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# Glutathione Metabolic Genes Coordinately Respond to Heavy Metals and Jasmonic Acid in Arabidopsis

**Chengbin Xiang and David J. Oliver[1]**

Department of Botany, Iowa State University, Ames, Iowa 50011-1020

**Glutathione plays a pivotal role in protecting plants from environmental stresses, oxidative stress, xenobiotics, and some heavy metals. Arabidopsis plants treated with cadmium or copper responded by increasing transcription of the genes for glutathione synthesis, γ-glutamylcysteine synthetase and glutathione synthetase, as well as glutathione reductase. The response was specific for those metals whose toxicity is thought to be mitigated through phytochelatins, and other toxic and nontoxic metals did not alter mRNA levels. Feeding experiments suggested that neither oxidative stress, as results from exposure to $H_2O_2$, nor oxidized or reduced glutathione levels were responsible for activating transcription of these genes. Jasmonic acid also activated the same suite of genes, which suggests that it might be involved in the signal transduction pathway for copper and cadmium. Jasmonic acid treatment increased mRNA levels and the capacity for glutathione synthesis but did not alter the glutathione content in unstressed plants, which supports the idea that the glutathione concentration is controlled at multiple levels.**

## INTRODUCTION

Glutathione (GSH), the tripeptide γ-glutamylcysteinylglycine, is the major source of non-protein thiols in most plant cells (Bergmann and Rennenberg, 1993). GSH plays a central role in protecting plants from environmental stresses, including oxidative stress due to the generation of active oxygen species, xenobiotics, and some heavy metals.

GSH is involved in quenching reactive oxygen species (Larson, 1988; Alscher, 1989; Foyer et al., 1994b). The ascorbate/GSH cycle reduces $H_2O_2$ to water (Foyer and Halliwell, 1976; Alscher, 1989). Ascorbate is also important in maintaining α-tocopherol in the reduced state and therefore links GSH to the dominant free radical scavenger in membranes (Hess, 1994). Plants detoxify many organic contaminants by conjugating them or their metabolites to GSH for storage or further metabolism (Lamoureux et al., 1994). These reactions are catalyzed by glutathione *S*-transferases (GSTs). Plants are protected from some metals, with cadmium and copper being the most studied, by a group of γ-glutamylcysteine (γ-EC) peptides, the phytochelatins (PCs). These molecules have the general structure $(\gamma\text{-Glu-Cys})_{2-11}$-Gly. They are formed by the polymerization of GSH catalyzed by the transpeptidase phytochelatin synthase (Grill et al., 1985, 1987; Chen et al., 1997). The PCs bind metals in the cytosol, and the PC metal complex is sequestered in the vacuole (Rauser, 1990).

GSH is synthesized from glutamate, cysteine, and glycine by a two-step ATP-dependent reaction (Meister and Anderson, 1983). The first reaction forms γ-EC from glutamate and cysteine by the enzyme γ-EC synthetase (Hell and Bergmann, 1990), which is encoded by *gsh1* (May and Leaver, 1995). GSH is then synthesized by the ligation of γ-EC and glycine in the reaction catalyzed by the enzyme GSH synthetase, which is encoded by *gsh2* (Wang and Oliver, 1996). When GSH is oxidized as part of its antioxidant activity, it forms glutathione disulfide (GSSG), the oxidized form of GSH. The glutathione reductases reduce GSSG back to GSH by using reducing equivalents from NADPH. Two genes encoding GSH reductase have been identified in Arabidopsis; one, *gr2*, encodes a plastidic isoform (Kubo et al., 1993), and the other, *gr1*, encodes a cytosolic enzyme.

Although the responses of several antioxidant enzymes, such as catalase, superoxide dismutase, and ascorbate peroxidase, to environmental stress have been studied in some detail (Bowler et al., 1989, 1992; Foyer et al., 1994a, 1994b; Sharma and Davis, 1994; Allen, 1995; Allen et al., 1997), little work has been done to show how the enzymes and genes responsible for GSH synthesis respond to oxidative and metal stress. Nevertheless, overexpression of *Escherichia coli* genes encoding GSH reductase and γ-EC synthetase has been exploited to manipulate tissue concentrations of γ-EC and GSH, and these proteins were found to increase antioxidant capacity and resistance to photoinhibition (Broadbent et al., 1995; Foyer et al., 1995; Strohm et al., 1995; Noctor et al., 1996, 1998; Arisi et al., 1997). The cloning of the genes for GSH synthesis and recycling (GSH metabolic genes) from Arabidopsis provides essential tools for the extensive analysis of their expression patterns under various environmental

[1] To whom correspondence should be addressed. E-mail doliver@iastate.edu; fax 515-294-1337.

stresses. Here, we report an analysis of the expression of the GSH metabolic genes of Arabidopsis in response to heavy metals and jasmonic acid (JA), a naturally occurring growth regulator that has important roles in plant development in addition to its amply demonstrated role in insect and disease resistance (reviewed in Creelman and Mullet, 1997a, 1997b). Our results show that the transcript levels of all of these genes were upregulated by both heavy metals and JA but not by $H_2O_2$, GSH, or GSSG. The upregulation was dependent on de novo protein synthesis. The elevated transcript accumulation was transcriptionally controlled. And more interestingly, it was found that these genes responded to heavy metals and JA in a coordinated manner. The possible role of $H_2O_2$, GSH, and JA as signal molecules was also investigated and is discussed.

