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Evidence for posttranscriptional activation of γ-glutamylcysteine synthetase during plant stress responses

(*Arabidopsis thaliana*/glutathione)

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ABSTRACT **Glutathione (GSH) is a key component of plant antioxidant defenses. We have sought to determine how the rate-limiting step in GSH biosynthesis, catalyzed by γ-glutamylcysteine synthase (γECS) is regulated in *Arabidopsis*. Functional complementation of a yeast mutant deficient in this enzyme with an *Arabidopsis* expression library yielded two cDNAs with sequence identical to the previously described *At*γECS. Nevertheless, the cellular concentration of GSH in these transformants was only 10% of wild-type concentrations and this was not a result of Cys availability. To explore the possibility that *Arabidopsis* γECS requires additional factors for full catalytic activity, we analyzed the GSH levels and the enzyme activities and transcript levels of both enzymes of the GSH biosynthetic pathway in *Arabidopsis* suspension cultures subjected to a variety of stresses that raise GSH levels. Our results demonstrate rapid posttranscriptional activation of *Arabidopsis* γECS. The implications of these findings for the mechanisms by which GSH concentrations are regulated during plant-stress responses are discussed.**

Plants have evolved defense mechanisms to minimize the accumulation of active oxygen species (AOS), which arise during environmental stress and as byproducts of metabolism. Among these defenses, the tripeptide thiol glutathione (GSH; γ-glutamylcysteinylglycine) plays a pivotal role. An important adaptive response of plants to conditions that increase AOS is a net increase in the cellular concentration of GSH (1). For heavy metal stress, this increase provides the substrates for the synthesis of phytochelatins (2).

GSH is synthesized in two ATP-dependent steps, catalyzed by γ-glutamylcysteine synthetase (γECS; EC 6.3.2.2) and glutathione synthetase (GSHS; EC 6.3.2.3). In some cases, increases in the concentration of GSH during stress responses have been correlated with an increase of γECS activity (2–4).

In mammals and yeast γECS catalyzes the rate-limiting step in GSH biosynthesis (5), and recent evidence suggests this may be true also for plants (6). For example, cadmium tolerance in tomato cell lines (2) and chilling tolerance in corn (3) correlate with increased γECS activity. Similarly, some herbicide safeners increase GSH levels in maize through enhancing γECS activity (4). More direct evidence came recently from the study of a cadmium-sensitive *Arabidopsis* mutant, *cad-2*, in which leaf glutathione content is decreased to 30% of that in the wild-type parental accession (M.J.M., unpublished data). GSH depletion in this mutant results from a 6-bp deletion in the *γECS* gene leading to an in-frame 2-aa deletion in the γECS protein that causes a 60% reduction in the extractable γECS activity compared with wild type. Whereas transformation of *cad-2* with a wild-type *γECS* gene restored cadmium resistance, GSH levels, and γECS activity to wild-type levels, GSHS activity did not change, demonstrating not only that the mutation in the *γECS* gene was responsible for the phenotype observed, but that γECS is the limiting enzyme of GSH biosynthesis *in planta* (M.J.M. unpublished data).

We have previously isolated cDNA clones encoding both *γECS* and *GSHS* from *Arabidopsis thaliana* (*At*γ*ECS* and *AtGSHS*, respectively) by functional complementation of *Escherichia coli* mutants deficient in the respective enzymes. Whereas the derived amino acid sequence of the cDNA encoding AtGSHS shares considerable sequence identity with the sequences from other species (7), the sequence of AtγECS shares only 15–18% identity with corresponding homologues (8). Furthermore, although extracts of *E. coli* mutants deficient in *γECS* expressing *At*γ*ECS* contained measurable γECS activity *in vitro*, the level of GSH in this transformed mutant was only 10% of the parental strain. These observations have led to questions about the identity of this *γECS* clone (9).

The aim of this work was twofold. First, we wished to isolate further clones encoding *At*γ*ECS* through functional complementation in a different heterologous system: *Saccharomyces cerevisiae*. Because mutants of *S. cerevisiae* deficient in γECS activity are unable to grow aerobically (10, 11), we have exploited this phenotype as a highly stringent screen for plant cDNAs that restore air tolerance. Second, our results led us to study the mechanisms by which γECS activity is controlled in *Arabidopsis*. Here, we describe the characterization of two cDNA clones that permit aerobic growth of a *γECS*-deficient mutant of *S. cerevisiae* and that have sequences identical to the described *At*γ*ECS* clone (8). Using *Arabidopsis* cell suspensions, we have demonstrated that increases in the level of GSH or phytochelatins that occur in response to different stresses are not a result of activation of *At*γ*ECS* or *AtGSHS* transcription. Rather, GSH accumulation results from posttranscriptional activation of γECS activity.

