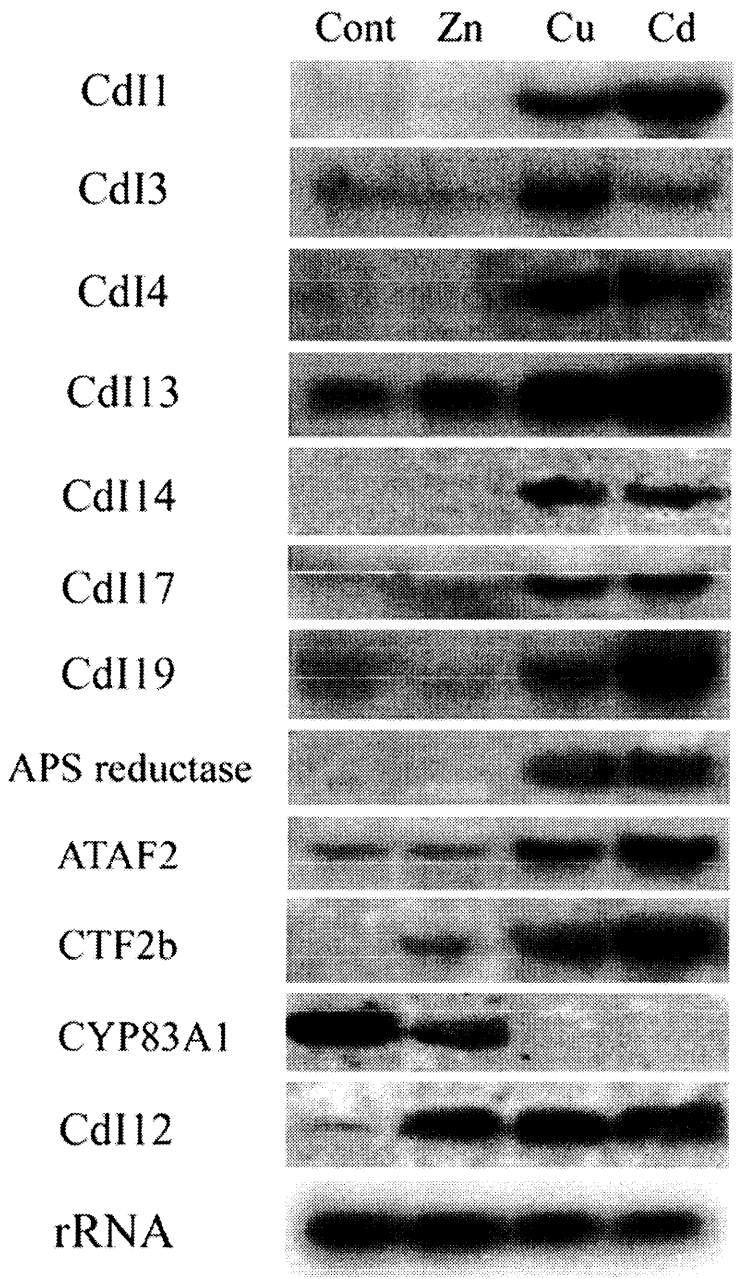


Figure 5. Effects of metal ions. After seedlings were treated with 100 μM $CuSO_4$ or $ZnCl_2$ or 500 μM $CdCl_2$ for 2 h, total RNA was extracted and subjected to northern hybridization with the indicated probes. Control samples were untreated seedlings.

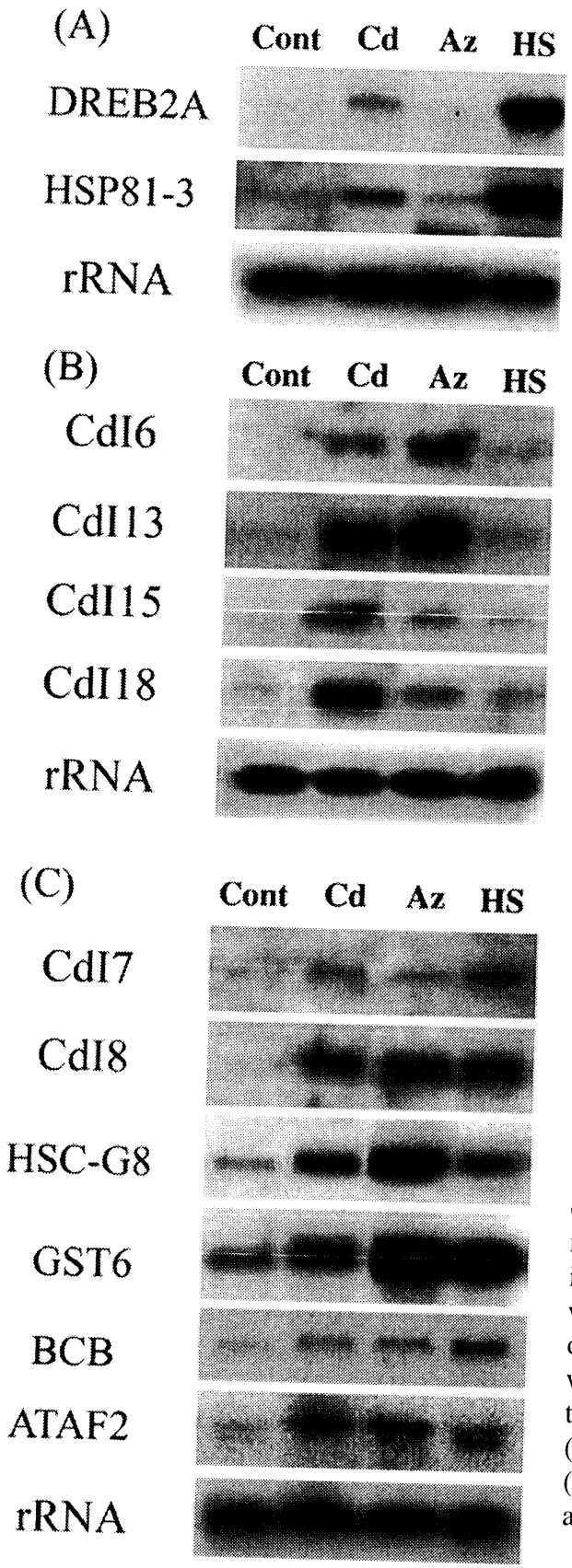


Figure 6. Effects of heat shock and azetidine-2-carboxylate. After seedlings were treated with 5 mM azetidine-2-carboxylate (Az) or kept at 37 °C for 2 h for heat shock treatment, total RNA was extracted and subjected to northern hybridization with the indicated probes. Control samples were kept at 23 °C for 2 h. Based on the response pattern, genes were classified into 3 groups; (A) those responding to heat shock; (B) those responding to Az stress. (C) those responding to both HS and AZ.

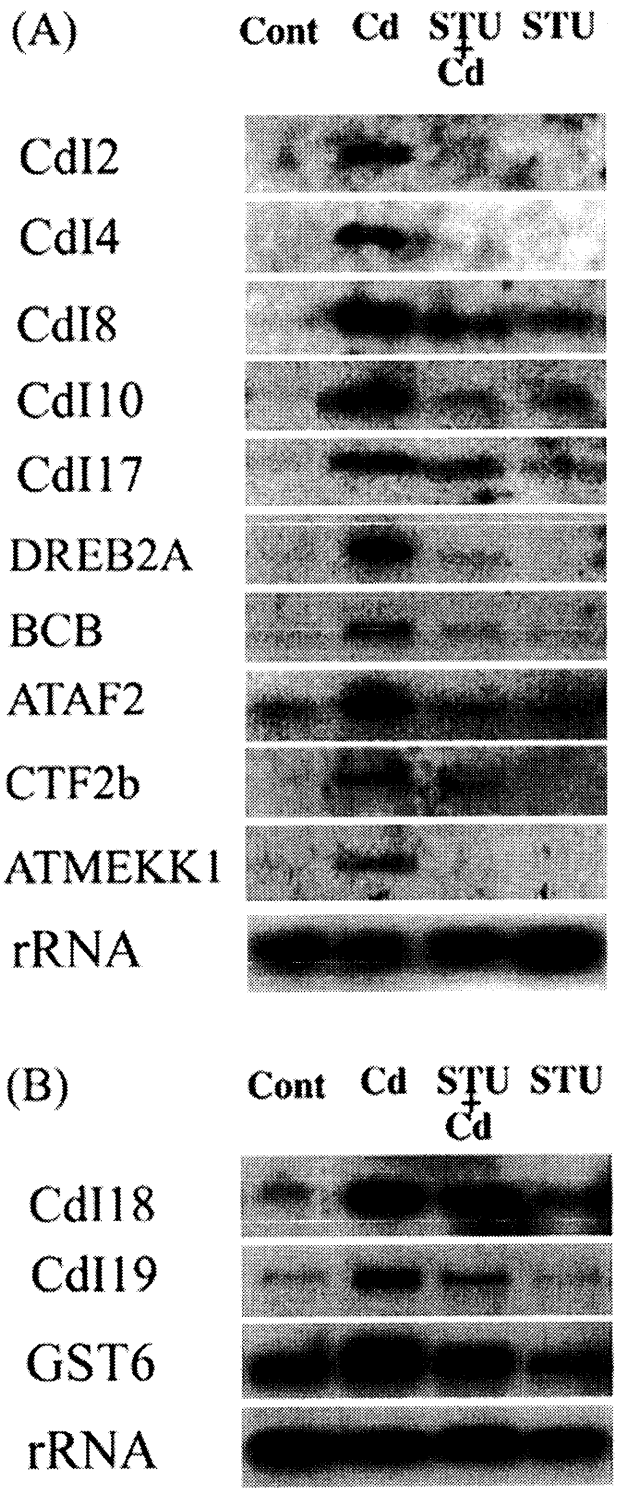


Figure 7. Effects of metabolic inhibitors. Seedlings were treated with 1.5 μM staurosporine (STU) for 2 h, and then treated with 500 μM Cd for 2 h. The level of transcripts for indicated gene was examined by northern hybridization. Based on the response pattern, genes were classified into 2 groups; (A) those inhibited by STU; (B) those attenuated by STU.