## RESULTS

### Dynamics of PC and GSH Synthesis in Response to Heavy Metal Challenge

A number of plant species have been shown to respond to heavy metals by synthesizing PCs (Grill et al., 1985, 1987; Steffens et al., 1986; Scheller et al., 1987). Arabidopsis accumulates PCs after exposure to $Cd^{2+}$ (Howden et al., 1995; Wang and Oliver, 1996). We used a liquid culture system to examine the kinetics and dose response of Arabidopsis plants to heavy metal exposure. This system, in which whole plants are grown in liquid culture, is ideal for these studies because exposing plants to the chemicals is straightforward and because most stress response genes show limited activity under control conditions. When the Arabidopsis cultures were exposed to 100 μM $CdCl_2$, PCs increased from undetectable levels to 0.4 mM after an 18-hr exposure (Figure 1A). Although the cellular GSH level initially decreased upon $Cd^{2+}$ challenge, it gradually returned to its initial level as the incubation continued. After exposure to various concentrations of $CdCl_2$ for 12 hr, cellular GSH level declined (Figure 1A). This decrease in GSH level was dependent on the $Cd^{2+}$ concentration, with greater cadmium concentrations causing larger decreases in GSH levels. A maximum twofold decrease in GSH was observed at the highest $Cd^{2+}$ concentration administered (400 μM). The level of GSSG was low in untreated plants and increased after treatment with 100 μM $Cd^{2+}$. Higher levels of GSSG were observed in samples treated with Cd concentrations $>$100 μM. The sum of GSH, GSSG, and PCs in the samples exposed to $Cd^{2+}$ for $>$6 hr was greater than that of the control, suggesting that GSH synthesis was promoted by $Cd^{2+}$ treatment.

The kinetics and dose response of Arabidopsis liquid cultures exposed to $Cu^{2+}$ are shown in Figure 1B. A decline in GSH level was observed after a 1-hr exposure to 100 μM $Cu^{2+}$, and a maximum decrease of twofold was seen after a 3-hr exposure. As with the plants exposed to $Cd^{2+}$, the cellular GSH level gradually recovered, approaching its initial level 18 hr after exposure. Dose–response analysis, in which the cultures were exposed to various concentrations of $Cu^{2+}$ for 12 hr, revealed a trend that was similar to that seen with Cd treatment, in which GSH declined to a greater extent at increasing concentrations of $Cu^{2+}$. A maximum decrease of threefold was observed at 400 μM of $Cu^{2+}$. Unlike the situation with cadmium, however, little PC was synthesized in cultures exposed to $\geqslant$100 μM concentrations of $Cu^{2+}$. The highest PC level (0.1 mM) was synthesized in the culture exposed to the lowest concentration of $Cu^{2+}$ (50 μM) in this experiment. This value was sixfold lower than the maximum induced by $Cd^{2+}$. These results suggest that although Cu induced PC formation, high $Cu^{2+}$ concentrations inhibited PC synthesis in Arabidopsis. $Cu^{2+}$ concentrations $<$50 μM may have induced higher PC accumulation.

The synthesis of GSH was clearly more tolerant to $Cu^{2+}$ toxicity than was PC synthesis. Only a 25% reduction in GSH level was observed for samples exposed to 200 μM $Cu^{2+}$ for 12 hr. It was also evident that $Cu^{2+}$ treatment caused oxidative stress, as shown by the two- to fivefold increase in GSSG level in cultures treated with $\geqslant$100 μM concentrations of $Cu^{2+}$. In copper-sensitive *Silene cucubalus* roots, PC synthesis dramatically decreased at $Cu^{2+}$ concentrations $>$20 μM, and both lipid peroxidation and GSSG increased in these roots (De Vos et al., 1992). The sum of nonprotein thiols (GSH, GSSG, and PCs) in cultures treated with 50 to 200 μM $Cu^{2+}$ for $\geqslant$6 hr was higher than that of the controls (Figure 1B), indicating that GSH synthesis was stimulated at these concentrations of $Cu^{2+}$.

### Coordinated Expression of GSH Metabolic Genes in Response to Heavy Metal Challenge

Arabidopsis plants grown in soil were responsive to $Cd^{2+}$ and $Cu^{2+}$ challenge. When sprayed with solutions of 100 μM $Cd^{2+}$ or $Cu^{2+}$, the plant leaves responded by increasing the steady state transcript level for *gsh1*, *gsh2*, and *gr1*. The increase in transcript level could be seen within 1 hr of treatment and plateaued between 3 and 6 hr. All three transcripts increased simutaneously after heavy metal treatment (data not shown).

Arabidopsis grown in liquid cultures behaved like Arabidopsis plants grown in soil in response to heavy metals. Kinetic and dose–response analyses of steady state transcript levels for *gsh1*, *gsh2*, and *gr1* from Arabidopsis liquid culture exposed to $Cd^{2+}$ and $Cu^{2+}$ are shown in Figures 1C and 1D. When exposed to 100 μM $Cd^{2+}$ or $Cu^{2+}$, plant tissues responded fairly rapidly by increasing the transcript levels of the genes encoding the GSH-synthesizing enzymes and the GSSG-reducing enzyme. Elevated transcript levels were evident 1 hr after exposure to $Cd^{2+}$ or $Cu^{2+}$, plateaued at $\sim$6 hr, and remained high for 18 hr. High levels of transcripts continued for a few days under this condition (Xiang and Oliver, 1998). The plant tissues were quite sensitive to heavy

