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Arabidopsis thaliana γ-glutamylcysteine synthetase is structurally unrelated to mammalian, yeast, and *Escherichia coli* homologs

(glutathione synthesis/functional complementation/expression cloning)

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ABSTRACT **A mutant of *Escherichia coli*, JTG10, deficient in γ-glutamylcysteine synthetase (γ-ECS; EC 6.3.2.2) is unable to synthesize glutathione (GSH) and is sensitive to 8-hydroxyquinoline. This phenotype was exploited for the isolation of *Arabidopsis thaliana* γ-ECS cDNAs by expression cloning, and clones were selected through functional complementation by growth on 8-hydroxyquinoline. High levels of γ-ECS activity were detectable in extracts derived from cultures of JTG10 expressing the *Arabidopsis* γ-ECS open reading frame, although these complemented mutants accumulated GSH to only 10% of the wild-type level. The derived amino acid sequence constitutes a polypeptide of 59.9 kDa and shows only 44–48% similarity with previously published sequences of rat kidney, human liver, yeast, and *E. coli* γ-ECS. When the γ-ECS cDNA was used as a probe, Southern blot analysis of *Arabidopsis* genomic DNA revealed that it is present as a low copy number gene. Furthermore, the *Arabidopsis* γ-ECS cDNA probe failed to hybridize to maize and tobacco genomic DNA at low stringency, suggesting that heterogeneity in γ-ECS structure exists between plant species. The activity of recombinant *Arabidopsis* γ-ECS was inhibited by buthionine sulfoximine and GSH, indicating that, while differences in the primary and secondary structure of γ-ECS from different sources exist, the enzymes may have similar active site structures.**

Mutations which affect the metabolism of the ubiquitous tripeptide thiol glutathione (GSH; L-γ-glutamyl-L-cysteinylglycine) are seriously debilitating for the host (1), since GSH plays a pivotal role in the control of redox status, the storage of reduced sulfur, the reduction of protein thiol groups, and the synthesis of DNA, and as an enzyme cofactor and an antioxidant (1–3). In plants, as in other organisms, GSH acts in concert with other antioxidants in the elimination of potentially damaging concentrations of reactive oxygen species which arise as by-products of metabolism and as a result of exposure to oxidative environmental stresses (for review see ref. 4). A crucial role for GSH has thus been proposed in the detoxification of air pollutants such as sulfur dioxide and ozone (5, 6), in the removal of xenobiotics such as herbicides (7) and heavy metals (8), and in the adaptation of plants to drought (9) and extremes of temperature (10). Significantly, GSH accumulates in response to oxidative stimuli in plants (4, 11), and despite the potential importance of this, little is known about the structural and kinetic properties of the enzymes responsible for its synthesis or the factors which regulate their activity.

GSH is synthesized in two ATP-dependent steps. In the first, catalyzed by γ-glutamylcysteine synthetase (γ-ECS; EC 6.3.2.2) the dipeptide γ-glutamylcysteine is produced from L-glutamic acid and L-cysteine:

$$\text{L-Glu} + \text{L-Cys} + \text{ATP} \rightarrow \text{L-}\gamma\text{-Glu-L-Cys} + \text{ADP} + P_i.$$

In the second step, catalyzed by glutathione synthetase (EC 6.3.2.3), glycine is added to the C-terminal site of γ-glutamylcysteine to form GSH:

$$\text{L-}\gamma\text{-Glu-L-Cys} + \text{Gly} \rightarrow \text{L-}\gamma\text{-Glu-L-Cys-Gly} + \text{ADP} + P_i.$$

The precise cellular location of GSH synthesis in plant cells is unclear; while it is known that chloroplastic and cytosolic forms of the enzyme exist, the proportion of the total cellular enzyme activity found in each compartment appears to vary in a species- and organ-specific manner (4).

As a first step to understanding GSH synthesis in plants, we report here the isolation of cDNA clones encoding γ-ECS from *Arabidopsis thaliana* by functional complementation of an *Escherichia coli* mutant deficient in this enzyme (12).† The structural and functional properties of *Arabidopsis* γ-ECS were compared with those of the enzyme from other sources.