Chapter III

Functional analysis of a heavy metal binding protein, CdI19, from *Arabidopsis*

ABSTRACT

Some metals such as Cd are nonessential and potentially highly toxic to organisms. Plant possesses homeostatic mechanism to minimize the damage from exposure to nonessential metal ions in different cellular compartments. Our previous study revealed that expression of a number of genes was induced by Cd stress in Arabidopsis, and that one of them, CdI19, encoding a putative metal binding protein with the core motif of M/LXCXXC. Upon introduction into yeast cells, it endowed marked toleration of Cd toxicity. In this chapter I describe that CdI19 directly interacts to Cd *in vitro*, and that it localized on plasma membranes as revealed by heterologous expression of fusion protein with green fluorescent protein. Northern blot analyses and histochemical assay using CdI19 promoter::GUS fusion indicated that the CdI19 transcripts were also induced by Hg^{2+}, Fe^{2+}, Cu^{2+} and Mn^{2+} and accumulated in the petiole, hypocotyl, peduncles and vascular bundles in root tissue. Homology search for Arabidopsis genome revealed more than 20 similar proteins with CdI19 to exist, possibly functioning in heavy metal detoxification. These findings are discussed in relation to the involvement of this gene product in heavy metal homeostasis or in detoxify on plasma membrane.

INTRODUCTION

Many transition metals are essential nutrition for plants, but regulation of these ions is also critical because of their toxicity for plant cells. Plants have evolved homeostatic mechanisms to maintain appropriate concentrations of essential metal ions and to remove nonessential ones. For this, plants possess various mechanisms to control accumulation, distribution and sequestration of the metals (Clemens, 2001; di Toppi and Gabbrielli, 1999; Zenk, 1996).

To avoid free metals ions to exist in tissues, chelators, such as small peptide, organic acids and amino acids play a crucial role. They contribute to metal detoxification by buffering cytosolic metal concentrations (Clemens, 2001). The principal classes of known metal chelators are phytochelatins (PC) and metallothioneins (MT). PC is a cysteine-rich peptide and enzymatically synthesized from glutathione to inactivate metal ions in response to toxic levels of heavy metals. Cd, Cu, Zn and Ag ions were chelated on the included thiol moieties and experimentally 97 % of Cd was bound to PC in cell-free extracts (Zenk, 1996). MTs are low molecular weight, cysteine rich proteins and mainly bind to Cu. Although Cu is an important plant micronutrient, it leads to the generation of harmful reactive oxygen species as well. Cytosolic concentration of free Cu would be typically maintained at exquisitely low levels (10^{-18} M) by metal scavenging systems including MT (Rae et al. 1999; O'Halloran and Culotta, 2000).

In the intracellular trafficking of metal ions, soluble metal receptor proteins are known as "metallochaperones". ATX1 was originally isolated as an antioxidant protein in yeast *Saccharomyces cerevisiae* (Lin and Culotta, 1995) and reported to deliver Cu ions to a copper transporter in the membrane of a post-Golgi vesicle in the cytoplasm (Pufahi et al. 1997). ATX1 binds a single Cu ion by two cysteines in MXCXXC sequence (M is methionine, X is any amino acid, and C is cysteine). This metal binding motif was found in many metal binding proteins, including CCC2, a P-type copper transporter (Yuan et al., 1995), MerP, a

bacterial carrier for mercury ions (Powlowski and Sahlman, 1999), and CCS, the copper chaperone for SOD1 (Culotta el al, 1997). However their action mechanism is largely unknown.

In order to determine the regulatory mechanism of metal trafficking, sequestration and detoxification, I examined properties of a putative metal binding protein, CdI19 from *Arabidopsis thaliana*. In the previous chapter, I reported CdI19 as one of genes induced by Cd stress. CdI19 protein contains a metal binding motif, CXXC, and a consensus isoprenylation site CaaX (C is cysteine, a is aliphatic amino acid, X is the carboxy-terminal residue). Isoprenylation is post-translational attachment of hydrophobic isoprenoid to proteins, and essential process in eukaryotes to confer proteins biochemical functions such as membrane attachment and protein-protein interaction (Clarke, 1992; Higgins and Casey, 1994; Zhang and Casey, 1996). I found that the structure of CdI19 protein changed by Cd *in vitro* and fluorescence of GFP-CdI19 fusion protein was observed on plasma membrane in BY2 cell. These results confirmed CdI19 being a metal binding protein and localizing on plasma membrane.

MATERIALS AND METHODS

Cloning of the CdI19 Gene and Sequencing analysis

A 370-bp cDNA fragment of CdI19 was first isolated by RT-PCR FDD. The missing 5' ends and 3' of cDNA sequence was obtained using 5' and 3' RACE system, respectively according to the manufacture's instructions (Marathon cDNA Amplification Kit; Clontech, CA, USA). After synthesis of double-stranded cDNA ligated adapter, PCR was performed with gene specific primers as follows: 5'- CGCCGCCAACAGCAACTTC –3' and 5'- GAAGTTGCTGTTGGCGGC –3'. The RACE product was subcloned into the pT7 blue cloning vector (Novagene, WT, USA). Nucleotide sequences were determined by the dideoxy chain-termination method (Big Dye Terminator Kit; ABI, CA, USA) with a sequencer (model 377, ABI). Nucleotide and predict amino acid sequences were analyzed and aligned Gene Works software (Intelli Genetics, CA, USA) and compared with the databases used the BLAST algorithm (Altschul et al., 1990).

Yeast culture, transformation and growth assays.

Genes were amplified from a cDNA library of *A. thaliana* by PCR. Appropriate forward- and reverse primers for amplification were designed having XbaI or SacI sites at their 5'ends. Resulting fragments were subcloned into the pT7 blue vector, and then directionally inserted into XbaI and SacI sites of the yeast expression vector pESC-URA (Stratagene, CA, USA). Transformation was performed with yeast cells of strain KMY1005 (which lacks *MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801*)(Mori et al., 1996).

Plasmid carrying CdI genes was transformed by the modified lithium acetate yeast transformation method (Elble, 1992). Yeast cells were grown in a 0.67 % yeast nitrogen base with a 2 % glucose supplement and appropriate amino acids. Growth assays were performed on plates in the presence of $CdCl_2$ for 60 h or in liquid SG medium with or without $CdCl_2$ for

30 h at 30 °C. Growth rate was measures at 600 nm in a 1cm-glass tube (Spectronic 20E Taitec Tokyo, Japan).

Site-directed Mutagenesis of CdI19

Site-directed mutagenesis of CdI19 gene was performed with standard protocol of Takara LA PCR™ in vitro Mutagenesis Kit (Takara, Kyoto, Japan). The mutations were performed in pT7 blue cloning vector. The desired fragments containing the mutation were identified by DNA sequencing on minipreps, digested by appropriate enzymes and ligated into the pBI121 (Clontech, CA, USA) or pGEX-4T-1 (Amersham, NJ, USA) plasmid.

Subcellular localization experiments with transgenic BY2 cell

pGFP-CdI19W and pGFP-CdI19M vector was constructed with XbaI and SacI digestion of a binary vector pBI121. The appropriate forward- and reverse primers were designed for PCR having XbaI or XhoI sites at their 5'ends and sGFP (S65T) fragment was obtained by PCR from CaMV35S-sGFP (S65T)-NOS3' (Chiu et. al. 1996). CdI19 cDNA fragment also amplified by the appropriate primers having XhoI or SacI sites at their 5'ends. The resulting fragments were subcloned into the pT7 blue vector. Then they were digested respectively by the restriction enzyme site of the primer's and released fragments were directionally inserted into XbaI and SacI sites of pBI121 vector. GFP fusion vectors were transformed into *Agrobacterium tumefaciens*. Encoding fusion proteins were expressed constitutively under the control of the CaMV 35S promoter in BY2 cell. 5 ml of BY2 cell suspension was used for transformation. At first 2 ml of full growth BY2 cell suspension transferred in 95 ml of fresh LS medium and after 4 days, 5 ml of suspension was placed in a plate and 0.1ml *Agrobacterium* culture (OD_{600}, 1.0) was added. Mixed culture incubated for 2 days and then BY2 cell was washed 3 times and suspended again by 1 ml of fresh LS medium. The

suspension was spread on a solid LS medium plate containing 100mg/l Kanamycin and 250mg/l Carbenicilin and then incubated for 3 weeks. Growing calli of BY2 cell was used for experiment. All incubations were at 27 °C under dark condition. The localization of GFP fusion protein was observed directly with a confocal laser-scanning microscope (LSM 510, Carl Zeiss, Tokyo, Japan). Samples were scanned and images of 10μm depth were produced. The images were collected with Zeiss Plan-NEOFLUAR lens 10X/0.3, 20X/0.3, 40X/0.3.