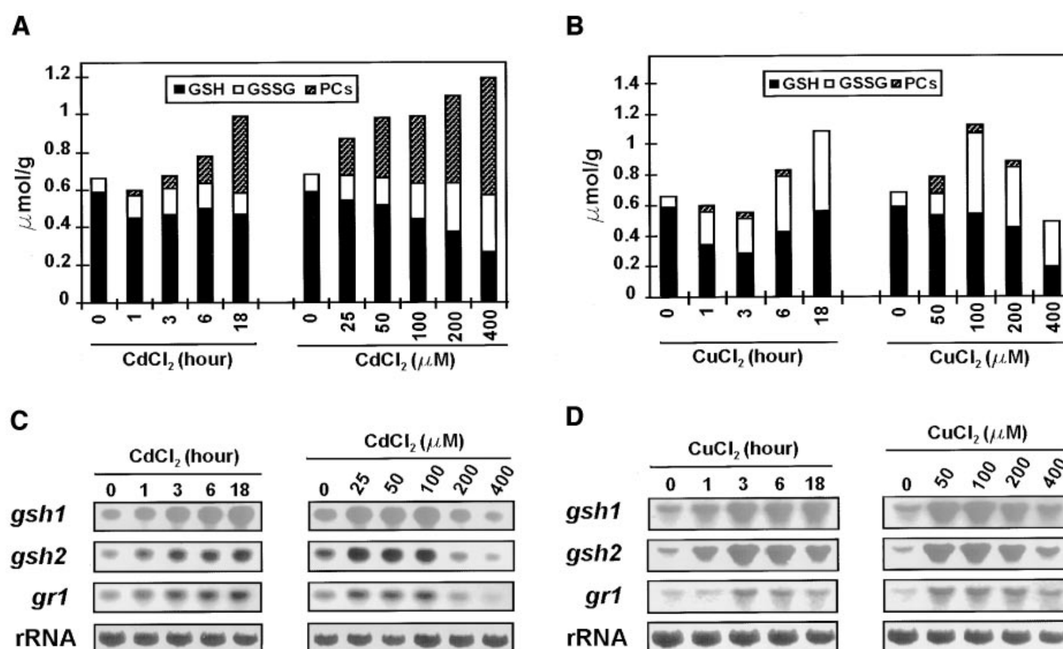


**Figure 1.** Dynamic Changes in Levels of GSH, GSSG, PC, and the Corresponding Metabolic Gene Transcripts in Arabidopsis Plants Grown in Liquid Culture in Response to $Cd^{2+}$ and $Cu^{2+}$.

**(A)** GSH and PC levels in response to $Cd^{2+}$ exposure. Two-week-old Arabidopsis plants grown in liquid cultures were treated either with 100 μM $CdCl_2$ for the indicated period of time or with various concentrations of $CdCl_2$ as indicated for 12 hr. Sample preparation, HPLC quantification of thiols, and GSSG assays were as described in Methods. The data represent the average results of two or three experiments. Variation between replicates was <5%.

**(B)** GSH and PC levels in response to $Cu^{2+}$ exposure. All treatments and thiol quantification were as described in **(A)**, except that $CuCl_2$ was used.

**(C)** RNA gel blot analysis of GSH metabolic gene transcripts in response to $CdCl_2$ exposure. Arabidopsis liquid cultures and $CdCl_2$ exposure treatments similar to those described in **(A)** were used for RNA analysis. RNA gel blotting was performed as described in Methods. The RNA filters were probed with cDNA inserts for *gsh1*, *gsh2*, cytosolic *gr1*, and 18S rRNA to ensure equal gel loading.

**(D)** RNA gel blot analysis of GSH metabolic gene transcripts in response to $CuCl_2$ exposure. All treatments, RNA isolation, and gel blotting and probing were as described in **(C)**.

metal exposure. Increased transcript accumulation was evident in cultures exposed to 25 μM $Cd^{2+}$ or 50 μM $Cu^{2+}$. The transcript levels increased in cultures exposed to up to 100 μM $Cd^{2+}$ or $Cu^{2+}$. However, cultures exposed to ⩾200 μM concentrations of $Cd^{2+}$ or $Cu^{2+}$ had decreased accumulations of *gsh1*, *gsh2*, and *gr1* transcripts. At concentrations >400 μM, the transcript levels were lower than in control plants. It is not obvious at this time whether the decrease in mRNA levels at high heavy metal concentrations or at long exposures was a specific regulatory event or whether it represented a general inhibition of cellular metabolism by heavy metal poisoning. The level of mRNA for at least one housekeeping gene, the $NAD^+$-dependent isocitrate dehydrogenase of the tricarboxylic acid cycle, was not decreased by high $Cd^{2+}$ treatment (data not shown).

Figure 2 shows the response of three GSH metabolic genes to various metal treatments. The steady state transcript levels were significantly increased only by $Cd^{2+}$ and $Cu^{2+}$ but not by toxic metals such as Zn, nontoxic metals such as Mg and Ca, and monovalent cations, suggesting that the response of these genes to Cd and Cu was very specific. The two metals that induce the mRNAs involved in GSH synthesis, Cd and Cu, were also the two that are most often considered to be protected against by PCs (Rauser, 1990).

It is informative to compare the effects of $Cd^{2+}$ concentration and treatment time on mRNA levels and on PC synthesis (Figure 1). Increasing Cd concentrations up to 100 μM and exposure time up to 18 hr with 100 μM Cd elevated the amount of mRNA for the enzymes of GSH synthesis as well as the amount of PC formed. At higher Cd concentrations, the steady state mRNA levels actually dropped, whereas the amount of PC continued to increase. This disconjunction between mRNA levels and the rate of final product accumulation suggests that at high metal concentrations, mRNA

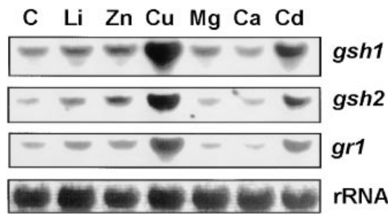


**Figure 2.** Steady State Transcript Levels of GSH Metabolic Genes in Response to Different Metal Ions.

Arabidopsis liquid cultures were treated with 100 μM LiCl (Li), $ZnCl_2$ (Zn), $CuCl_2$ (Cu), $MgSO_4$ (Mg), $CaCl_2$ (Ca), or $CdCl_2$ (Cd) for 12 hr. The control culture (C) did not receive any metal treatment. The RNA filter was probed as given for Figure 1C.

levels do not limit PC and, by extension, GSH synthesis, implying that post-transcriptional regulation might be involved.

In every instance that we analyzed for heavy metal induction, the levels of mRNA for all three of the genes involved in GSH metabolism, *gsh1*, *gsh2*, and *gr1* (as well as *gr2*; data not shown), appeared to be regulated in a coordinated fashion (Figures 1C, 1D, and 2). This suggests that common signal transduction pathways and regulatory mechanisms are shared by all three genes in responding to heavy metals.