MATERIALS AND METHODS

Yeast Strains. The parental *Saccharomyces cerevisiae* strain used in this study and for the generation of the disruption mutant was W303a (MATa, *ade2*-1, *can1*-100, *trp1*-1, *ura3*-1, *his3*-11, *leu2*-3, 112). Yeast transformation was carried out as described (12). Strains were grown in minimal medium [0.67% yeast nitrogen base (Difco), 2% glucose] supplemented with

Abbreviations: AOS, active oxygen species; AT, aminotriazole; FC, fenchlorazole; γECS, γ-glutamylcysteine synthetase; GSH, glutathione; GSHS, glutathione synthetase; MQ, menadione; NPT, nonprotein thiol.

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individual amino acids as required. Plates contained the same media together with 1.5% agar.

Construction of Yeast Disruption Mutants and Screening of cDNA Library. The *γECS* disruption plasmid (*γECS*::*LEU2*) was kindly provided by T. Lisowsky (13). The plasmid was linearized and transformed into W303a, and Leu prototrophic colonies were selected on minimal plates supplemented with 100 μM GSH. The disruption mutants, designated YΔ*γECS*, failed to grow when GSH was omitted from the medium. An *Arabidopsis* cDNA library in the yeast expression vector pFL61 was kindly provided by M. Minet (14). To select *Arabidopsis* cDNAs that allowed aerobic growth in the absence of GSH, YΔ*γECS* cells were transformed with the cDNA library, and Ura/Leu/GSH prototrophic colonies were selected on minimal plates after 7–14 days of growth at 30°C. Four independent transformations were made with an average transformation efficiency of 10^5 colonies/μg plasmid. Plasmids from individually isolated yeast clones were rescued into *E. coli* as described (15), and the insert was sequenced on both strands on an ABI373A automatic DNA sequencer (Applied Biosystems) using gene-specific primers and fluorescent dye terminators. The DNA and amino acid sequence analyses were done with a software package by the Genetics Computer Group (Madison, WI). To confirm the GSH-independent aerobic growth phenotype, YΔ*γECS* cells were transformed with the plasmids rescued in *E. coli* and colonies were selected on minimal plates.

Plant Material and Stress Treatments. An *Arabidopsis* cell suspension culture (Columbia, Col-0) was kindly provided by M. Axelos (Toulouse, France) and maintained as described (16). For stress treatments, exponentially growing cultures were incubated with sublethal doses of aminotriazole (AT), menadione (MQ), cadmium (Cd), and the herbicide safener fenchlorazole [FC; 1-(2, 4-dichlorophenyl)-5-trichloromethyl-1*H*-1,2,4-triazole-3-carboxylic acid; AgrEvo, Frankfurt] at concentrations that had been shown previously to increase the cellular concentration of GSH (ref. 17; J.-P. Reichheld, personal communication; R.S.-F., unpublished data). Samples of 1 or 5 ml of the culture (70–100 mg fresh weight/ml) were taken for determination of GSH (or phytochelatins) and RNA extraction or enzyme activity measurement, respectively, at the indicated time points after the addition of the drug.

GSH and Phytochelatin Determination. Preparation of extracts from *Arabidopsis* suspensions for the determination of GSH was as described (18). Extracts for the determination of GSH in yeast were prepared by lyticase digestion (Boehringer Mannheim) for 30 min in 50 mM potassium phosphate buffer, pH 7.6, followed by vigorous vortexing in 5 mM sulfosalicylic acid/1 mM EDTA for 5 min with three cycles of rapid freeze-thawing. Phytochelatins were determined in *Arabidopsis* extracts as nonprotein thiols (NPT) as described (19).

RNA Analysis. Total RNA was extracted by using TRIzol according to the manufacturer's instructions (Boehringer). Northern blot analysis was essentially as described (8), using radioactively labeled fragments of *AtγECS* (ref. 8; this work) and *AtGSHS* (7) as probes.