Preparation of Glutathione S-Transferase Fusion Proteins

The expression plasmid for this study was constructed with pGEX-4T-1 plasmids. A 675-bp (1 to 675) fragment of the CdI19 cDNA were prepared by PCR. Primers having EcoRI or XhoI sites at their 5'ends were used and sequences were 5' - GGAATTCATGGGTGAGAAAAAGGAAGAAAC – 3' and 5' - CTCGAGGTCATCCTTTTTGGCCGGAA – 3', respectively. Amplified fragment was cloned into the EcoRI - XhoI sites of the pGEX-4T-1 and the plasmids were transformed to *E. coli DH5α*. After 4 h induction with 1 mM IPTG, the protein was isolated by batch purification of glutathione sepharose 4B according to the manufacture's instructions (Amersham). GST fusion protein bound to glutathione sepharose was cleaved by incubation with thrombin protease for 24 h at 22 °C. Approximately 1 - 2 mg of pure CdI19 protein was obtained per liter of bacterial culture. Protein concentration was determined by the Bradford method (Bio-Rad, CA, USA) with bovine serum albumin as a standard.

Circular dichroism (CD) measurement

The apo-protein was prepared by dialysis. 3 ml of saturated urea solution containing 10 mM of EDTA was added to 3 ml of recombinant protein solution. Samples were dialyzed by three steps for 2 h in 50 mM Tris-HCl (pH7.0) buffer containing 1 mM DTT and 1 mM EDTA with

200 ml of 4 M, 2 M and no urea. Finally protein solution was changed again and dialyzed over a 12 h with 500 ml of 50 mM Tris-HCl (pH7.0) buffer containing 1 mM DTT. All dialysis was performed at 4 °C under stirring. CD measurements were performed with this protein solution and used 1mm quartz cell on Jasco spectropolarimeter (J-720W) at 25 °C.

Assay of GUS localization and histochemical staining

By using the primers 5' - GCATGCAACTGAGGAGATCTTAGCGG – 3' and 5' - TCTAGACGTAGTAGTAGTAGTAGAGAGAATC – 3', a 1 kb fragment immediately upstream of the CdI19 coding region was amplified from genomic DNA of Arabidopsis. The fragment was cloned into a XbaI site at the ATG initiation codon and a HindIII site at the 5' end corresponding sites of pBI121. The plasmid verified by DNA sequencing was transferred to *Agrobacterium tumefaciens (C58).* Transformant of Arabidopsis was produced by infiltration (Clough and Bent, 1998) in 200 ml of *A. tumefaciens* suspension and selected on a solid 1/2 MS medium plate containing 50 mg/l Kanamycin and 250mg/l Carbenicilin.

Transformants were assayed for GUS activity using 1 mM 5-bromo-4-chloro-3-indolyl--glucuronic acid (X-Gluc) as substrate. Plants were treated for 4 h by 500 µM $CdCl_2$ and then incubated in 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 0.3 % (w/v) Triton X-100 and 1.9 mM X-Gluc for 12 h at 37 °C. The reaction was stopped by 70 % ethanol and chlorophyll was removed by incubation in 100% ethanol. 3 transgenic strains were independently used for assay and similar results were obtained.

RNA-blot hybridization

Surface sterilized seeds of *A. thaliana* were cultivated in half strength 10 ml Murashige-Skoog medium supplemented with 1% sucrose for 2 weeks in a greenhouse at 23 °C under a continuous light (10 - 15 seeds per bottle). Two-week-old plants floated on 10ml MS medium

were subjected to metal ions treatments by addition of 5 or 50 μl of 0.1 M solution corresponding ion ($FeCl_2$, $CuCl_2$, $HgCl_2$, $CoCl_2$, $MnCl_2$, $CaCl_2$). After 4 h samples were harvested and total RNA was isolated by the acid guanidinium-phenol-chloroform method (AGTC) described in chapter II.

Total RNA (20 μg) was size-fractionated by electrophoresis on a 1.2 % agarose gel containing 2.2 M formaldehyde and 1x MOPS (3-[N-morpholino] propanesulfonic acid) buffer, and transferred to nylon membranes (Hybond-N, Amersham) in 20x SSC by the capillary blotting method. After crosslinking by irradiation with UV, hybridization was carried out at 42 °C for 16 h with gentle shaking in a solution containing 1 mM EDTA, 0.5 % SDS, 50 mM Tris-HCl (pH 7.5), 1x Denhardt's solution, 3x SSC, 50 % formamide, 10 % dextran sulfate. The cDNA probe was used full-length fragment prepared by PCR amplification from the pT7 blue-CdI19 vector with the pair of M13 primers. After phenol/chloroform extraction and ethanol precipitation, it was labeled with [α-32P] dCTP with a random labeling kit (BcaBEST Labeling Kit, Takara). Membranes were washed with a solution containing 0.1x SSC and 0.1 % SDS at 62 °C, and subjected first to a bioimage analyzer (BAS-2500, Fuji, Tokyo, Japan) and then to autoradiography with x-ray film.

RESULTS

Yeast transformation analysis

Among genes that were described in chapter II, cDNA cloned for HSC-G8, BCB, CYP83A1, CTF2b, ATMEKK1 and CdI19 were obtained from data base, transformed into *S. cerevisiae* in order to examine their effects on yeast growth. Cd tolerance of the *S. cerevisiae* strain carrying these genes was tested in SG medium containing 50 μM $CdCl_2$. The effect of Cd was monitored in terms of growth rate by measuring absorbance at 600 nm. While Cd strongly suppressed the growth of *S. cerevisiae* as shown by control cells transformed with the empty vector, the strains carrying ATMEKK1 and CdI19 demonstrated resistance as evidenced by their fast growth rates (Fig. 1A, C). The experiments were repeated 5 times independently and similar results were obtained. It should be noted that the growth rate of transformants in the absence or lower concentration of Cd was slower than the others (Fig. 1B), suggesting that constitutive expression of these genes may exert some toxicity regarding yeast growth.

Sequence Analysis of the Protein Encoded by CdI19

Since CdI19 showed the highest tolerance to Cd, its full-length cDNA was isolated and characterized. 1.7kb sequence containing CdI19 ORF was obtained from the results combining the sequence of 5' and 3' RACE PCR on total cDNA (Fig. 2A). The nucleotide sequence of the CdI19 cDNA contained ORF with the potential to encode a polypeptide of 392 amino acid residues with a predicted molecular mass of 42.2 kD and an isoelectric point of 9.16(Fig. 2A). The protein was identical with a putative protein that is similar to ATFP3 farnesylated protein (protein ID = CAB83295.1) on chromosome 5.

The deduced amino acid sequence of the protein showed several interesting features which was predicted to have farnesylated (isoprenylated) motif at C-terminal (Dykema, et al. 1999) and 2 heavy metal binding regions (HMR)(Pufahl et al. 1997)(Fig. 2B). The protein is

highly hydrophilic and has a flexible region enriched with Gly between HMR and farnesyl motif. Two HMRs were shown to have βαββαβ secondary structure by prediction of protein secondary structure using SSThread (Ito et al. 1997).

Cellular localization

Farnesylation is the post-translational attachment of a hydrophobic isoprenoid. The moiety confers an essential biochemical function to proteins. To determine the cellular location of CdI19, GFP-CdI19 fusion vector (pGFP-CdI19W) driven by CaMV 35S promoter was constructed and transformed into BY2 cells. As control, transgenic BY2 strains expressing only GFP was used. For functional analysis GFP-CdI19 was constructed in which Cys-389 residue was replaced with Gly in farnesylated motif (Fig. 3). GFP fluorescence was observed by laser scanning microscopy. In cells expressing GFP alone, the entire cytoplasm except vacuole is diffusely labeled and the nucleus appears bright green, indicating that GFP enters the nucleus readily (Fig. 4A). GFP fluorescence of GFP-CdI19W was visible only at the plasma membrane. Consistent with an association of the isoprenylated fusion protein, the cells expressed GFP-CdI19M showed different fluorescence image not to localize at plasma membrane (Fig. 4B, C). Results suggest strongly CdI19 to localize at plasma membrane by its farnesylated motif.