## Coordinated Expression of GSH Metabolic Genes in Response to JA

To determine whether the increase in mRNA levels for these three genes was a general stress response or specific for the heavy metals, we exposed Arabidopsis seedlings grown on agar plates to a number of chemicals that normally are associated with stress or stress response pathways. Exposure to $H_2O_2$, diamide, 2,4-D, salicylic acid, and methyl viologen did not increase the levels of *gsh1*, *gsh2*, or *gr1* mRNA, as shown in Figure 3. They did, however, increase the level of mRNA for a GST, *parA*, which is known to be activated by multiple stimuli (van der Kop et al., 1996). JA, unlike the other compounds tested, increased the level of all three mRNAs involved in GSH metabolism. This JA-induced accumulation of GSH metabolic gene transcripts was further confirmed on soil-grown plants. When plants grown in soil were sprayed with 100 μM JA, increased accumulation of transcripts was evident 1 hr after the spray and plateaued after ~6 hr. The response to JA showed similar kinetics and sensitivity to the $Cd^{2+}$- and $Cu^{2+}$-induced responses in this experiment (data not shown).

JA-induced accumulation of GSH metabolic gene transcripts was further analyzed in Arabidopsis liquid cultures. These analyses are shown in Figure 4. The accumulation of transcripts was induced within 1 hr. Transcript levels plateaued between 3 and 6 hr after JA application. The induction was sensitive to as little as 10 μM JA and was dose dependent up to 500 μM, which was the highest concentration tested in the experiment. We also compared the transcript and GSH levels in these treatments (Figure 5A). Although the transcript levels for all of the genes involved in GSH metabolism were increased by JA treatment, the same treatment did not increase GSH content compared with the control. This suggests that under these conditions, other levels of regulation were involved in controlling cellular GSH concentrations.

These results demonstrate unequivocally the coordination among the GSH metabolic genes in responding to heavy metals and JA. These data also provide evidence supporting the role of JA in defense against abiotic stresses through increased expression of GSH metabolic genes.

## Accumulation of Steady State Transcript Levels Is Dependent on de Novo Protein Synthesis and Is Regulated at the Transcriptional Level

Cycloheximide is known to inhibit GSH synthesis (Scheller et al., 1987), to prevent the induction of GSH reductase activity by paraquat and $H_2O_2$ (Pastori and Trippi, 1992), and to block the JA-induced accumulation of the vegetative storage protein *VspB* and the protease inhibitor *Pin2* transcripts (Creelman and Mullet, 1997a). In our studies with Arabidopsis (Figure 5), it is clear that the inhibition of GSH synthesis by cycloheximide was at the mRNA level. All transcripts of GSH metabolic genes fell below the control level in cycloheximide-treated plants. In addition, cycloheximide blocked the response of these plants to Cd, Cu, and JA in that none of these treatments resulted in increased *gsh1*, *gsh2*, or *gr1*

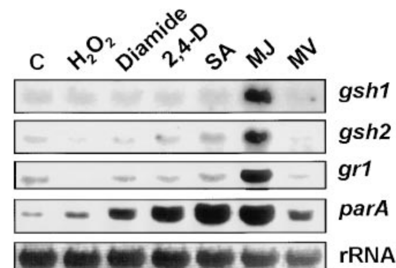


**Figure 3.** Steady State Transcript Levels of GSH Metabolic Genes in Response to Stress Agents.

Two-week-old seedlings germinated on agar plates were treated with 5 mM $H_2O_2$, 2 mM diamide, or 100 μM 2,4-D, salicylic acid (SA), methyl jasmonate (MJ), or methyl viologen (MV) for 4 hr at room temperature. RNA gel blot analysis was performed as given for Figure 1C.

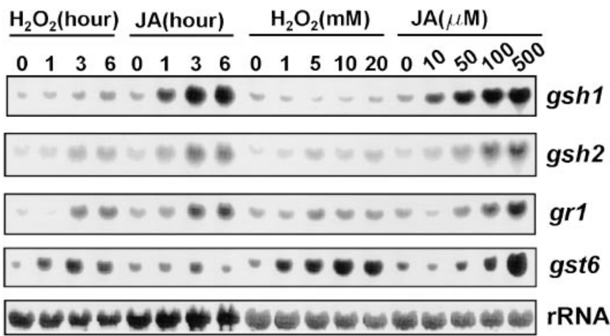


**Figure 4.** RNA Gel Blot Analysis of the Steady State Transcript Level of GSH Metabolic Genes in Response to $H_2O_2$ and JA.

Arabidopsis liquid cultures were treated with 100 μM JA and 5 mM $H_2O_2$, as indicated, for 0, 1, 3, or 6 hr or with various concentrations of $H_2O_2$ (mM) or JA (μM) for 3 hr. RNA gel blot analyses were performed as given for Figure 1C, except that one additional probe for GST 6 (*gst6*) was used.

transcript levels. The coordinated shutdown of JA-induced transcripts by cycloheximide may suggest a general characteristic of JA-modulated genes.

The accumulation of steady state transcripts may have resulted from either activated transcription or decreased turnover of mRNA. To understand the mechanisms behind the increased transcript accumulation, we conducted nuclear run-on transcription assays. The results indicate that the upregulation of GSH metabolic genes by JA and the heavy metal Cd was largely controlled at the transcriptional level (data not shown). The turnover of mRNA played a minor role, if any at all. To further confirm the mechanism of the upregulation, we also conducted experiments in which actinomycin D was used to inhibit RNA polymerase II. The results from these experiments (data not shown) are in agreement with the data from nuclear run-on assays, which show that transcriptional activation was largely responsible for the observed transcript accumulation for GSH metabolic genes. Taking cycloheximide sensitivity into account, we concluded that the transcriptional regulation was dependent on de novo protein synthesis, suggesting that rapidly turning over protein factors control transcription of these genes.

### $H_2O_2$ Does Not Induce the Accumulation of GSH Metabolic Gene Transcripts

It has been shown that GSH synthesis is regulated by oxidative stress. Smith et al. (1984, 1985) and May and Leaver (1993) have demonstrated that exogenously applied and endogenously produced $H_2O_2$ increase GSH concentrations. We also observed a twofold increase in cellular GSH level in samples treated with 5 mM $H_2O_2$ in our liquid culture system (data not shown). These observations showed that $H_2O_2$ induced GSH synthesis and suggested that GSH protected the cells from oxidative damage. However, the mechanism behind $H_2O_2$-induced GSH synthesis is unclear.