Enzyme Activities. Crude cell extracts were prepared from yeast spheroplasts after lyticase digestion for 30 min in 100 mM Tris·HCl/10 mM $MgCl_2$/5 mM EDTA, pH 8.0, in the presence of a protease inhibitor mixture (Boehringer) at a ratio of 1:5 (cell volume/buffer volume). Extracts from suspension-cultured *Arabidopsis* cells were prepared by grinding 500 mg cells in the above extraction buffer at a 1:1 ratio of buffer to packed cell volume. Desalted protein extracts were prepared and enzyme assays were carried out as described (4). For HPLC analysis of reaction products, samples were derivatized with monobromobimane and separated on a Vydac C18 silica column (Alltech Associates) on a Vista 5500 HPLC (Varian) coupled to a scanning fluorescence detector (Waters). Bimane conjugates were detected by excitation at 380 nm and emission at 480 nm. Protein was determined as described (20).

RESULTS

Isolation and Characterization of *Arabidopsis γECS* Clones by Complementation of GSH-Deficient Yeast. A yeast mutant lacking a functional *γECS* was generated by disruption of the yeast *γECS* gene with a *LEU2* gene. Leu prototrophs were selected on minimal medium supplemented with 100 μM GSH, and one of these was taken for further analysis and designated YΔ*γECS*. Disruption of the yeast *γECS* gene was confirmed by PCR analysis (data not shown). The GSH level of this strain was under the limit of detection of the method used, indicating that it was unable to synthesize GSH as a result of the gene disruption. YΔ*γECS* was unable to grow aerobically on minimal medium, as reported previously (10, 11). Under anaerobic conditions, the effects of GSH deficiency were suppressed (data not shown), indicating the toxicity of oxygen in yeast when GSH is limiting. The air sensitivity of YΔ*γECS* and the suppression of this phenotype by GSH was exploited for the selection of *Arabidopsis* cDNAs that restore viability under aerobic conditions through provision of GSH.

YΔ*γECS* cells were transformed with an *Arabidopsis* cDNA library in the yeast expression vector pFL61 (14). Seven Leu/Ura prototrophs, which grew aerobically on minimal medium, were obtained in four independent transformations from a total of 5×10^5 transformants. To confirm that this phenotype was linked to the presence of the cDNA, plasmid was isolated from each transformant and reintroduced into YΔ*γECS*. All of these transformants were able to grow aerobically on minimum medium (data not shown). Partial sequence analysis revealed that all these cDNAs were derived from the same gene, corresponding to the *AtγECS* cDNA we have described previously (8). Two of these clones (clones 1 and 4) were fully sequenced on both strands. Clone 1 differed only from clone 4 by the presence of an N-terminal chloroplast-targeting peptide, found also on the previously described cDNA (8). In addition, sequencing of clones 1 and 4 revealed an error in the published sequence: an additional base in the published cDNA created a frame shift in the C-terminal region of the derived polypeptide, and the entry in the GenBank database (accession no. Z29490) has been amended accordingly.

To investigate whether the air-tolerant phenotype of the mutant strain transformed with clones 1 and 4 was a result of the ability of the cDNAs to raise GSH levels, we determined the concentration of GSH in extracts of all of the yeast strains used in this study. Surprisingly, the levels of GSH in extracts of clones 1 and 4 were very low: only 6% and 11% of the level in the parental strain, respectively (Fig. 1). A possible reason for the low GSH levels in extracts of the transformants may be pleiotropic impairment of the pathway for assimilation of sulfur, as has been reported for some oxidative stress-hypersensitive yeast mutants (21). Although supplementation of 100 μM Cys, Met (which can be converted into Cys by cystathionine synthetase), *O*-acetyl Ser (a direct precursor of Cys), and thiosulfate (which provides sulfide) to the growth medium all raised the extractable GSH in extracts of the parental strain, these compounds had no significant effect on the levels of GSH in extracts of the transformants (Fig. 1). To obtain unequivocal proof that the protein encoded by clones 1 and 4 was indeed a *γECS*, we measured γECS activity in extracts of all of the yeast strains used in this study by postreaction derivatization of thiols with monobromobimane, separation of the bimane-thiol conjugates by C18 HPLC, and detection of the fluorescent conjugates by excitation at 380 nm and emission at 480 nm (4). The γECS activity in extracts of clone 1 and clone 4 was 16% and 24% of the activity in extracts of the parental strain, respectively (Table 1). Measurements of the GSHS activities showed that a pleiotropic consequence of the mutation in the yeast *γECS* gene in the strain employed in this study is a reduction in the activity of GSHS, although the