Cd binding

Recombinant proteins containing metal binding regions of wild type (ΔCdI19W) and mutated CdI19 (ΔCdI19M) were expressed in *E-coli* and purified (Fig. 5). UV-CD spectra were measured at 25 °C. By addition of Cd, the spectra of ΔCdI19 changed strongly, indicating a complex formation between protein and Cd. Such a spectrum change was greatly reduced

with ΔCdI19M (Fig. 6). Similar spectra pattern was observed with Cu and Hg ions (Fig. 6). Mn, Co and Ca gave the less spectra change than Cd, Cu and Hg (Fig. 6, 7). These results indicate CdI19 indeed binds to metals by its CXXC motif.

Accumulation of CdI19 mRNA

Cd is proposed to displace Ca^{2+}, Cu^{2+} and Fe^{2+} in proteins (Stohs et al., 2000). Some proteins having CXXC motif were reported to bind Cu^{1+}, Hg^{2+}, or Co^{2+} (Huffman and O'Halloran, 2001; Portnoy et al., 1999; Dykema et al., 1999). In order to examine whether or not these metals also induce CdI19, northern hybridization assay was performed to the seedlings treated with them. Results showed transcripts were increased by treatments with them except Co^{2+} (Fig. 8). Interestingly, CdI19 treatments were induced by 50 μM Mn^{2+} but had no effects on CdI19 protein structure (Fig 7). Other metals Fe^{2+}, Cu^{2+} and Hg^{2+} also induced transcript accumulation but the effect was observed only at 500 μM.

Tissue specific expression

To know tissues-specific expression of CdI19, CdI19 promoter::GUS fusion transgenic Arabidopsis plants were constructed. GUS activity was analyzed in 1-week and 1-month old transformants that were treated with 500 μM Cd for 4 h prior to assay. Results showed petioles, hypocotyls, peduncles, vascular bundles and root meristems to be stained intensely (Fig. 9). Hypocotyls of 1-week old seedling especially showed a strong GUS staining (Fig. 9H). In the absence of Cd, only hypocotyl showed weak induction but other tissues, petioles, peduncles, vascular bundles and root meristems showed little GUS activity.

DISCUSSION

In this chapter, I described properties of a metal binding farnesylated protein, CdI19. It possessed two putative metal binding domains, each consisting of M/LXCXXC. Proteins containing this motif are found in groups participating in metal ion metabolism. Examples are MerP, ATX1, CCC2, CCS and Wilson's and Menkes disease copper transporter ATPase. Bacterial MerP transports Hg across the plasma membrane. ATX1, CCC2 and CCS bind to Cu and deliver it to other metalloproteins. When ATX1 binds metal ions by two cysteine sulfurs in the metal binding region, its conformation changes (Huffman and O'Halloran, 2001). Interactions between recombinant CdI19 protein and metal ions were analyzed by UV-CD spectroscopy, which gives information of protein secondary structure by changing UV-CD spectrum. Spectra of recombinant CdI19 proteins (ΔCdI19W) changed by adding Cd, Cu and Hg ions, but did not by Mn, Co and Ca. This indicates the protein to selectively bind certain metal ions. When Cys resides in metal binding region was replaced with Gly, spectrum change was greatly reduced, indicating that CdI19 protein also binds metal ions on its thiol moieties of CXXC motif.

Northern blot analysis showed that, in addition to Cd, CdI19 transcripts were induced by Hg^{2+}, Fe^{2+}, Cu^{2+} and Mn^{2+} treatments. This supports that CdI19 functions in binding these metals in vivo. However, since CD spectra did not indicate CdI19 to bind Mn, it possibly responds to limited species of metal ions to regulate the metal homeostasis.

Harmful or excessive metal ions are likely to enter cells through cation transporters of root tissues with broad substrate specificity (Thomine et al., 2000; Grotz et al., 1998). Absorbed ions, both essential and non-essential, are released into xylem sap as mineral nutrients and transferred to upper-ground parts. As I described in chapter I, root meristems are one of the most sensitive tissues to heavy metals, and to cope with damages, metallochaperons are proposed to cooperatively function to maintain concentration of metal

ions by binding and releasing them in the cell. This idea is consistent with our results of CdI19 promoter::GUS transgenics in Arabidopsis showing that, upon exposing to Cd for 4 h, GUS activity was observed petioles, hypocotyls, peduncles, vascular bundles and root meristems. It is likely that CdI19 in these tissues functions to trap metal ions and protect cells from their toxicity.

Another notable feature of CdI19 protein is farnesylation. Farnylation is one of the post-translational attachments of hydrophobic isoprenoid to proteins, and essential process in eukaryotes to confer protein biochemical functions such as membrane attachment and protein-protein interaction. Fluorescence of GFP-CdI19 fusion protein in tobacco BY2 cell demonstrated the localization of this protein on plasma membrane. Since fluorescence of mutated protein GFP-CdI19M that lack the farnesylation site was not observed on plasma membrane, it was concluded that CdI19 protein is anchored at plasma membrane through farnesylated region. A unique characteristic CdI19 is combination of transition metal binding and farnesylated motif. This indicates that CdI19 functions as a metal chaperon. Since, on introduction into yeast cells, overexpression of CdI19 rescued yeast cells against Cd stress, it is highly probable that CdI19 indeed participates in detoxification of Cd in plant cells.

More than 20 genes encoding proteins similar to CdI19 were identified in Arabidopsis genome, and some were found to encode proteins found in soybean, rice and maize. Among these proteins, ATAF3 from Arabidopsis was the only reported one to be farnesylated and to bind metals (Dykema et al., 1999). Since they are not found in yeast genome, and since ATX1 and other metallochaperons from yeast lack the conserved isoprenylation site (Himelblau et al., 1998), metal binding and farnesylated proteins may present only in plant kingdom, forming a new protein family engaging in heavy metal homeostasis and detoxification.

Finally, it is worthy to mention that transgenic plants expressing CdI19 can be used for phytoremediation of polluted environment with heavy metals. A preliminary trial with

Arabidopsis is now under process to see whether or not the gene transfer confers Cd accumulation in plant body.

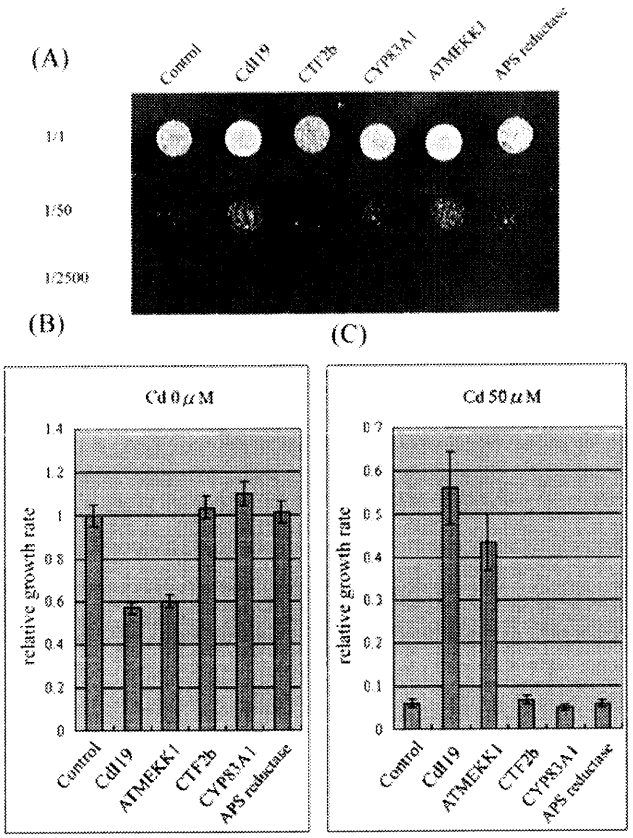


Figure 1. Growth of wild-type yeast cells expressing CdIs. Transformed cells were grown in liquid SD medium for 16 h, and absorbancy at 600 nm of culture was adjusted to 0.8 followed by dilution to 1/50 and 1/2500. A 10 µl aliquot of each culture was spotted onto a solid plate containing 50 µM Cd, and incubated at 30 °C for 3 days before photography (A). A 50 µl aliquot of these cultures adjusted to the fixed absorbancy at 600 nm was added to 3 ml liquid SG medium without (B) or with 50 µM $CdCl_2$ (C), and incubated at 30 °C for 24 h (B) or 30 h (C).