To determine whether $H_2O_2$ played any role in regulating the GSH metabolic genes in our system, we performed kinetic and dose–response analyses using Arabidopsis liquid cultures. Incubating plants in 5 mM $H_2O_2$ for up to 6 hr and in up to 20 mM $H_2O_2$ for 1 hr failed to induce the accumulation of GSH metabolic gene transcripts to the same extent as heavy metals and JA (Figure 4). Although one might argue that the exogenously applied $H_2O_2$ would not be effective in vivo, the $H_2O_2$ applied to the liquid culture did increase transcript levels for *gst6*, a gene known to be responsive to $H_2O_2$ (Chen et al., 1996). Moreover, treatment

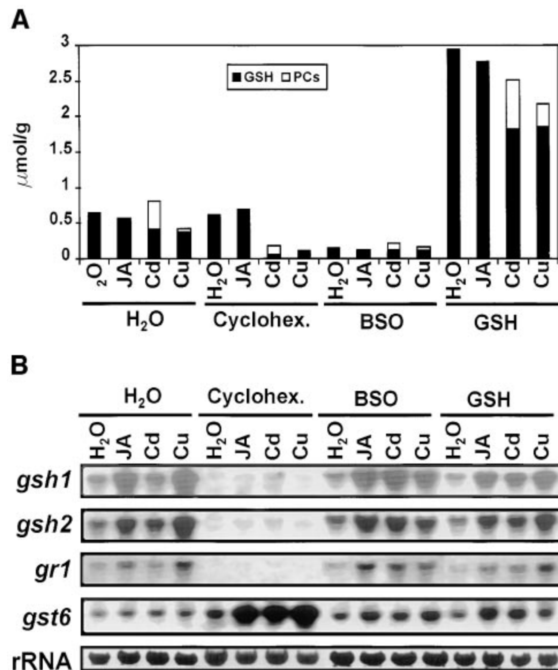


**Figure 5.** Effects of Cycloheximide, Butathione Sulfoximine, and GSH on the Synthesis of GSH and PCs and the Steady State Transcript Levels of GSH Metabolic Genes in Response to JA, $Cd^{2+}$, and $Cu^{2+}$.

**(A)** Effects of cycloheximide (Cyclohex.), butathione sulfoximine (BSO), and GSH on the synthesis of GSH and PCs. Arabidopsis liquid cultures were pretreated with 1 mM cycloheximide, 1 mM BSO, or 2 mM GSH, as indicated, for 2 hr, followed by incubation with 100 μM JA, $CdCl_2$, or $CuCl_2$ for 12 hr. Sample preparation for GSH and PC quantitation was as described for Figure 1A.

**(B)** Effects of cycloheximide, BSO, and GSH on the steady state transcript levels of GSH metabolic genes. Similar treatments as given in **(A)** were used for RNA gel blot analysis, as described for Figure 4.

with a catalase inhibitor, aminotriazol, did not induce accumulation of GSH metabolic gene transcripts (data not shown). These observations suggest that $H_2O_2$ does not induce transcription of GSH metabolic genes to a substantial degree. Preliminary data from a study using the *gsh1* promoter and a translational fusion to the β-*glucuronidase* reporter in transgenic Arabidopsis suggest that translational regulation is likely to control the level of GSH1 protein (C. Xiang and D.J. Oliver, unpublished data).

### GSH and GSSG Levels Do Not Control the Expression of GSH Metabolic Genes

GSH induces several defense genes (Dron et al., 1988; Wingate et al., 1988). GSH also regulates plastid gene transcription (Link et al., 1997). To determine whether GSH was involved in signaling the expression of genes for its own synthesis in our system, we used buthionine sulfoximine (BSO) to manipulate cellular GSH levels. BSO treatment decreased GSH concentrations in Arabidopsis plants. These decreased GSH levels did not induce gene transcription (Figure 5B). If depletion of cellular GSH played a role in signaling, one would have expected to see increased transcript levels in plants treated with BSO. Likewise, one would expect decreased transcript levels upon heavy metal induction if cellular GSH levels were elevated by supplementing GSH in the culture medium before the metal treatment. Both GSH and GSSG are taken up by plant cells (Schneider et al., 1992; Jamaï et al., 1996). Although GSH application increased cellular GSH levels, these higher GSH levels failed to alter the expression of *gsh1*, *gsh2*, and *gr1* after metal or JA treatment. BSO and GSH failed to modulate gene expression, although the cellular GSH content was indeed manipulated as expected (Figure 5A). In addition, 2 mM GSSG also failed to increase the transcript levels of these genes when it was added to the liquid cultures, despite the fact that it increased cellular GSSG levels (data not shown). These results suggest that GSH, GSSG, and the GSH/GSSG ratio did not play any direct roles in the signal transduction pathway controlling GSH synthesis.

### JA Potentiates and Enhances GSH-Synthesizing Capacity

To determine whether JA potentiates and enhances GSH-synthesizing capacity, we conducted an experiment in which Arabidopsis liquid cultures were first treated with JA for 3 hr. $Cu^{2+}$ was then administered, and the plant tissues were sampled at various times to measure GSH levels. The results shown in Figure 6 demonstrate that JA does potentiate and enhance the capacity for GSH synthesis. Although JA treatment alone did not alter GSH levels, JA-pretreated plants produced more GSH when challenged with Cu.

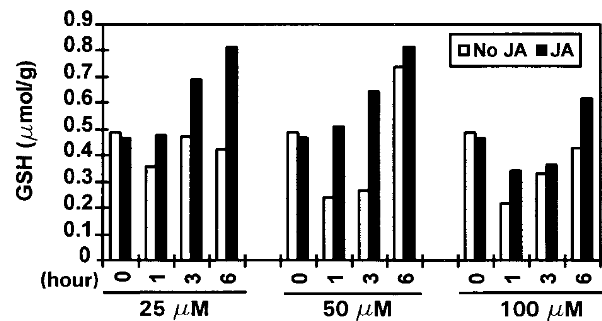


**Figure 6.** JA Potentiates and Enhances GSH-Synthesizing Capacity.

Arabidopsis liquid cultures were pretreated with 100 μM JA for 3 hr before $CuCl_2$ was added to final concentrations of 25, 50, and 100 μM, as indicated. Incubation with JA and Cu was terminated at 1, 3, and 6 hr after the addition of $CuCl_2$, and samples were analyzed for GSH, as described for Figure 1A.

