Cloning Heavy Metal Tolerance Genes

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ABSTRACT

Engineering nonfood plants to mine heavy metals from contaminated sites offers an economical solution for remediation. However, plants generally have a low tolerance for these toxins. That is the reason why few plants can thrive near mine sites. To enhance the ability of plant to populate toxic sites, we sought to define the genes involved in heavy metal tolerance. This work began with two complementary approaches: 1) to clone genes that complement cadmium hypersensitive mutants of the fission yeast *Schizosaccharyomes pombe*; and 2) to isolate genes that confer cadmium hypertolerance to wild type *S. pombe*. Through this second approach, we have identified a set of genes that confer enhanced tolerance to metals and/or oxidative stress. A few of these genes have been engineered into *Arabidopsis thaliana*. The plants carrying the tolerance genes are more resistant to heavy metals and oxidizing agents.

INTRODUCTION

Heavy metals and radionuclides are persistent environment contaminants. Conversion to a biologically inert form is possible for only a few of the toxic elements. For most of these pollutants, effective remediation requires the physical extraction from soil and water systems. Since conventional engineering technologies are prohibitively expensive, a biological approach has been proposed. A wide variety of bacterial, fungal, algal and plant systems are capable of concentrating toxic metals from soils or waters. However, no cost-effective way exists to retrieve small organisms from the soil. On the other hand, contaminant-laden plants are easy to harvest, especially if the metals accumulate in shoots. Metals may be reclaimed or disposed of after concentrating the plant biomass.

Phytoextraction is conceptually simple, but plants generally do not accumulate large amounts of toxic metals and radionuclides. Although plant uptake of inorganics is a prerequisite, increasing metal uptake is <u>not in itself a sustainable solution</u>. The plants that take up a high amount of metals lack the mechanisms to tolerate the higher metal content. The sparse vegetation of toxic mine sites is consistent with plants capable of metal uptake but inadequate in metal detoxification. The few plants that populate mine sites have evolved new mechanisms that either exclude or tolerate the toxic metals. As the key for phytoextraction technology is to overcome the inability of plants to detoxify high metal content, we sought to find the genes that can confer metal hypertolerance.

RESEARCH PROGRESS

Approach #1: We cloned and characterized several genes that complement a number of Cd hypersensitive mutants of *Schizosaccharomyces pombe*. This fission yeast was chosen as a model organism as it shares similar Cd sequestration mechanisms with higher plants. The rationale for this strategy was that by defining the adaptive mechanisms responsible for metal tolerance, insight would be gained for the genetic manipulation of metal tolerance pathways. The research progress on these genes has been described in last year's report.

Approach #2: This strategy began with the shotgun transformation of cDNA libraries into fission yeast, with the intent of isolating genes that when hyperexpressed from a multi-copy plasmid, can confer

high level tolerance to a wild type strain. Initially, we transformed a cDNA library made from *S. pombe* into wild type cells and screened for Cd tolerance. This allowed us to refine the screening protocol prior to testing heterologous cDNA libraries. Table 1 lists 18 cDNA clones from *S. pombe* that confer hypertolerance to Cd. Only two of them are from pathways that are in metal detoxification. A large number of them fall into biosynthetic or regulatory pathways. Some are novel proteins with domains that could have similarity to known proteins. Half of the cDNAs in this list can also confer hypertolerance to oxidative stress.

We subsequently screened plant libraries made from *Arabidopsis thaliana*, whose complete genome sequence will be publicly available soon, and *Brassica juncea*, a known metal hyperaccumulator. *B. juncea* and *Arabidopsis* are in the same family of plants, so we expect *B. juncea* genes to be quite homologous to those available in the *Arabidopsis* genome database. Table 2 lists 6 independent clones from *B. juncea*, and 2 from *A. thaliana*. Again, many of these genes were subsequently found to also confer resistance to oxidative stress agents, as well as to other metals. Figure 1 exemplifies a cDNA that confers hypertolerance to metals and oxidative stress. Like the *hmt1*$^+$ cDNA that codes for a vacuolar transporter of phytochelatins, Bj6, isolated from *B. juncea* (Table 2) is as tolerant to Cd. Unlike *hmt1*$^+$, however, it also confers resistance to diamide. Since oxidative damage commonly occurs upon exposure to heavy metals and radionuclides, we also broadened our search to isolate plant genes that confer resistance to oxidizing agents. From the *B. juncea* library, 25 cDNAs have been isolated (Tables 3a, 3b). So far, we have not yet tested these clones for cross-resistance to toxic metals.