(A)

```
GTTATATCATCACCATCAAACACAT CATTAATCATACACACATAAAAAAA AGAGGGTCTATATATTCCTCATCTT GCCCTTTTTCCTTATTCTCCAAACC  -101

AGACCAAAAATATATTCTCTCGGTT TTTTCCAGAGCGTATATTCAAATTC TTCCTTAAAAAGTAAAAATTAATCT GATTCTCTCTACTACTACTACTACG    -1

ATGGGTGAGAAAAAGGAAGAAACGG CGACGAAACCTCAAGGAGAGAAGAA ACCTACCGATGGTGGAATCACCACC GTCGTTATGAAGCTTGATATGCATT   100
M  G  E  K  K  E  E  T  A    T  K  P  Q  G  E  K  K    P  T  D  G  G  I  T  T    V  V  M  K  L  D  M  H  C

GTGAAGGTTGCGGCAAAAAAATCAA ACGCATCTTCAAACATTTCAAAGGT GTGGAAGATGTGAAGATTGATTATA AGAGTAACAAATTGACGGTGATCGG   200
E  G  C  G  K  K  I  K    R  I  F  K  H  F  K  G    V  E  D  V  K  I  D  Y  K    S  N  K  L  T  V  I  G

AAACGTAGATCCAGTGGAAGTTCGT GATAAAGTGGCCGACAAAATTAAGA GACCAGTCGAACTTGTCTCTACAGT AGCGCCGCCGAAGAAAGAGACACCT   300
N  V  D  P  V  E  V  R    D  K  V  A  D  K  I  K  R    P  V  E  L  V  S  T  V    A  P  P  K  K  E  T  P

CCTTCATCAGGCGGTGCAGAGAAGA AGCCTTCTCCGGCAGCGGAGGAGAA ACCGGCTGAGAAGAAACCAGCCGCC GTTGAGAAACCCGGTGAGAAAAAAG   400
P  S  S  G  G  A  E  K  K    P  S  P  A  A  E  E  K    P  A  E  K  K  P  A  A    V  E  K  P  G  E  K  K  E

AAGAGAAGAAGAAAGAGGAAGGAGA GAAGAAAGCTTCTCCTCCACCACCA CCTAAAGAGAGTACTGTGGTTTTGA AGACCAAGTTACATTGTGAAGGTTG   500
E  K  K  K  E  E  G  E    K  K  A  S  P  P  P  P    P  K  E  S  T  V  V  L  K    T  K  L  H  C  E  G  C

CGAACACAAAATCAAAAGAATAGTC AACAAAATTAAAGGGGTTAATTCGG TTGCTATTGATAGTGCCAAGGATTT GGTTATAGTTAAGGGGATCATTGAC   600
E  H  K  I  K  R  I  V    N  K  I  K  G  V  N  S  V    A  I  D  S  A  K  D  L    V  I  V  K  G  I  I  D

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CGGCTCCAGCTGGCGGTGAGAAGAA GGACAAAGTTGCTGGTGAAAAGAAA GAGATTAAAGATGTTGGAGAAAAGA AAGTCGACGGTGGTGGTGAGAAGAA   800
A  P  A  G  G  E  K  K    D  K  V  A  G  E  K  K    E  I  K  D  V  G  E  K  K    V  D  G  G  G  E  K  K

GAAAGAAGTTGCTGTTGGCGGCGGT GGAGGAGGAGGTGGAGGTGGAGGAG ACGGTGGTGCTATGGATGTGAAGAA ATCGGAGTATAACGGTTACGGTTAT   900
K  E  V  A  V  G  G  G    G  G  G  G  G  G  G  G  D    G  G  A  M  D  V  K  K    S  E  Y  N  G  Y  G  Y

CCTCCACAGCCGATGTATTACTACC CAGAAGGACAAGTATACGGTCAACA ACATTACATGATGCAAGGTCAATCA TCGCAATCGTATGTACAAGAACCGT  1000
P  P  Q  P  M  Y  Y  Y  P    E  G  Q  V  Y  G  Q  Q    H  Y  M  M  Q  G  Q  S    S  Q  S  Y  V  Q  E  P  Y

ATTCGAACCAAGGATACGTGCAAGA ATCGTATATGAACCAAGGTTACGGT CAAGGGTATGGACAAGAAGCTCCAC CACCACCATATATGAACCAACAAGG  1100
S  N  Q  G  Y  V  Q  E    S  Y  M  N  Q  G  Y  G    Q  G  Y  G  Q  E  A  P  P    P  P  Y  M  N  Q  Q  G

TTACGCAGATCCTTATGGTCACATG CGTGCGCCTGAGTTGTTTAGTGATG AGAATCCAAATGGGTGTTCTGTGAT GTGAGTAAAACAAATGGGGAGGAAA  1200