## DISCUSSION

### Demand-Driven GSH Synthesis and Multilevel Regulation of GSH Homeostasis

GSH synthesis is promoted by oxidative stress (Smith et al., 1984; Smith, 1985; Alscher, 1989; May and Leaver, 1993). Additional support comes from a recent study of catalase-deficient tobacco plants created by sense and antisense technology (Chamnongpol et al., 1996; Willekens et al., 1997). Catalase-deficient plants show a remarkable increase in total GSH content under high light (Willekens et al., 1997). All of these studies have unequivocally demonstrated that GSH synthesis is driven by increasing demand for GSH in response to oxidative stress and GSH conversion to PCs.

Multilevel regulation of GSH homeostasis has been implicated in a number of studies (reviewed in May et al., 1998). To date, at least five levels of control of steady state GSH concentrations have been identified. These are (1) substrate availability, (2) rate limitation of GSH synthesis by γ-EC synthetase activity, (3) feedback inhibition of GSH formation at γ-EC synthetase, (4) post-transcriptional regulations, including translational controls, and (5) control of the transcription of the genes for GSH synthesis.

Control by substrate availability and γ-EC synthetase activity has been well documented in transgenic poplars by Foyer and co-workers (Foyer et al., 1994a, 1995, 1997; Strohm et al., 1995; Noctor et al., 1996, 1997, 1998; Arisi et al., 1997). They have shown that the amount of γ-EC synthetase but not the amount of GSH synthetase controls GSH level in these transgenic plants. In plants overexpressing γ-EC synthetase, the amount of GSH can be increased substantially by feeding cysteine, suggesting that under these conditions, the availability of this amino acid limits synthesis of this tripeptide.

Several groups have presented data suggesting that the activity of enzymes involved in GSH metabolism increases after heavy metal or oxidative stress. The induction of GSH reductase was demonstrated in response to ozone treatment with spinach (Tanaka et al., 1988). Guy and Carter (1984) showed that hardened spinach exhibited two GSH reductase isoforms that were absent in unhardened spinach, indicating de novo synthesis of new GSH reductase isoforms in response to cold stress. Pastori and Trippi (1992) reported that oxidative stress caused by paraquat or $H_2O_2$ would stimulate de novo GSH reductase synthesis in maize, probably at the level of translation of preexisting mRNA. GSH synthetase activity was stimulated by Cd treatment (Ruegsegger et al., 1990; Bergmann and Rennenberg, 1993). These studies suggest that GSH reductase and the GSH synthesis enzymes are also increased by de novo protein synthesis after stress treatment. In this study, we have directly addressed the control of GSH metabolism by demonstrating demand-driven changes in the level of gene transcription in response to stresses.

The role of feedback inhibition by GSH on γ-EC synthetase has been extrapolated from in vitro results. Although data presented by Foyer's group (Arisi et al., 1997) suggest that this feedback control is not particularly important in controlling GSH levels, some of our data (see below) suggest that GSH levels may be regulated in a manner expected by this mechanism.

The increase in GSH levels after treatment with $H_2O_2$ seems to suggest that an unidentified control mechanism still exists. Results from Smith et al. (1984, 1985) and May and Leaver (1993) as well as our results clearly show that $H_2O_2$ treatment raises the GSH concentration in plants. Given the results presented here that $H_2O_2$ treatment does not increase the level of mRNAs for GSH synthesis, it is clear that this does not result from increased gene transcription. Similarly, the feedback control described above would not result in increased GSH levels. Some additional control mechanism must be functioning, possibly at the translational or post-translational level. Our unpublished data show that translational control is likely involved. An oversimplified multilevel modulation scheme for regulating GSH levels is depicted in Figure 7.

In this scheme, unstressed plant cells establish GSH homeostasis through a low level of GSH metabolic enzymes and feedback inhibition of γ-EC synthetase by GSH. This steady state GSH level is maintained by a low level of transcription, protein synthesis, and enzyme activity. Under condition of minimal stress, the turnover of GSH and the enzymes of GSH synthesis are limited. Thus, whereas cycloheximide decreased the amount of mRNA for *gsh1*, *gsh2*, and *gr1* to nearly undetectable