MATERIALS AND METHODS

Bacterial Strains and cDNA Library. *E. coli*, γ-ECS-deficient strain JTG10 (F^-, thr^-, leu^-, $proA^-$, his^-, $argB^-$, thi^-, $strA^-$, $gshA^-$, Tn*10*:Kan^r), and the parental strain, AB1157, were a gift from B. Demple (12). The *Arabidopsis thaliana* cDNA plasmid expression library in λYES-R was a gift from R. Davis (13).

Isolation and Characterization of cDNA Clones. Preparation of JTG10 for electroporation and electrotransformation was carried out essentially as described by Van Camp *et al.* (14). After electroporation using 1 μg of the cDNA library, cells were plated on minimal medium containing 0.8% agar and 1× M9 salts (ref. 15, Appendix A3) supplemented with 8-hydroxyquinoline (8-HQ) at 10 $\mu g \cdot ml^{-1}$, ampicillin at 100 $\mu g \cdot ml^{-1}$, kanamycin at 50 $\mu g \cdot ml^{-1}$, 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), and glucose at 1 $g \cdot liter^{-1}$. Colonies which appeared after 4 days of incubation at 37°C were selected for further analysis. The sequences of complementing plasmid inserts were determined on both strands by subcloning cDNA inserts from pYES-R in pBluescript II KS(+) (Stratagene) and using the dideoxynucleotide chain-termination method (16). Sequence analysis was performed by using the University of Wisconsin Genetics Computer Group software package. The five cDNA clones analyzed were named pM1A, pM2A, pM5A, pM8A, and pM10A. The

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Abbreviations: BSO, buthionine sulfoximine; γ-ECS, γ-glutamylcysteine synthetase; γ-EC, γ-glutamylcysteine; GSH, glutathione; 8-HQ, 8-hydroxyquinoline; IPTG, isopropyl β-D-thiogalactopyranoside.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. Z29490).

same cDNA inserts subcloned in pKS(+) were named pM1B, pM2B, pM5B, pM8B, and pM10B. The longest cDNA clone, pM2B, was further analyzed.

GSH Assay. GSH was extracted from leaves and roots or bacterial pellets in 2 vol of 5% (wt/vol) sulfosalicylic acid and the concentration was determined as described (11).

Enzyme assays. Extracts were prepared from *E. coli* by treatment with lysozyme to 100 $\mu g \cdot ml^{-1}$ and two cycles of freezing and thawing. Protein was determined according to the method of Bradford (17). The extracts were desalted on Sephadex G-50 in 100 mM Tris·HCl, pH 8.2/40 mM $MgCl_2$ to remove γ-EC, cysteine, and GSH, which could interfere with subsequent assays. The standard assay reaction mixtures for the determination of γ-ECS activity contained 100 mM Tris·HCl at pH 8.2, 10 mM L-glutamate, 10 mM L-α-aminobutyrate, 40 mM $MgCl_2$, 5 mM ATP, and enzyme extract in a final volume of 0.15 ml. The release of inorganic phosphate (P_i) in the presence and absence of substrates was determined as described by Chifflet *et al.* (18). Enzyme activity is expressed as nmol of P_i released per min per mg of protein.

Southern and Northern Blot Analysis. Genomic DNA was isolated from leaves of *Arabidopsis thaliana* (var. Landsberg erecta) according to Dellaporta *et al.* (19). Ten-microgram samples of DNA were digested with *Eco*RI, *Bam*HI, *Hin*dIII, or *Hin*cII, fractionated by agarose gel electrophoresis, and transferred to Hybond N (Amersham) (18). Filters were incubated at 65°C with a 1975-bp fragment of the coding sequence of pM2A produced by digestion with *Xho* I and labeled with [α-^{32}P]dCTP. The hybridization solution for Northern and Southern blots contained 5× SSC (1× SSC is 150 mM NaCl/15 mM sodium citrate, pH 7.0), 1% SDS, 5× Denhardt's solution (1× is 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 0.1% tetrasodium pyrophosphate, and denatured salmon sperm DNA at 200 $\mu g \cdot ml^{-1}$. Filters were washed in 5× SSC/1% SDS/0.1% tetrasodium pyrophosphate for 30 min at 55°C (low stringency) or to a final stringency of 0.1× SSC/0.1% SDS at 65°C (high stringency) and exposed to X-Omat AR film (Kodak). Plants for root RNA isolation were grown in sterile liquid culture in 250-ml Erlenmeyer flasks with 50 ml of Murashige and Skoog basal medium containing 1% sucrose at 18°C with an 8-h light, 16-h dark cycle and constant swirling at 100 rpm. RNA was extracted from roots and leaves by using a guanidinium isothiocyanate extraction method (20), subjected to denaturing formaldehyde/agarose gel electrophoresis, and transferred to Hybond N (ref. 15, pp. 7.46–7.48). Filters were probed with a 1975-bp *Xho* I fragment from pM2A and hybridization was carried out as above.