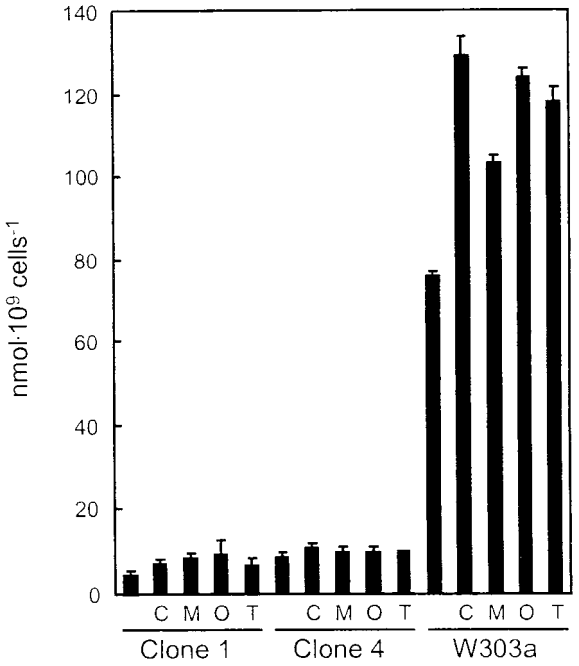

FIG. 1. GSH content of the different yeast strains. The parental yeast strain W303a as well as W303a in which the *γECS* gene had been disrupted with *LEU2* and transformed with the plasmids (clones 1 and 4) were grown in liquid minimal medium for 36 h at 30°C. Cells were sedimented by centrifugation and GSH was extracted and measured as described in *Materials and Methods*. GSH content of the disruption mutant YΔ*γECS* was undetectable by HPLC analysis. GSH content in the extracts is expressed as nmol/10^9 cells. The supplements (100 μM) to the medium are: C, Cys; M, Met; O, *O*-acetyl Ser; T, thiosulfate. Bars represent means ± SE (n = 3).

presence of the plant cDNA did not change GSHS activity in the mutant background (Table 1). The higher GSH level and higher γECS activity of clone 4 compared with clone 1 were probably linked to the presence of the chloroplast transit peptide in clone 1, which would not be cleaved in yeast and may impair protein folding.

Stress-Mediated Activation of γECS. The limited activity of the *AtγECS* gene product both *in vitro* and *in vivo* and the inability of enhanced substrate supply to increase intracellular GSH concentrations in exponentially growing mutant yeasts transformed with clones 1 and 4 raised the question of whether the *AtγECS* gene product required additional plant-specific factors for full activity. To test this hypothesis, we used a system based on an *Arabidopsis* cell suspension, in which GSH accumulation can be provoked by mild oxidative stress (18).

Table 1. Glutathione biosynthetic enzyme activity in yeast strains

Strain*	γECS activity†	GSHS activity†
W303a	11.48 ± 1.85	50.32 ± 2.39
Clone 1	1.86 ± 0.02	19.47 ± 1.88
Clone 4	2.72 ± 0.51	23.46 ± 1.18
Mutant	0.59 ± 0.00	22.05 ± 0.16

*W303a, parental strain; clone 1 and clone 4, mutant strains transformed with the plasmids harboring the corresponding cDNAs; mutant, W303a in which the *γECS* gene has been disrupted with the *LEU2* gene.

†γECS and GSHS activity in extracts of the respective yeast strains expressed as nmol product/min per mg protein. Values are the means of three experiments ± SE.