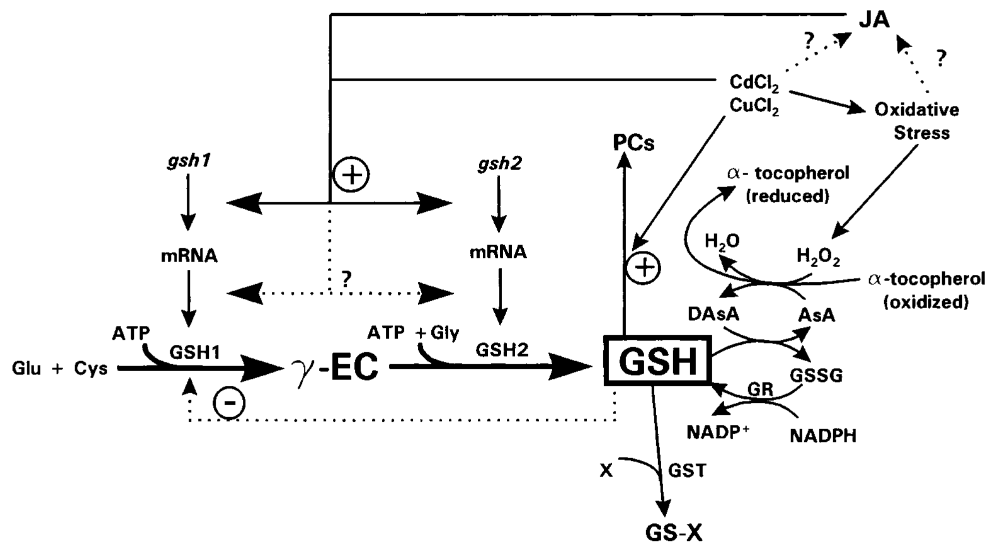


**Figure 7.** Demand-Driven GSH Synthesis and Multilevel Regulation of GSH Homeostasis.

GSH homeostasis is dynamically established between synthesis and consumption. GSH is synthesized by γ-EC synthetase and GSH synthetase using Glu, Cys, Gly, and ATP as substrates. GSH is consumed by (1) reduction of dehydroascorbate (DAsA) to ascorbate (AsA) by DAsA reductase, (2) detoxification of xenobiotics (X) by GSTs to form GSH conjugates (GS-X), and (3) formation of PCs by PC synthase upon heavy metal exposure. In addition, degradation of GSH also contributes to the balance of GSH. GSH feedback inhibits γ-EC synthetase. Metals are required for PC synthase activity. Cd, Cu, and JA transcriptionally activate *gsh1*, *gsh2*, *gr1*, and *gr2*. JA may or may not lie in the signal transduction pathway for the heavy metal response. The circled plus signs indicate positive effects, and the circled minus sign indicates negative regulation. Question marks indicate uncertain but possible regulatory mechanisms.

levels, cycloheximide treatment had little or no effect on the steady state GSH level. There must be some GSH turnover even under these conditions, however, because BSO inhibition of γ-EC synthetase decreased the GSH level. As soon as environmental stress was imposed in the form of Cd or Cu on cycloheximide-treated tissue, however, the amount of GSH plummeted, demonstrating that the capacity to synthesize GSH was extremely restricted.

When plants were challenged to a stress environment, the homeostasis was perturbed, and GSH was rapidly consumed in combating the imposed stress. In response, plant cells have to replenish GSH by synthesizing more GSH to maintain a high GSH/GSSG ratio. This is manifested at multiple levels. First, the feedback inhibition of γ-EC synthetase by GSH would be immediately alleviated by the decreased GSH level, thus restoring GSH synthesis by the preexisting enzymes. However, the GSH-synthesizing capacity of preexisting enzymes can never significantly increase without de novo enzyme synthesis. To meet the ever-increasing demand for GSH as imposed stress becomes more severe, plant cells respond with increased steady state mRNA accumulation, which is controlled by transcriptional activation and possibly by enhanced translation of preexisting mRNA. Consequently, more enzyme is made through de novo protein synthesis, leading to an enhanced GSH-synthesizing capacity, thereby elevating GSH levels. There must be controls to switch these mechanisms on and off accordingly with the changing status of the stress. The identity of the signal molecule(s) and the nature of signal transduction are largely unknown, although our results suggest that $H_2O_2$, the concentrations of GSH and GSSG, and the GSH/GSSG ratio are not in the signal transduction pathway.

### Insights into JA-Induced Cross-Tolerance to Oxidative Stress

Cross-tolerance, the induced tolerance to additional biotic and abiotic stresses after exposure to a specific oxidative stress, is a widespread defense mechanism in higher plants. Arabidopsis preexposed to ozone becomes resistant to *Pseudomonas syringae* (Sharma et al., 1996). Tobacco preexposed to UV light and ozone also shows induced resistance to tobacco mosaic virus (Yalpani et al., 1994). $H_2O_2$-induced stress tolerance has been demonstrated in numerous reports (Matsuda et al., 1994; Prasad et al., 1994, 1995; Foyer et al., 1997). Wounding- and JA-induced cross-tolerance to ozone stress has also been demonstrated in tobacco (Örvar et al., 1997). Our results of JA-induced upregulation of GSH metabolic genes may provide partial explanations for JA-induced cross-tolerance. In our study, JA strongly stimulated the expression of the genes for GSH synthesis and recycling, potentially leading to enhanced synthesis of GSH and providing protection against oxidative and ozone stress.

Exogenously applied JA greatly increased transcript levels, but an increase in GSH level was never observed under those conditions (Figure 5). This is not surprising because GSH homeostasis is tightly controlled. Under the induction condition, there is no demand for increasing GSH level because no heavy metals or other oxidative stresses were imposed. If JA does enhance the capacity for GSH synthesis when there is no demand for GSH, it is expected that GSH synthesis should be faster and more responsive when an oxidative stress is imposed upon the organism. Indeed, results shown in Figure 6 support this hypothesis.

### Diverse Stress Stimuli Converge on Promoters of Coordinately Expressed GSH Metabolic Genes

Hausladen and Alscher (1993) proposed that multiple regulatory "circuitry" may be involved in the sensing and processing of oxidative stress signals. Signals originating from diverse oxidative stress stimuli are transduced to the nucleus to activate the expression of genes that specify the downstream events. Redox state, GSH, and reactive oxygen species have been proposed as central components of signal transduction in both abiotic and biotic stresses (reviewed in Foyer et al., 1997). Whether these proposed signal molecules regulate the expression of GSH metabolic genes under oxidative stress is debatable because our results do not favor reactive oxygen in the form of $H_2O_2$ or the redox potential as measured by GSH/GSSG ratio as signals in our system.

JA levels in plants rapidly and transiently increase in response to wounding (Creelman et al., 1992), water deficit (Creelman and Mullet, 1995), mechanical stimulation (Falkenbation et al., 1991; Weiler et al., 1993), elicitors (Gundlach et al., 1992; Doares et al., 1995), and the short polypeptide systemin (Pearce et al., 1991). JA also mediates some of the UV-induced defense responses (Conconi et al., 1996). The JA signal transduction pathway has yet to be determined and is being studied using JA-insensitive Arabidopsis mutants. Promoter analyses with JA-inducible genes *Pin2* (Kim et al., 1992) and *VspB* (Mason et al., 1993) have revealed that the JA-responsive domain contains a G-box element (Williams et al., 1992), which is a binding site for basic leucine zipper transcription factors. We have isolated and sequenced the promoter of Arabidopsis *gsh1*. Several putative G-box elements are located in the promoter (C. Xiang and D.J. Oliver, unpublished data). It is conceivable that such JA-responsive elements must reside in the JA-inducible gene promoters.