Expression in plants. The collection of cDNAs described in Tables 1, 2, 3a, and 3b is currently being introduced into *Arabidopsis*. Figure 2 outlines the experimental plan. T_1 progeny will be subjected to the initial testing. The T_1 progeny is a segregating population that may or may not harbor the transgene (denoted TG). The genotype of the T_1 plants will be reveal only upon examination of the T_2 progeny, at which these plants can be retested. Extensive testing will be possible after bulking up T_3 seed from homozygous T_2 lines.

To date, we have conducted the initial testing on a few lines. Bj6 and Bj4 (Table 2) represent two distinct members of the same gene family, as their sequences share extensive homology but are not identical. In these tests, multiple T_1 plants from independent transgenic lines were used. Due to the differences in genome location and pattern of DNA integration, gene expression could vary among different transgenic lines. Hence, some differences among the lines would be expected. In addition, the T_1 plants are derived from self-fertilized T_0 (primary transformant) plants, which means that the transgenes could be in either a hemizygous or a homozygous state. Despite these possibilities of line to line variation and gene dosage effects, the class of Bj4 and Bj6 plants showed consistent differences compared to the class of empty vector control plants.

Late flowering. Independently derived transgenic lines of Bj6 and Bj4 flowered far later than the 5 independent lines of the empty vector control. Figure 3 exemplifies this observation, in which the Bj4 and Bj6 plants had not yet flowered compared with the 5 rows of control plants whose flowering stalks crowd into the Bj4 and Bj6 plants. Each row represents an independent transgenic line. As plants tend to flower earlier when stressed, this could indicate that the transgenic lines are more stress tolerant. Consistent with this interpretation, subjecting plants to severe stress (beyond the protective capacity of plants) causes all sickly plants to flower synchronously (such as 1 mM diamide).

Metal and oxidative stress. Figures 4 -7 show 43 day-old seedlings that had been treated with the designated toxin for the previous 26 days. Each figure shows a representative empty vector control plant (C) and two representatives of each of Bj4 and Bj6 plants retrieved from hydroponic growth. In the presence of added Cd (Figure 4) or Cu (Figure 5), there are indications of enhanced tolerance in Bj4 and Bj6 plants. They grow slightly better than the control plants, but a clear difference is less certain. This

may be due to Cd and Cu concentrations that are too high, where the toxic effects overwhelm all plants. Further tests are underway with various metal concentrations. In contrast, there is a noticeable difference with the zinc concentration tested. Figure 6 shows Bj4 and Bj6 plants with greater biomass in both the roots and the aerial parts. Bj4 and Bj6 showed different degrees of enhanced tolerance when grown with added diamide. The most noticeable difference is seen between Bj4 and the empty vector control. Figure 7 shows that root and aerial biomass is significantly enhanced in the Bj4 transgenic plants, and slightly enhanced in Bj6 lines.

Larger plants. We have not completed our test on At11 (Table 2). An interesting observation, however, is that the T_0 plants in the greenhouse are unusually large. A clear interpretation is not yet possible, but it is a sign of healthy plants, and would be consistent with a higher level of stress tolerance.

Bj4/Bj6 not inducible by Cd. A popular approach in functional genomics is the use of high throughput methods to detect the appearance and disappearance of mRNA species. This technology has led to the assumption that mRNA abundance would indicate function. Surprisingly, this correlation is not found with Bj4 / Bj6. Using Bj6 as a hybridization probe, which cross hybridizes with Bj4, we failed to detect a difference in steady-state mRNA in the presence or absence of Cd (Figure 8). This indicates that Bj4 / Bj6 would not have been discovered by methods that monitor mRNA abundance.