Y  A  D  P  Y  G  H  M    R  A  P  E  L  F  S  D  E    N  P  N  G  C  S  V  M    .

ATGTAAATAACTAAAACAATATAGG GTGAGAATGTTAGCTATGACAAAAA TGGTATACTACGAAAGCACGGAAAC TTCCGAGTCCGGTAATCTAAACCGG  1300

CAAGATTGGCCGGTTAGAACCGGTT TTTGAGAGGCGTGGATTGGTTGGTT GGTTTATTTCTTCTATATAGGTACT ATAATTTTGTATCGTAATAATCTTC  1400
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(B)

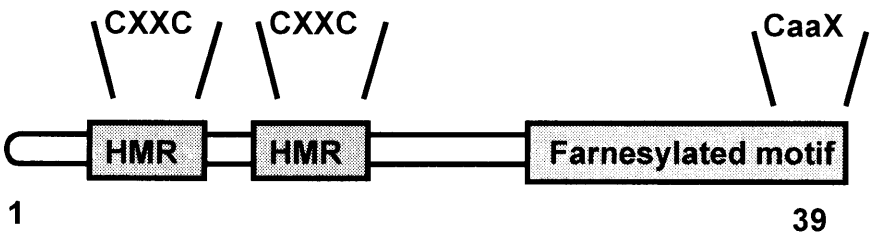

Figure 2. Sequence of the CdI19 cDNA (A) and schematic illustrating of CdI19 protein (B). CdI19 protein has two metal binding regions and farnesyl motif. The underlines are TATA box, core sequence of metal binding regions (C is cysteine and X is any amino acid) and farnesylated motif (a is aliphatic amino acid and X is the carboxy-terminal residue).

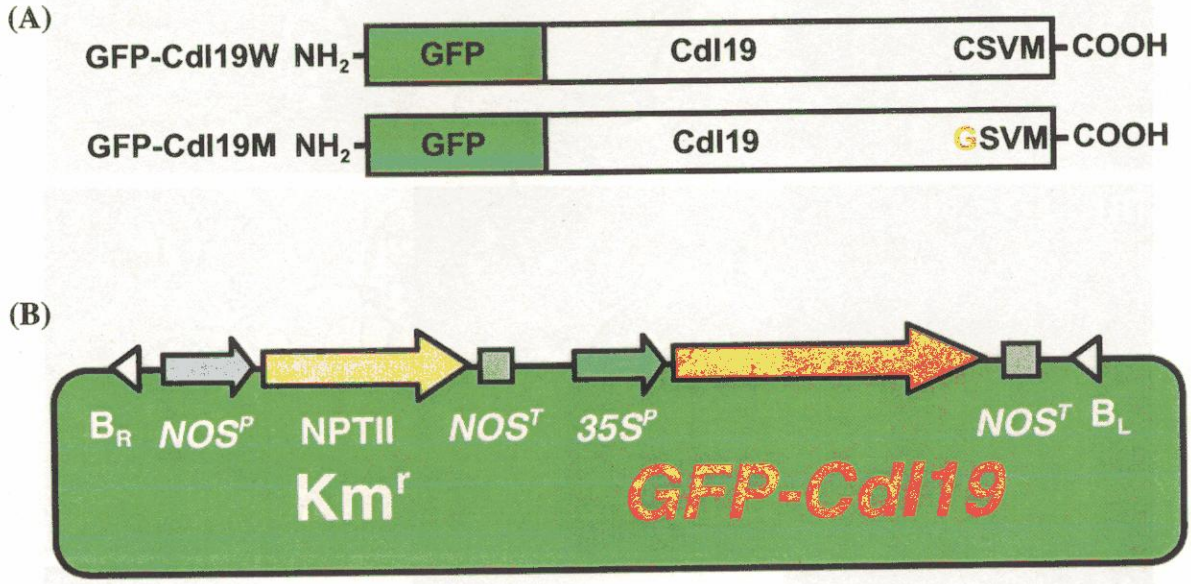

Figure 3. Schematic diagrams of GFP-CdI19 fusion proteins (A) and pGFP-CdI19 vector (B). Mutant protein was displaced Cys-389 with Gly. GFP-CdI19 fusion was driven by CaMV 35S promoter.

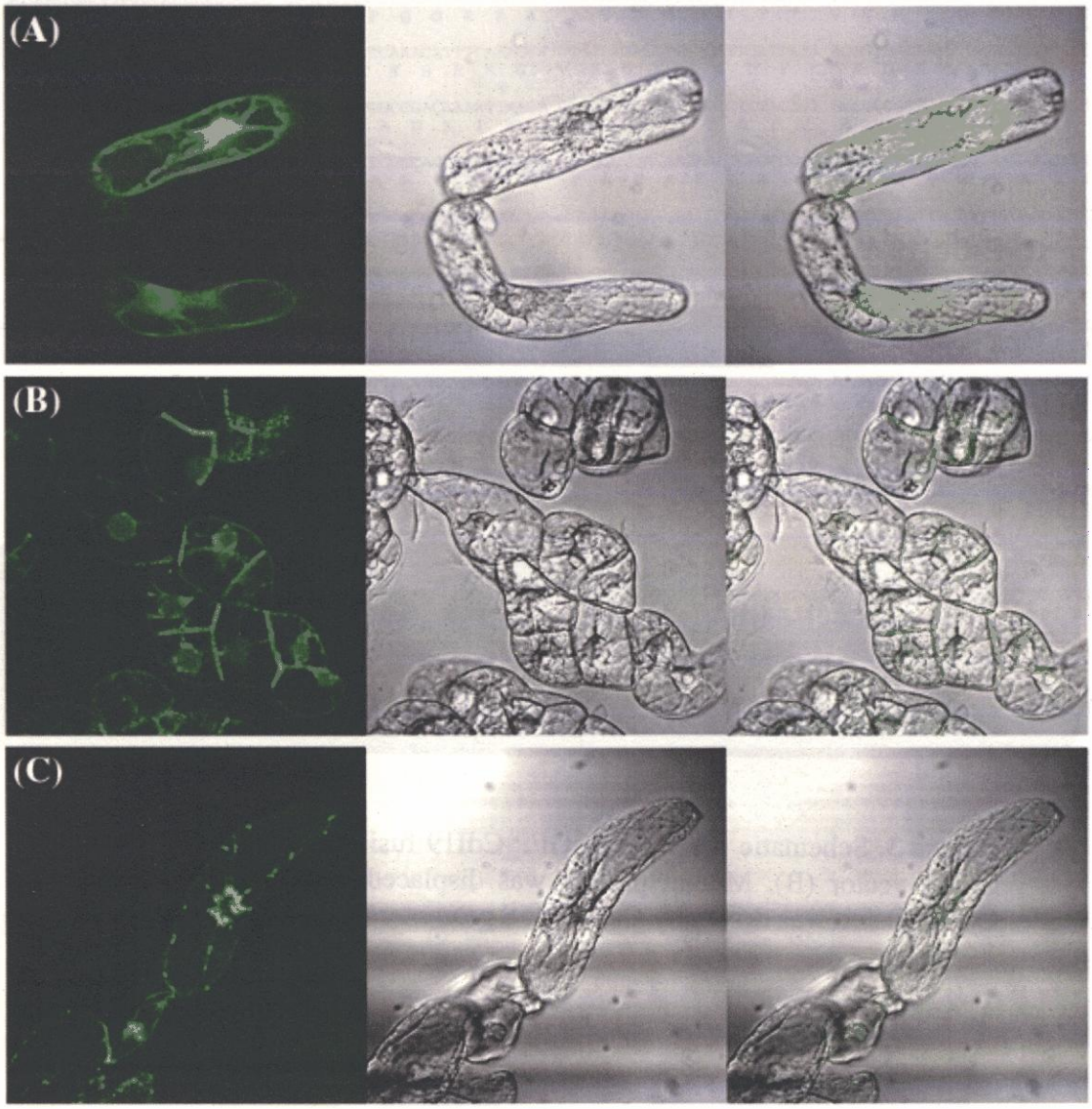

Figure 4. Cellular localization of GFP-CdI19. BY2 cells express GFP alone (A), GFP fused CdI19 (B), GFP fused mutated CdI19 (C). The fluorescence was obtained with a confocal laser-scanning microscope. Images are fluorescence, normal and overlapping ones of both images.

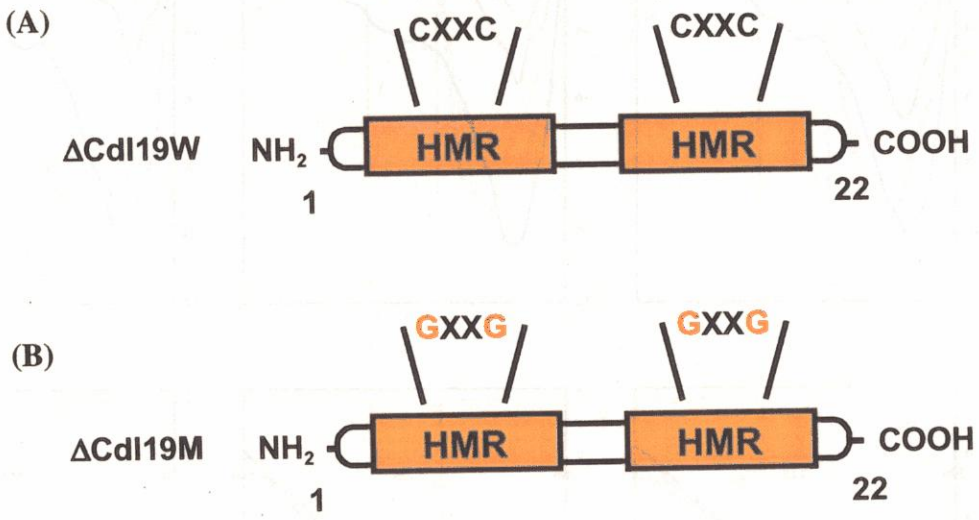

Figure 5. Illustration of recombinant CdI19 proteins containing metal binding regions of wild type (A, ΔCdI19W) and mutant (B, ΔCdI19M). ΔCdI19M was displaced Cys with Gly in metal binding regions. Proteins were expressed in E. coli.

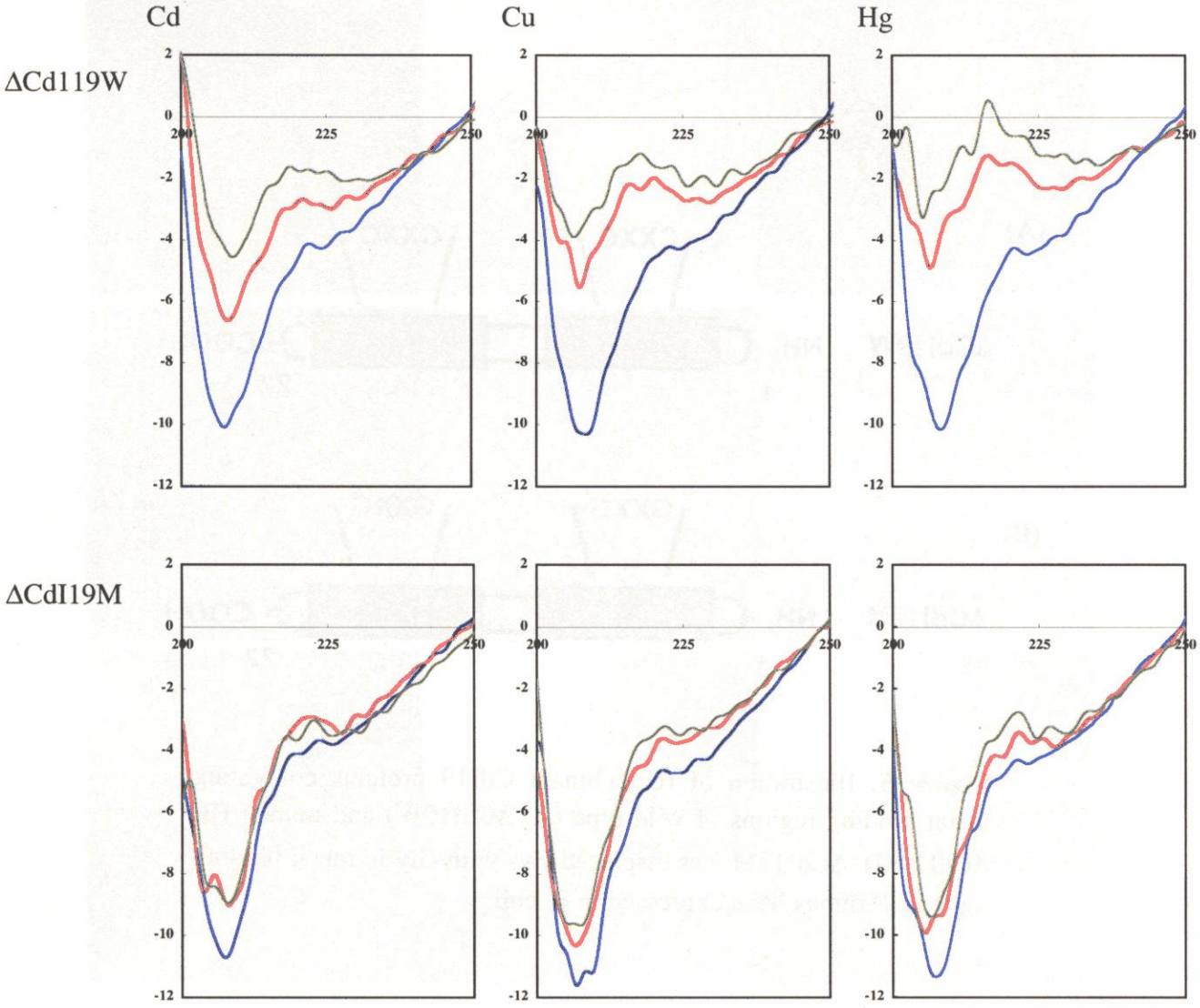

Figure .6 Circular dichroism spectra of recombinant protein, ΔCdI19W and ΔCdI19M with Cd, Cu and Hg. Each metal ions was added to 30 μM protein solution. Final concentration of metal ions was 0 (blue), 300 (red) and 500 (green) μM. X, Y axis is wavelenght (nm) and Θ (mdeg), respectively.

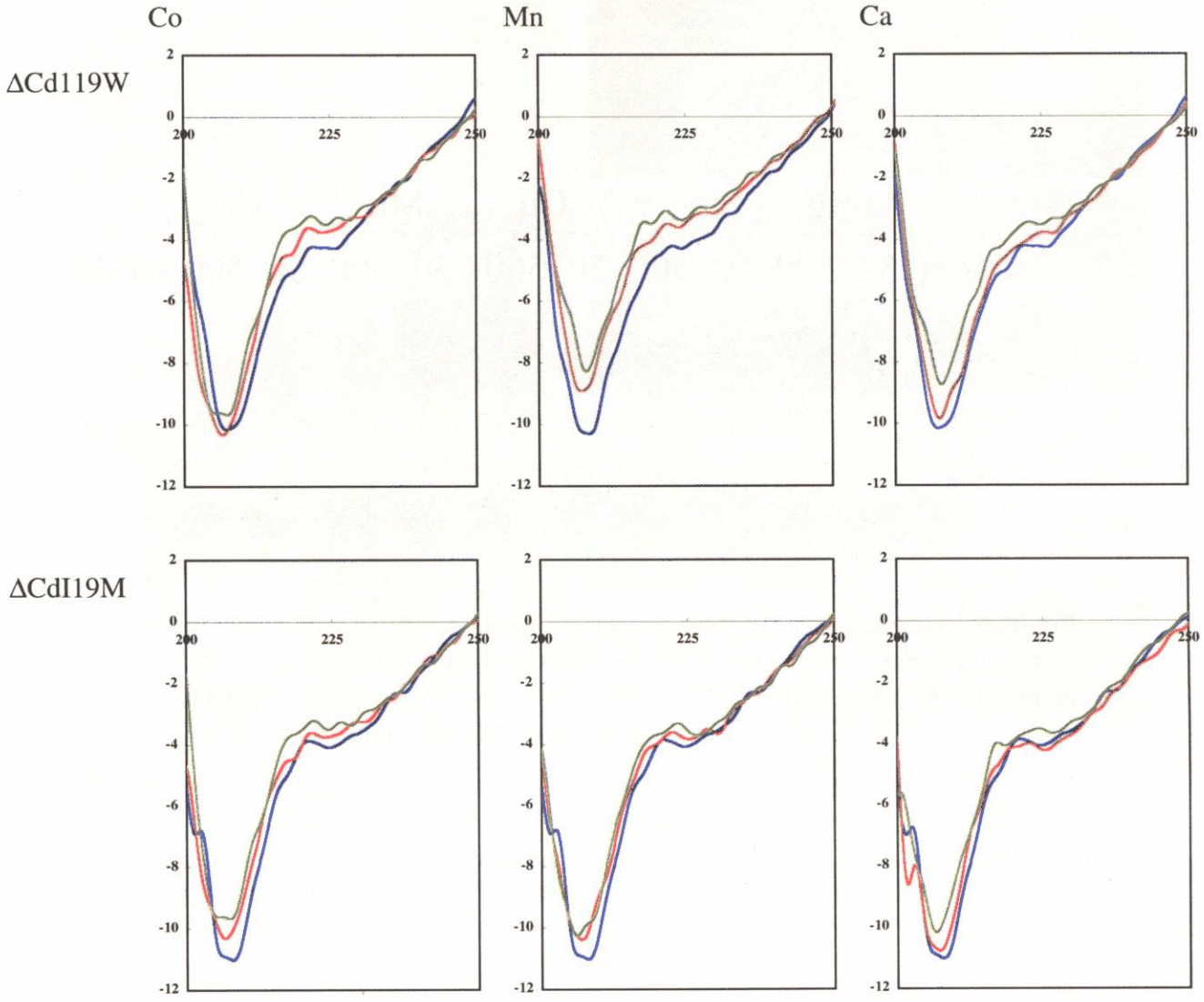

Figure 7. Circular dichroism spectra of recombinant protein, ΔCdI19W and ΔCdI19M with Co, Mn and Ca. Each metal ions was added to 30 μM protein solution. Final concentration of metal ions was 0 (blue), 300 (red) and 500 (green) μM. X, Y axis is wavelenght (nm) and Θ (mdeg), respectively.

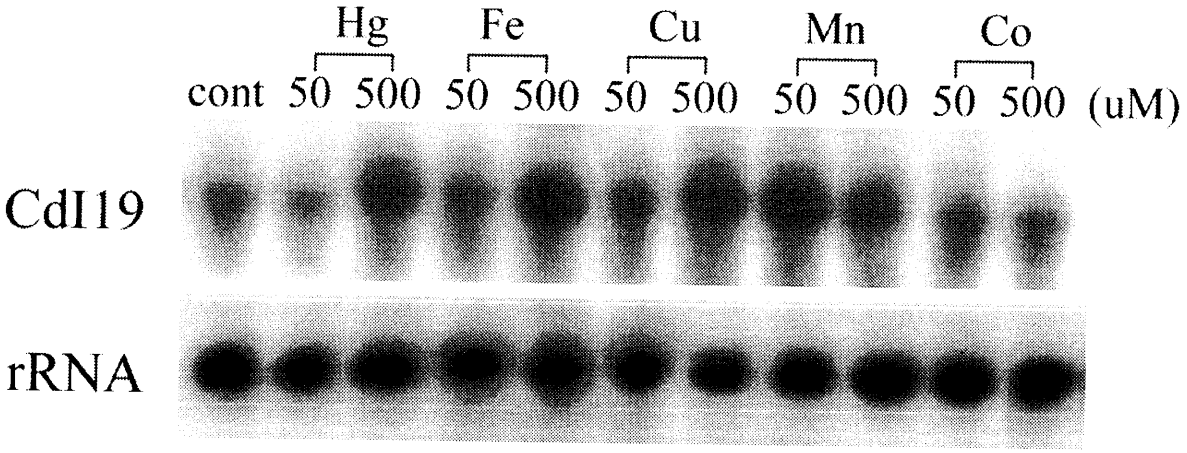

Figure 8. Accumulation of CdI19 transcripts upon treatments with heavy metals. 2-week-old plants were subjected to indicated concentration of metal ion treatments for 4 h. Total RNA (20μg) was extracted, size-fractionated by electrophoresis and subjected to northern hybridization analysis. 5S rRNA was used for standard of RNA loading.