Exponentially growing *Arabidopsis* cell cultures were treated with compounds that are known to elicit a rapid accumulation of GSH (J.-P. Reichheld, personal communication; R.S.-F., unpublished data) or phytochelatins (measured as NPT). Supplementation of 2 mM AT (an inhibitor of catalase) (18) and treatment with the safener FC (10 μM) both caused marked increases, with similar kinetics, in the cellular concentration of GSH (Fig. 2*A*). After 1 h, the intracellular GSH level had risen to approximately 1.2-fold the initial level, reaching a maximum at 4 h (1.7-fold above control cultures at the initial value). The addition of 20 μM MQ (a superoxide-generating quinone) also caused a substantial increase in the cellular concentration of GSH, reaching a maximum after 6 h (2.2-fold the initial value). However, the kinetics of this response were different from those observed for AT and FC treatments: changes in the level of GSH were only detectable 2 h after the onset of treatment and thereafter increased linearly (Fig. 2*A*). The accumulation of NPT after treatment with 20 μM Cd followed similar kinetics although increases in the intracellular NPT were more significant than those of GSH after MQ treatment: 3.4-fold the initial value (Fig. 2*B*). Although specific treatments elicited specific changes in the metabolism of

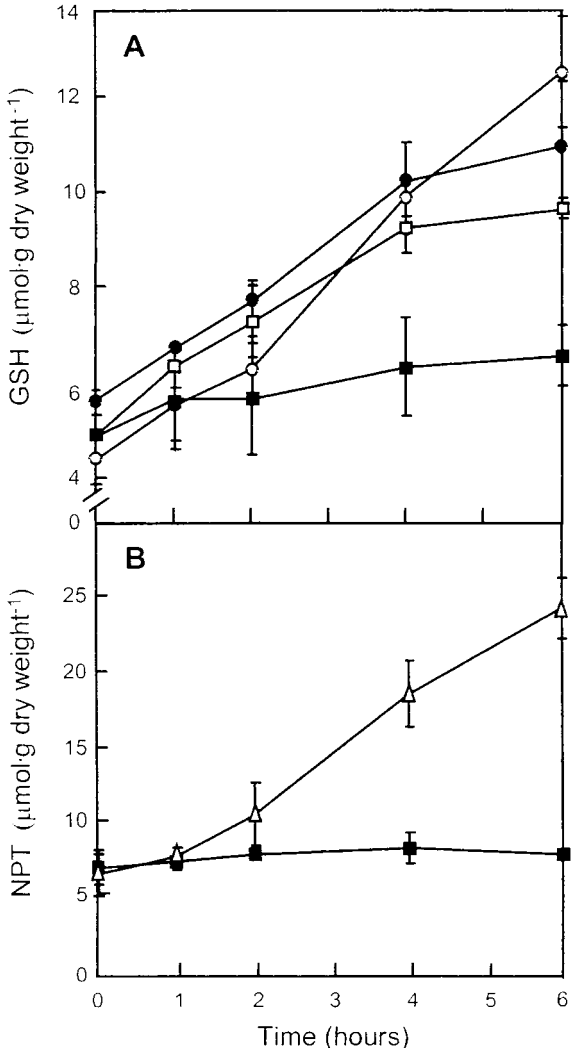

FIG. 2. (*A*) GSH content in exponentially growing *Arabidopsis* cell suspensions incubated with no supplement (solid squares) or in the presence of 2 mM AT (open squares), 10 μM FC (solid circles), or 20 μM MQ (open circles). Samples were taken at the indicated time points. (*B*) NPT content in *Arabidopsis* suspensions grown identically (solid square, control) or supplemented with 20 μM Cd (open triangles). Bars represent SE (n = 2).

GSH, a common feature of these responses was that they were rapid.

To elucidate the molecular basis for these kinetically different responses, we determined the steady-state level of mRNA corresponding to *At*γ*ECS* and *AtGSHS* by Northern blot analysis. Throughout the course of all experiments, whatever the treatment, steady-state mRNA levels for both genes did not vary significantly compared with those of the control (Fig. 3). These results indicated that posttranscriptional modifications of the GSH biosynthetic pathway may be responsible for the changes in the cellular concentration of GSH (or NPT) measured. To address this possibility, the activities of γECS and GSHS were measured in extracts obtained from *Arabidopsis* cell samples taken at 0, 1, 2, and 4 h after drug administration. For all treatments the activity of GSHS remained relatively invariant (1.5–2 nmol/mg per min). In contrast, significant increases in the activity of γECS were evident for all treatments compared with the control (Fig. 4). Interestingly these increases in the γECS activity paralleled the increase in the cellular concentration of GSH (or NPT) for each treatment (Fig. 2*A*). At 1 h after AT and FC treatments, a substantial increase in γECS activity was measured, reaching a plateau at 2 h, whereas with MQ and Cd treatment increases in γECS activity were detectable only after 2 h (Fig. 4). Thus, the two GSH accumulation kinetics matched the two types of γECS activity activation.