IMPLICATIONS

The ability to engineer plants to tolerate the toxic effects of heavy metals and the associated oxidative stress is a prerequisite for re-vegetating toxic waste sites, and for phytoextraction or phytostabilization of the contaminants of concern. We have only begun to examine the first few clones from our cDNA collection. It is likely that some of these clones will not work in a heterologous system. For example, in previous attempts, the *hmt1* gene product (vacuolar transporter of PC-Cd) was not detectable in our transgenic tobacco plants. On the other hand, we are confident that a hypertolerance phenotype will be conferred by a good percentage of these genes, as they were functionally selected for this trait. If this proves true, it would be important to expand our functional tests to other model plants. In that regard, this laboratory has extensive experience in constructing transgenic plants including tobacco, maize, rice, wheat and cotton.

We are also expanding our testing into animal cell lines. Our concerned with Cd and other oxidatives stress agents is because of their toxicity to humans. Cd and other toxic metals interfere with many cellular enzymatic activities. They also induce oxidative stress by forming reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^\cdot$) that oxidize DNA, proteins, lipids, and carbohydrates. Similar to Cd, radionuclides also cause oxidative damage. If the expression of certain cDNAs can alleviate this stress, we may be able to uncover new genetic mechanisms that protect animal cells from harmful effects of toxic metals and radionuclides.

INFORMATION ACCESS

Cunningham, S., Ow, D.W. (1996) Promises and prospects of phytoremediation. *Plant Physiology* 110 : 715-719.

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Perego, P, VandeWeghe, J, Ow, DW, Howell, SB (1997) The role of determinants of cadmium sensitivity in the tolerance of *Schizosaccharomyces pombe* to cisplatin. *Molecular Pharmacology* 51 : 12-18.

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VandeWeghe, J., Ow, DW (1999) A fission yeast gene for mitochondrial sulfide oxidation. *Journal of Biological Chemistry* 274:13250-13257.

Table 1. *S. pombe* clones conferring Cd-hypertolerance in *S. pombe*
(Yes = enhanced tolerance; No = no effect)

Relevance	Gene	Potential role	Cd	Diamide	*tb*-HOOH	Status
Metal Detoxification	4L1	γ-Glutamyl-cysteine synthase	YES	NO	NO	Selecting T_0 transformants
	1A1	Cu/Cd metallothionein	YES	NO	YES	Also Cu^R T_1 hydroponics test
Signal Transduction	4L4	Ca binding	YES	NO	NO	T_0 seeds
	4B4	Signal transduction	YES	YES	NO	T_0 seeds
Transcription & translation	4F4A	Transcription factor	YES	YES	YES	T_0 seeds
	3A1	Translation protein	YES	YES	NO	T_0 seeds
	4E2C	Translation protein (may titrate Cd)	YES	NO	NO	Not yet in agro vector
Biosynthetic Pathways	1F2	Antioxidant synthesis	YES	NO	NO	Selecting T_0 transformants
	1D1	Pentose phosphate pathway	YES	NO	NO	T_0 seeds
	4B2	Pentose phosphate pathway	YES	NO	NO	T_0 seeds
	4G2	Peroxisome function	YES	YES	YES	T_0 seeds
	4J1	Oxidoreductase?	YES	NO	YES	T_0 seeds
Unknown	3B4	TCA cycle intermediate synthesis?	YES	NO	NO	Not yet in agro vector
	2A1	Translation role?	YES	NO	NO	T_0 seeds
	1F1	Unknown	YES	NO	NO	T_1 seed
	4J5	Heat-shock role?	YES	YES	YES	T_0 seeds
	4K1D	no match in database	YES	YES	YES	Selecting T_0 transformants
	4K3A	no match in database	YES	YES	YES	T_0 seeds