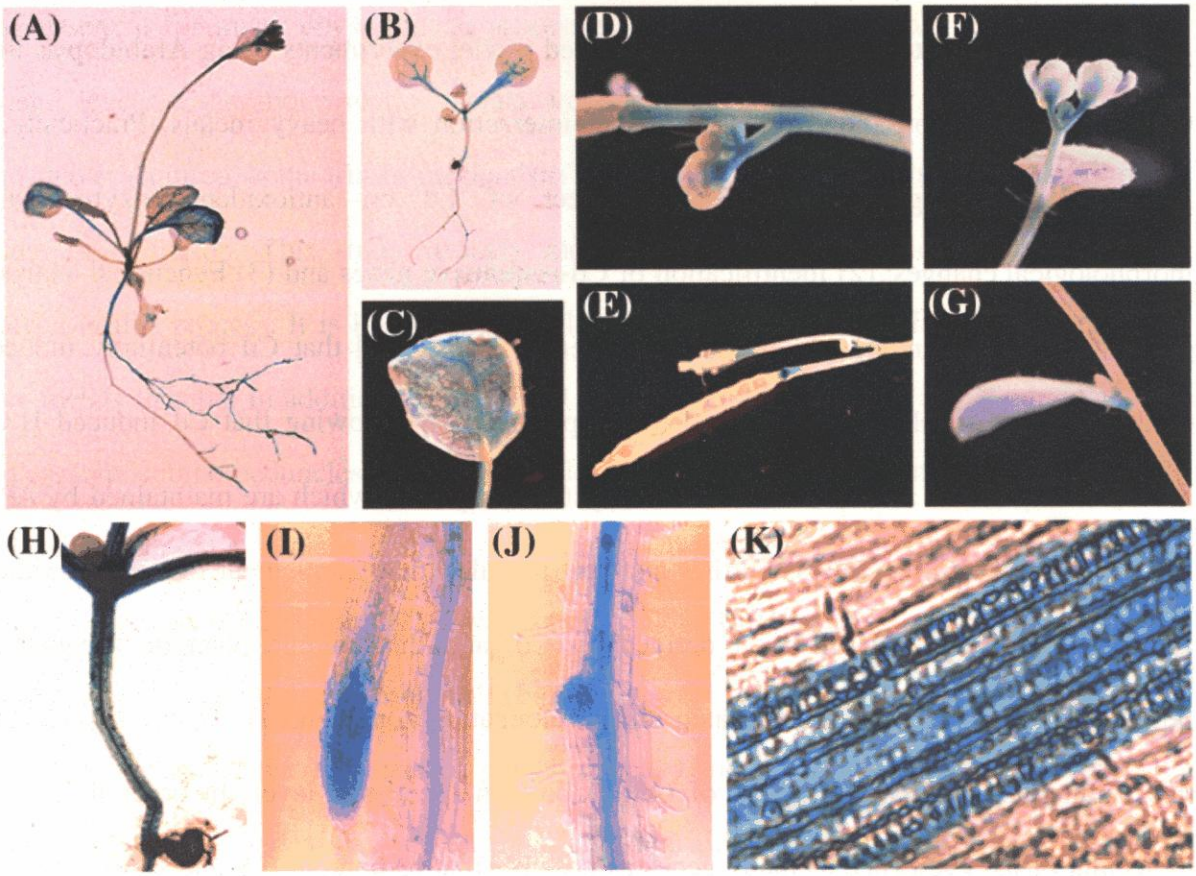

Figure 9. Histochemical localization of GUS activity in transgenic *Arabidopsis* expressing CdI19 promoter::GUS fusion gene. Plants were treated with 500 μM for 4 h prior to assay. A and C trough G are 1-month-old, B and H trough K are 1-week-old plant. A and B, whole plant; C, leaf; D, E and F, peduncle; G, petiole; H, hypocotyls; I and J, root meristems and vascular bundles; K, vascular bundles of mature root.

Concluding Remarks

The long-range aim of my study is to create plants that can remediate heavy metal polluted soil and water. To this end, I conducted model experiments using Arabidopsis and Cd, and found various new aspects of plant interaction with heavy metals. Practically, I performed following experiments. (1) Effect of Cd on antioxidant enzymes and morphological changes; (2) Identification of Cd-responsive genes and (3) Functional analysis of a heavy metal binding protein. It has generally considered that Cd potentially induces oxidative stress in plants. I confirmed this experimentally, showing that Cd induced H_2O_2 accumulation due to an imbalance of redox systems, a part of which are maintained by APX and SOD enzymatic activities. Further analysis revealed that supplement of an antioxidant agent reduced H_2O_2 accumulation derived by Cd, and that soluble phenolic substances, possibly antioxidant, were accumulated in root elongation zone (Fig. 1). To my knowledge, this finding is the first clear case of Cd toxicity on the tissue level. In order to see its molecular basis, I screened as many genes as possible that respond to Cd, and confirmed that 31 genes showed changes in their transcript population under Cd stress, and that 15 responded not only to Cd but also to oxidative stress. These findings support strongly the idea that Cd toxicity is primarily caused by oxidative stress (Fig. 1).

However, I found Cd stress to be much more complicated. Although oxidative stress is one of the serious components of Cd stress, many others such as protein denaturation should be considered. For example, decrease of enzyme activity by displacing Ca^{2+}, Zn^{2+} and Fe^{2+} with Cd in proteins looks to be serious. Indeed, inhibition of root elongation was not restored by reduction of oxidative stress, but by excess Ca ions. These results suggest that, in order to protect molecular machinery and to give high Cd tolerance to plants, it is important to reduce dissolved and free Cd^{2+} ions that are incorporated into plants rather than to directly depress

the toxicity derived from Cd.