Given the rapidity with which γECS was activated after AT treatment of the *Arabidopsis* culture, we wished to investigate in finer detail the kinetics of this response. To do this, AT was added to the *Arabidopsis* suspension culture to a final concentration of 2 mM and samples were taken every 15 min during 1 h. An increase in the activity of γECS was already detectable at 15 min and the activity progressively reached a maximum at 45 min (Fig. 4 *Insert*).

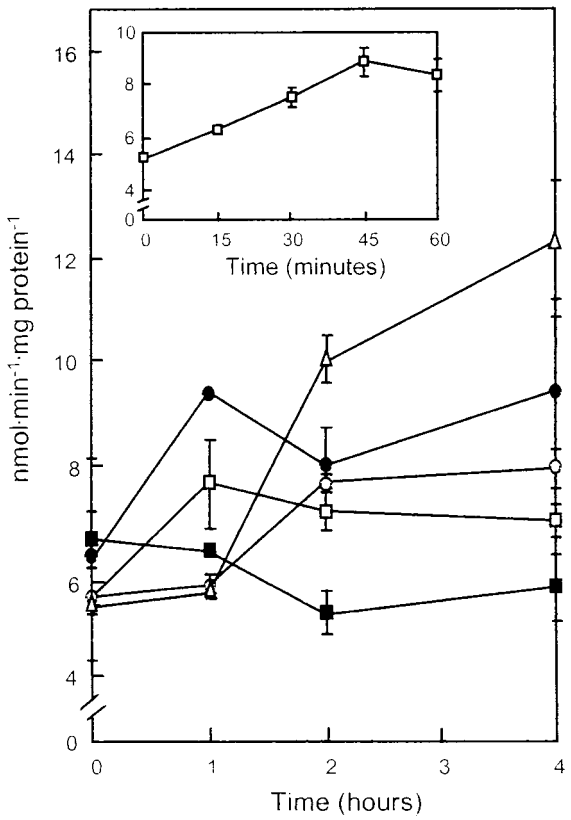

FIG. 4. γECS activity in exponentially growing *Arabidopsis* suspension cultures grown under control conditions or subjected to 2 mM AT, 20 μM MQ, 20 μM Cd-sulfate, or 10 μM FC treatments. At the times indicated after the onset of treatment, extracts were prepared for measurement of γECS activity. GSHS activities were measured in the same extracts and were not found to vary between treatments and with time (data not shown). Symbols are as in Fig. 2. (*Inset*) γECS activity in exponentially growing *Arabidopsis* suspension cultures subjected to 2 mM AT. Enzyme activities are expressed as nmol product/min per mg protein.

DISCUSSION

Most cellular processes tolerate only minor perturbations in redox potential, and in most organisms GSH functions to provide adequate redox buffering. Given the impact of environmental stress on the redox status of the cell and consequent risk of metabolic imbalance, stress tolerance will depend not only on the severity of the stress but also on the capacity for inducible mechanisms to increase the supply of antioxidants and thus sustain efficient redox buffering. The efficiency with which stress is perceived and the speed with which restoration of redox homeostasis is deployed may be essential for stress tolerance. The importance of these concepts is borne out through the finding that among the targets of the stress sensors of yeast and animals is the gene encoding γECS, and inactivation of these sensors leads to oxidative stress hypersensitivity (22, 23). Similar mechanisms may exist in plants, because increases in GSH levels are often observed in response to oxidative stimuli, and in some cases increases in the intracellular GSH correlate with tolerance to a number of stresses (3, 18, 24). A major objective of the present study was to gain a better knowledge of how GSH synthesis is regulated under stress. An additional priority was to clarify open questions