Table 2. 6 independent *B. juncea* (Bj) and 2 *A. thaliana* (At) cDNAs that confer Cd hypertolerance in *S. pombe.*
(Yes = enhanced tolerance; No = no effect)

Relevance	**Gene**	**Potential role**	**Cd**	**Cu**	**Zn**	**Diamide**	**Status**
Novel	Bj3	Unknown	YES	YES	YES	YES	T_1 hydroponics test T_2 seeds, 3 lines
Novel	Bj4	Unknown, Same as Bj8	YES	YES	YES	YES	T_1 hydroponics test T_i plants Cd^R, Cu^R, Zn^R $diamide^R$ **slow to flower**. T_2 seeds, 4 lines
Novel	Bj8	Same as Bj4	YES	YES	YES	YES	T_1 hydroponics test **slow to flower**
Novel	Bj6	Unknown, identical to Bj1 (not shown)	YES	YES	YES	YES	T_1 hydroponics test T_i plants Cd^R, Cu^R, Zn^R $diamide^R$ **slow to flower** T_2 seeds, 5 lines
Oxido- reductase	Bj7	Unknown, several possibilities	YES	?	?	?	T_1 hydroponics test
Novel	Bj11	Unknown	YES	?	?	?	T_1 hydroponics test
Novel	Bj12	Unknown	YES	?	?	?	T_0 seeds
Novel	At3	DNA-binding?	YES	YES	YES	YES	T_1 hydroponics test
Novel	At11	Unknown	YES	?	?	?	T_1 hydroponics test T_0 seeds **T_0 plants larger**

Table 3a. *B. juncea* cDNAs (BDT) conferring oxidative-stress hypertolerance in *S. pombe* (13 of 25).
(Yes = tolerance confered to yeast; No = no effect)

Relevance	**Gene**	**Potential role**	**Diamide**	**Status**
Unknown	BDT1	sugar metabolism	YES fast	Further analyses in yeast In Agro vector, to transform
Unknown	BDT2	needs additional sequencing	YES slow?	Further analyses in yeast
ROS detoxification signaling	BDT3	oxidoreductase	YES	Further analyses in yeast
Unknown	BDT5	sugar metabolism; antisense	YES	Further analyses in yeast T_0 seeds
Unknown	BDT29	Homologous to drought	YES fast	Further analyses in yeast T_0 seeds
Unknown	BDT30	Novel	YES	Further analyses in yeast T_0 seeds
Unknown	BDT33	transaminase	YES	Further analyses in yeast T_0 seeds
Unknown	BDT36	synthesis of methionine derivative	YES	Further analyses in yeast In Agro vector, to transform
Unknown	BDT37	Novel	YES fast ?	Further analyses in yeast T_0 seeds
Unknown	BDT40	needs additional sequencing	YES / ?	Further analyses in yeast
Senescence, Metal status	BDT41	MT-like	YES slow?	Further analyses in yeast
Translation	BDT42	similar to a translation protein	YES	Further analyses in yeast
Pentose phosphate pathway	BDT43	Dehydrogenase	YES	Further analyses in yeast

Table 3b. *B. juncea* cDNAs (BDT) conferring oxidative-stress hypertolerance in *S. pombe* (12 of 25).
(Yes = tolerance confered to yeast; No = no effect)

Relevance	**Gene**	**Potential role**	**Diamide**	**Status**
Unknown	BDT44	Novel	YES fast	Further analyses in yeast
Unknown	BDT45	Possible pathogen defense signal	YES	Further analyses in yeast
Unknown	BDT47	no cDNA insert or too small to detect in restriction digests	YES slow?	Further analyses in yeast
Unknown	BDT48	needs additional sequencing	YES slow?	Further analyses in yeast
Nitrogen metabolism	BDT49	N assimilation	YES	Further analyses in yeast
Unknown	BDT50	Homologous to a novel protein in *Arabidopsis*	YES	Further analyses in yeast
Unknown	BDT51	Vacuolar function	YES fast	Further analyses in yeast In Agro vector, to transform
Unknown	BDT52	needs additional sequencing	YES	Further analyses in yeast
Unknown	BDT53	needs additional sequencing	YES slow?	Further analyses in yeast
Radiation damage ?	BDT54	ribonucleoside metabolism	YES	Further analyses in yeast In Agro vector, to transform
Unknown	BDT55	homologous to 2 proteins with unknown functions	YES fast	Further analyses in yeast
Unknown	BDT56	needs additional sequencing	YES	Further analyses in yeast