In this context, the function of CdI19 protein was of interest. The CdI19 protein probably localizes on plasma membrane and binds specifically some heavy metals. Despite being essential for many physiological processes, particular heavy metals are also toxic at elevated levels. Other nonessential metals are potentially highly toxic. Livings including plants have acquired homeostasis mechanism to minimize heavy metals damages from in process of evolution. This study strongly indicated that CdI19 in one of such proteins involved in this process. It is known that similar proteins function to deliver and to remove heavy metals in cells. In addition to Cd, CdI19 readily binds Cu and Hg, and accumulates in petioles, hypocotyls, peduncles, vascular bundles and root meristems. Since CdI19 localizes at plasma membrane, which essential biochemical reactions are undertaken, and where harmful metal ions initially trapped. It was concluded that CdI19 plays a critical role in metal ion transport and sequestration within cell (Fig. 1).

Study on molecular response of plants to heavy metal ions is important, because it provides not only understanding of the inorganic nutrition metabolism in plants, but also possible technology to create transgenic plants for phytoremediation. Yet the system involved in metal transport, sequestration and tolerance in plant and other organisms is beginning to be understood. It is my hope that present work will be of help to advance the knowledge in this field.

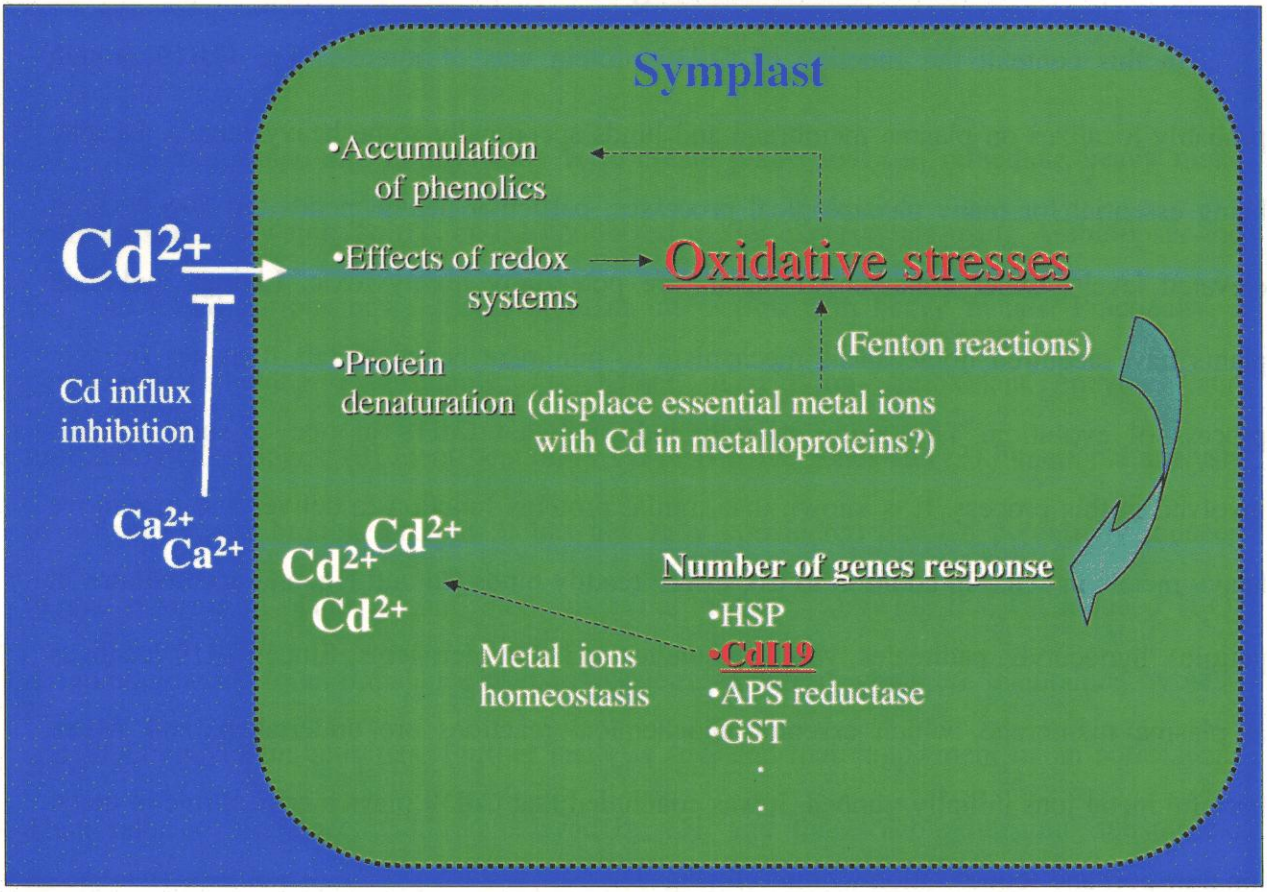

Figure 1. Model illustration of Cd effects in plants. Cd is transported into plant cells through Ca channels located on plasma membrane. Excess Ca may interfere unfavorable Cd uptake. In cells, Cd induces oxidative stress due to an imbalance of redox systems, protein denaturation and accumulation of soluble phenolic substances, possibly antioxidant. Protein denaturation is presumably due to displacing Ca^{2+}, Zn^{2+} and Fe^{2+} with Cd in proteins,which can also generate oxidative stress. To cope with such stresses, plants activate a set of genes. CdI19 is one of such genes. It readily binds heavy metal ions, localizes at plasma membrane and plays a critical role in metal ion transport and sequestration within cell.

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SUZUKI Nobuaki, March, 2002

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Appendix

List of publication

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鈴木伸昭　佐野浩（2002年出版予定）「重金属汚染土壌　植物による汚染物質の除去、そのメカニズム」　植物代謝工学ハンドブック（新名惇彦・奈良先端科学技術大学院大学教授監修）第6章2節 株式会社エヌ・ティー・エス.