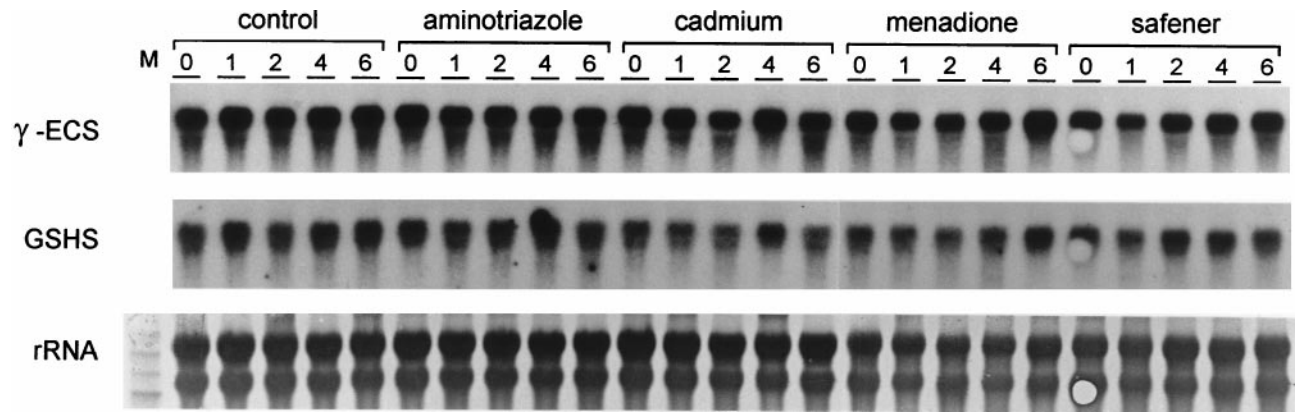

FIG. 3. Steady-state levels of mRNA corresponding to *At*γ*ECS* and *AtGSHS* during stress treatments. Stress treatments were as described in Fig. 2. RNA was extracted at the time points (hours) indicated after the onset of treatment. The levels of γ*ECS* and *GSHS* mRNAs were visualized by Northern blot analysis, and the membrane was stained previously with methylene blue to control equal RNA loading.

concerning the identity of the *At*γ*ECS* clone isolated previously (8, 9).

Disruption of the γ*ECS* gene of yeast renders it inviable when grown aerobically on minimal media, although such mutants can be rescued by exogenous supply of GSH (10, 11). This phenotype was exploited in a screen for *Arabidopsis* cDNAs that rescue the mutant phenotype through restoration of the endogenous GSH pool. By using this approach, we isolated cDNAs that restored growth of the mutant in the absence of exogenous GSH. Two of these cDNAs were taken for further analysis and were found to encode the same amino acid sequence as the previously described *At*γ*ECS* cDNA (8). Thus, complementation approaches using two different heterologous hosts, two different cDNA libraries, and two highly stringent screening strategies yielded the same clone. The identity of the cDNAs as γ*ECS* was unequivocally proven by measurement of γECS activity of YΔγ*ECS* transformed with clones 1 and 4 (Table 1). Although the γ*ECS* null mutant phenotype could, in theory, be used to isolate cDNAs that suppress the phenotype by GSH-independent bypass of the mutation, we have never obtained such clones. This would suggest that the only way to rescue such mutants is through expression of γ*ECS* sequences.

The cDNA clones isolated encoded AtγECS, but the level of GSH in the yeast transformants was only 10% that of the wild type. Although many hypotheses could be envisaged to explain the discrepancy between the level of GSH in the transformed mutants and the parental strain, it is known that GSH biosynthesis is held out of equilibrium by substrate availability, notably Cys (5, 6). Increased supply of intermediates of the sulfur assimilation pathway did not significantly alter the GSH level in the transformants. A number of lines of evidence suggest that the discrepancy therefore may arise through features intrinsic to the protein encoded by *At*γ*ECS* itself: the level of GSH correlates with the extractable γECS activity whether *At*γ*ECS* is expressed on a multicopy plasmid in γ*ECS*-deficient *E. coli* (8) or yeast (this study) and never exceeds 10% of the wild type. Furthermore, expression of a genomic clone corresponding to *At*γ*ECS* in a γ*ECS*-deficient *Arabidopsis* mutant raised the cellular concentration of GSH up to 2.3-fold of that of the wild type (M.J.M., unpublished data). The regulation of plant γECS thus appears to be rather complex, and plant-specific factors, absent in heterologous hosts, may be necessary for full catalytic activity.

To address the possibility of the existence of factors that interact directly with γECS and modulate its activity, we studied the regulation of GSH biosynthesis in *Arabidopsis* cell suspensions. We and others have described previously a variety of treatments that lead to increases in cellular GSH (Fig. 2). Under the conditions used, none of the treatments altered the steady-state level of mRNA corresponding to *At*γ*ECS* or *AtGSHS* (Fig. 3), showing the absence of stress-mediated transcriptional regulation in contrast to the situation in animals (25). Rather, increases in the level of GSH correlated with increases in the activity of γECS (Fig. 4) but not GSHS. This demonstrates the existence of posttranscriptional mechanisms that specifically and rapidly activate γECS, the limiting enzyme on GSH biosynthesis. Such a response would allow adequate supply of GSH to accommodate the demands placed on redox buffering under stress.