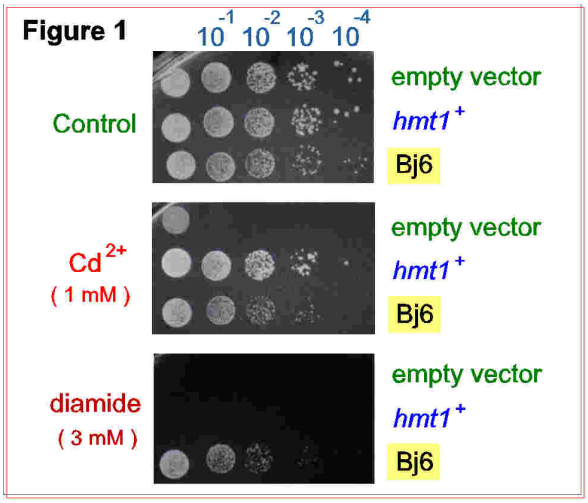


Figure 1
10^-1 10^-2 10^-3 10^-4
Control
empty vector
hmt1+
Bj6
Cd2+
(1 mM)
empty vector
hmt1+
Bj6
diamide
(3 mM)
empty vector
hmt1+
Bj6

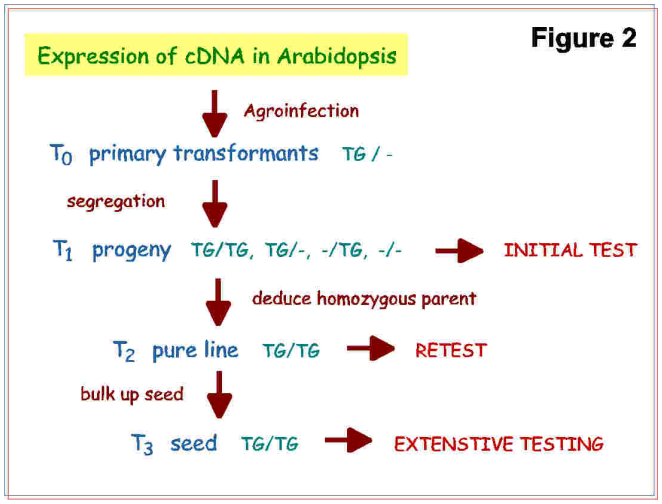


Figure 2
Expression of cDNA in Arabidopsis
Agroinfection
T0 primary transformants TG / -
segregation
T1 progeny TG/TG, TG/-, -/TG, -/-
INITIAL TEST
deduce homozygous parent
T2 pure line TG/TG
RETEST
bulk up seed
T3 seed TG/TG
EXTENSTIVE TESTING