The heavy metal signal transduction pathway in higher plants is not known. It is not clear whether JA is involved in heavy metal–induced gene expression in Arabidopsis. It has been shown that heavy metals such as Cd and Cu do not stimulate JA production in *Rauvolfia serpentina* cell cultures (Blechert et al., 1995). If this is also true in Arabidopsis, heavy metal–induced gene expression may be mediated by a different signaling pathway from JA. It is quite likely that these different oxidative stress stimuli transduce their signals through different pathways and finally converge on the same *cis*-acting elements. A promoter analysis being

currently undertaken may reveal whether distinct *cis*-acting elements responsive to heavy metals and JA, respectively, reside in the *gsh1* promoter.

Metal- and antioxidant-responsive elements have been identified in the promoter of the human γ-EC synthetase heavy subunit gene (Mulcahy and Gipp, 1995). The expression of the γ-EC synthetase gene is Jun dependent, indicating that AP-1 transcription factors participate in the regulation of GSH metabolism (Sekhar et al., 1997). Similar to mammalian cells, baker's yeast *GSH1*, encoding γ-EC synthetase, is also regulated by transcription factor yAP1p (Wu and Moye-Rowley, 1994), which is the yeast counterpart of mammalian AP-1. In response to elevated levels of Cu, Zn, and other metals, budding yeast also activates the expression of its single metallothionein gene, *CUP1* (Fogel and Welch, 1982). *CUP1* is transcriptionally induced by a copper-activated transcription factor, *ACE1* (Thiele, 1988). ACE1 is constitutively expressed and becomes active for specific DNA interaction upon the binding of Cu through its cysteine-rich metal-binding domain (Fürst et al., 1988). Whether these elegant regulatory mechanisms have been exploited by higher plants awaits further investigation.

## METHODS

### Plant Growth Conditions, Liquid Culture, and Stress Treatments

*Arabidopsis thaliana* (ecotype Columbia) plants were grown at 22°C and 60% humidity under continuous white fluorescent light (75 μmol $m^{-2}$ $sec^{-1}$). Healthy 1-month-old plants were sprayed with 100 μM heavy metal and jasmonic acid (JA) solutions containing 0.1% Triton X-100. Rosette leaves were sampled for RNA isolation after the indicated period of time. For JA treatment, plants were kept in a desiccator after being sprayed. Arabidopsis seeds were surface sterilized in 70% ethanol for 2 min, soaked in 50% bleach for 15 min, and then rinsed four times with sterile water. The sterilized seeds were germinated on agar plates (half-strength Murashige and Skoog salts, B5 vitamins, 0.4 g of Mes per liter, pH 5.8, solidified with 2 g of Phytogel per liter; all tissue culture supplies were purchased from Sigma). Liquid cultures were initiated by inoculating 125-mL flasks containing 40 mL of medium (half-strength Murashige and Skoog salts, B5 vitamins, 2% sucrose, and 0.4 g of Mes per liter, pH 5.8; Sigma) with ∼50 surface-sterilized Arabidopsis seeds. The cultures were kept on a rotary shaker at 80 rpm and under continuous dim light. Stress treatments were conducted 10 to 14 days after inoculation. Stress agents were directly administered into the culture medium. At the end of the treatment, plant tissues were washed twice with deionized $H_2O$. All liquids on the surface of plant materials were blotted with paper towels. The fresh weight of plant materials was recorded before acid extraction for thiol analysis.

### Quantitation of Phytochelatin and Glutathione

Quantitation of phytochelatins (PCs) and glutathione (GSH) was essentially as described (Steffens et al., 1986; Fahey and Newton, 1987). Briefly, PCs and thiols were acid extracted from fresh tissues in 2 volumes of 0.15 N HCl. The homogenate was centrifuged at 12,000*g* for 5 min. An aliquot of supernatant was then used for derivatization with monobromobimane and quantified fluorometrically after separation by reversed-phase HPLC. Standards were derivatized and quantified under identical conditions. Glutathione disulfide (GSSG) was measured using glutathione reductase and 2-vinylpyridine, as described previously (Griffith, 1980). PC standards were synthesized on an advanced Chemtech (Des Plaines, IL) model 396 oligopeptide synthesizer using N-αFMOC-γ-carboxyglutamate (Anaspec Corp., San Jose, CA).

### RNA Gel Blot Analysis

Total RNA was isolated from fresh tissues, as described previously (Xiang et al., 1996). Thirty micrograms of total RNA was denatured and fractionated in a 1% agarose–formaldehyde gel, blotted onto a Zeta Probe nylon membrane (Bio-Rad), probed with $^{32}P$-labeled cDNA inserts, and reprobed after stripping in boiling 0.1% SDS solution. All probes were labeled using a random priming labeling kit (Gibco BRL). Both prehybridization and hybridization for all blots were performed at 65°C in a hybridization solution (0.05 M $NaH_2PO_4$, pH 7.4, 1.0 mM EDTA, 7% SDS, and 1% casein). After overnight hybridization, filters were sequentially washed in 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS, 0.2 × SSC and 0.1% SDS, and 0.1 × SSC and 0.1% SDS for 10 min each at room temperature. After a final wash in 0.1 × SSC and 0.1% SDS at 65°C for 15 min, the filters were exposed to x-ray film for autoradiography. All of the cDNA inserts used in this study except *gsh2* were polymerase chain reaction amplified from an Arabidopsis cDNA library (λPRL2) and confirmed by sequencing before they were used as probes. The GenBank accession numbers are Z29490 for *gsh1*, U22359 for *gsh2*, U37697 for *gr1*, D14049 for *gr2*, H77062 for *parA*, and X95295 for *gst6*.

### Nuclear Run-on Transcription Assays

Nuclear run-on transcription assays were performed as described previously (DeRocher and Bohnert, 1993). Arabidopsis liquid cultures were treated with heavy metals and JA as given for RNA gel blot analysis. Nuclei were isolated, and in vitro run-on transcription was performed in the presence of $^{32}P$-UTP. Subsequently, labeled mRNA was purified and hybridized to cDNA inserts (1 μg) immobilized on nylon membrane. The hybridization and wash conditions were the same as for the RNA gel blot analysis. The filter was exposed to x-ray film for autoradiography.

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