Most importantly, we provide evidence that the kinetics of changes in the activity of γECS is adapted to the type of stress eliciting the response. The activation of γECS and subsequent increases in the cellular GSH induced by AT and FC occurred within the first hour of treatment followed by a plateau; those induced by Cd and MQ displayed a distinct lag phase followed by a linear increase. It is important to note that AT and FC, which elicit responses of similar kinetics, both contain a 1,2,4-triazole moiety. Considerable increases in the pool of GSH have been measured in whole plants in response to chilling (3), heat shock (24), pathogen attack (26–28), AOS accumulation (18), air pollution (29, 30), and drought (31). A concerted effort has been made to identify the molecules that elicit this common response. Attention has been focused on molecules common to each stress, notably H_2O_2. Here, we present evidence that the situation is much more complex than previously envisaged. Discrete signal transduction pathways exist that permit discrimination between stresses of different nature and that activate appropriate responses adapted to specific stimuli. These results imply the existence of one or more proteins that act as stress sensors and relay this information to molecules that interact directly with γECS. In theory, a single sensor protein could be able to discriminate between stresses, and evidence for such mechanism recently has been demonstrated in the responses of yeast and animals to oxidative stress arising from different stimuli (32). As an adaptive response, such layers of complexity are of obvious importance, given the central role of GSH in the maintenance of cellular integrity under stress. Candidates that could directly and rapidly activate γECS are protein kinases or phosphatases, as is known for animal γECS (33). Indeed, sequence analysis of *At*γ*ECS* using the PROSITE program identified potential amino acid motifs that may be phosphorylated by protein kinase A, protein kinase C, and CAM-dependent kinase. The latter is of particular interest because the motif is located in the proposed active site of the enzyme (Fig. 5).

In conclusion, although we have unequivocally proven the identity of the *At*γ*ECS* clone, this work raises the question as to why the primary structure of *Arabidopsis* γECS has evolved to be so distinct from that of other organisms. This is all the more surprising given that we have also demonstrated γECS to be the rate-limiting step in a pathway responsible for the production of an evolutionarily conserved molecule of such central importance in so many cellular processes. The combination of genetic and biochemical approaches will permit structure–function analysis and the identification of the molecules that interact with different regions of the protein. This will allow us to determine whether the divergence of the *Arabidopsis* γ*ECS* gene reflects the existence of specific structures adapted to meet the demands for GSH supply in *Arabidopsis*. The same may be true for other plant species as well because cDNAs encoding γECS from tomato share considerable sequence identity to the clones described here (34).

We gratefully acknowledge Paul De Vos for helping with yeast experiments, Michel Minet for providing the pFL61 expression library,

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T. brucei 315 MGCNCL.QLTMQLPNEAQARHIYDQLGILCPLFLALSSATPFQKGILCESDVRWLTITASVD.DR
A. thalia 247 MLRTCTVQVNLDFSSEADMIRKFRAGLALQPIATALFANSPFTEGKPNGFLSMRSHIWTDTDKDR
R. norveg 245 MGNCCL.QVTFQACSISEARYLYDQLATICPIVMALSAASPFYRGYVSDIDCRWGVISASVD.DR
S. cerevi 262 MGCSCL.QVTFQAPNINKARYLYDALVNFAPIMLAFSAAAPAFKGWLADQDVRWNVISGAVD.DR
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FIG. 5. Comparison of the amino acid sequence in the active site of γ*ECS* from different organisms. The derived amino acid sequence of γ*ECS* from *Trypanosoma brucei* (*T. brucei*), *Arabidopsis thaliana* (*A. thalia*), *Rattus norvegii* (*R. norveg*), and *Saccharomyces cerevisiae* (*S. cerevi*) corresponds to the active site prediction of Lueder and Phillips (35). Identical amino acids are shaded, the potential calmodulin-dependent kinase motif on the *A. thaliana* sequence is underlined, and the putative active-site cysteine is marked with an asterisk. Numbers to the left indicate the amino acid position on each sequence.

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