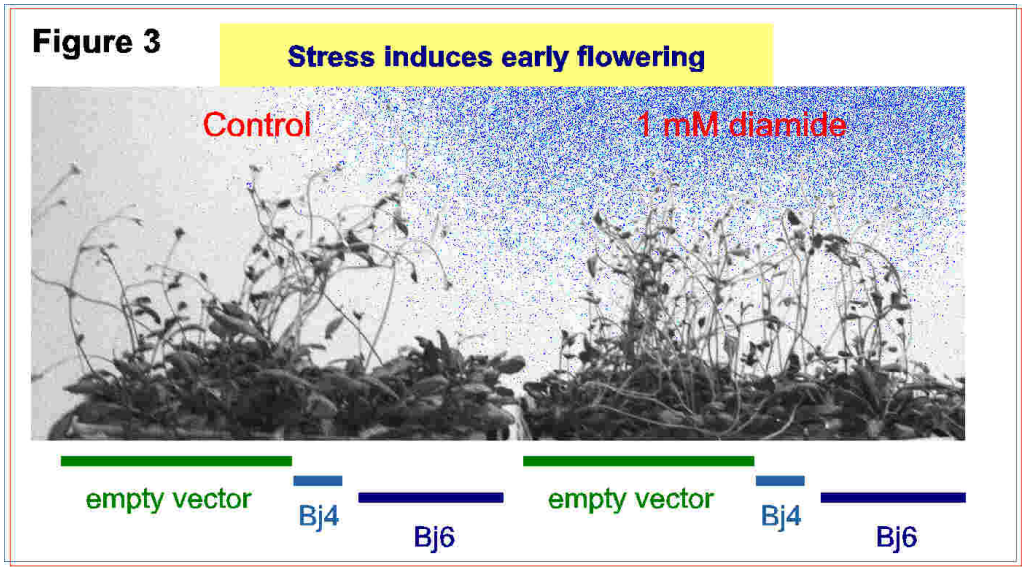
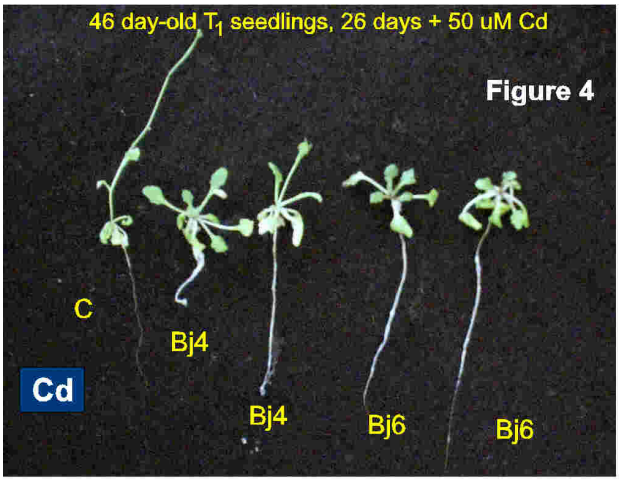
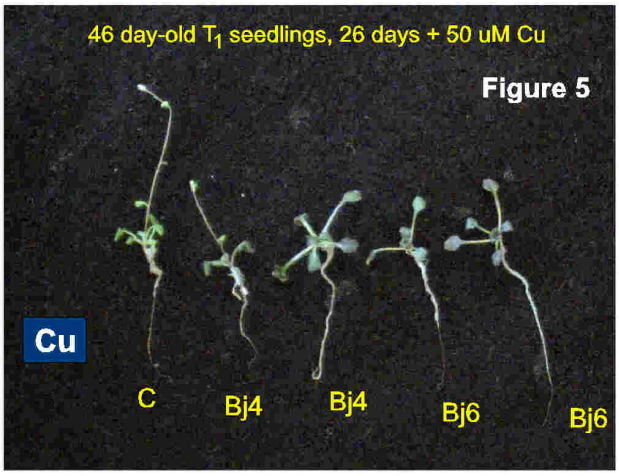
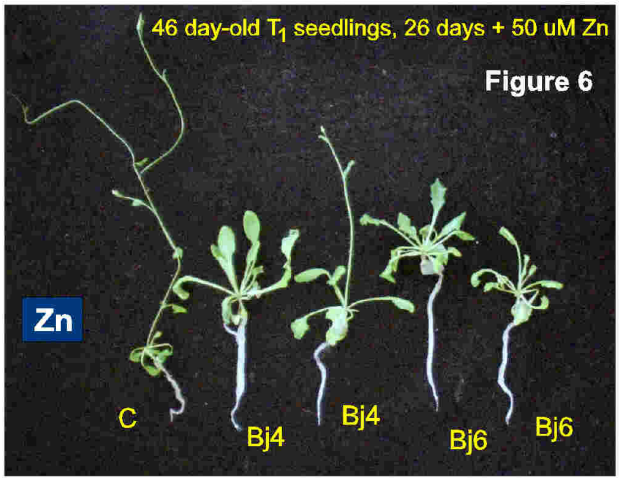
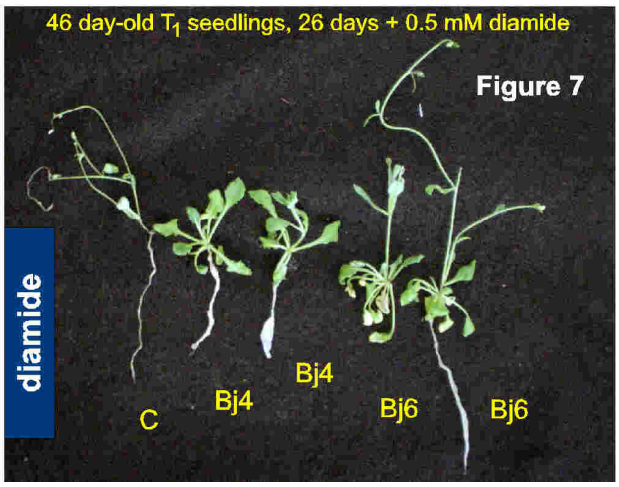


Figure 3
Stress induces early flowering
Control
1 mM diamide
empty vector
Bj4
Bj6
empty vector
Bj4
Bj6


46 day-old T_1 seedlings, 26 days + 50 uM Cd
Figure 4
C
Bj4
Cd
Bj4
Bj6
Bj6


46 day-old T_1 seedlings, 26 days + 50 uM Cu
Figure 5
Cu
C
Bj4
Bj4
Bj6
Bj6


46 day-old T_1 seedlings, 26 days + 50 uM Zn
Figure 6
Zn
C
Bj4
Bj4
Bj6
Bj6


46 day-old T_1 seedlings, 26 days + 0.5 mM diamide
Figure 7
diamide
C
Bj4
Bj4
Bj6
Bj6

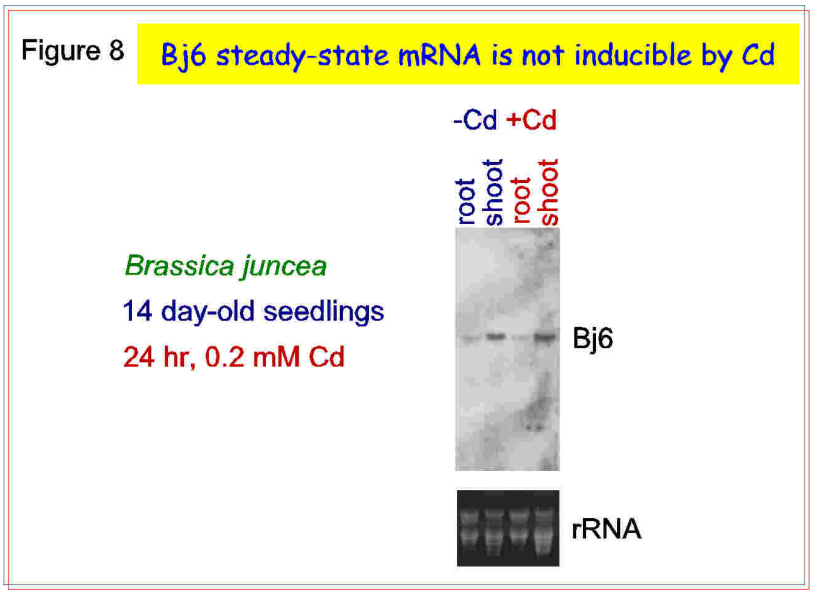


Figure 8
Bj6 steady-state mRNA is not inducible by Cd
-Cd +Cd
root
shoot
root
shoot
Brassica juncea
14 day-old seedlings
24 hr, 0.2 mM Cd
Bj6
rRNA