RESULTS

Isolation of *Arabidopsis* γ-ECS cDNA Clones. Suitable DNA probes or antibodies were not available to screen a plant cDNA library for γ-ECS sequences. We therefore used an alternative strategy based upon the complementation of a γ-ECS-deficient *E. coli* mutant, JTG10 (12) in which the gene encoding γ-ECS (*gshA*) has been disrupted by transposon mutagenesis, resulting in an inability to synthesize GSH. Absence of γ-ECS in *E. coli* renders it sensitive to xenobiotic compounds that are removed by a GSH-dependent mechanism (21). The mutant JTG10 was unable to grow on minimal medium containing 8-HQ at 10 $\mu g \cdot ml^{-1}$ while the parental strain, AB1157, formed viable colonies on the same medium (data not shown). JTG10 was transformed with 1 μg of an *Arabidopsis* cDNA expression library (13), and clones that expressed a putative *Arabidopsis* γ-ECS were selected by their ability to grow on minimal medium containing 8-HQ at 10 $\mu g \cdot ml^{-1}$, ampicillin at 100 $\mu g \cdot ml^{-1}$, kanamycin at 50 $\mu g \cdot ml^{-1}$, and 1 mM IPTG. Transposon-mediated inactivation of the bacterial *gshA* gene was selected by the inclusion of kanamycin in the medium at 50 $\mu g \cdot ml^{-1}$. Growth results from IPTG-dependent expression of cDNA inserts encoding enzymes that complement the γ-ECS deficiency of JTG10.

Thirteen colonies appeared after 3 days of growth at 37°C. Plasmid DNA from these colonies was prepared and used to retransform JTG10. Only five of these plasmids permitted growth on minimal medium supplemented with 8-HQ at 10 $\mu g \cdot ml^{-1}$ (Fig. 1). Curing experiments in which one of the plasmids (pM2A) was lost from JTG10 by repeated subculturing on medium lacking ampicillin demonstrated that, in the absence of the plasmid, the mutant lost its resistance to 8-HQ (Fig. 1). Restriction enzyme analysis of the pYES-R plasmids giving rise to the 8-HQ-resistant phenotype and Southern blot hybridization using an *Xho* I fragment of pM2A as a labeled probe demonstrated that all five plasmids contained inserts with similar restriction patterns and significant cross-homology (data not shown). These data demonstrate that 8-HQ resistance in the mutant JTG10 is dependent upon the presence of the plasmid pM2A.

GSH Content and γ-ECS Activity of *E. coli*. Analysis of the GSH levels in the complemented mutant showed that the presence of pM2A raised the intracellular concentration of GSH in JTG10 from below the limit of detection to 0.4 nmol per 10^9 cells, and this could be enhanced to 0.5 nmol per 10^9 cells by growing the bacteria in the presence of 1 mM IPTG, albeit to levels much lower than in the parental strain, AB1157 (5.5 nmol per 10^9 cells). Presumably GSH accumulates in the absence of IPTG because multiple copies of the *lac* promoter titrate out endogenous *lac* repression (22). Curing JTG10 of the plasmid reduces the levels of GSH to below the limit of detection. Attempts to raise the level of GSH in the complemented mutant by provision of cysteine and glutamate in the growth medium were without effect.

γ-ECS activity was determined by measuring the release of P_i from ATP in the presence of the substrates L-glutamate and L-α-aminobutyrate. While L-cysteine is the natural substrate for γ-ECS, it is convenient to replace it by L-α-aminobutyrate, since the latter amino acid does not undergo spontaneous oxidation and is an effective substrate for the enzyme (23). Under the conditions described, release of P_i was linear over 10 min in the presence of 10–100 mg of protein in the assay mixture. P_i release was proportional to the amount of extract or substrates and was not detectable in assays with boiled enzyme or without Mg^{2+}, glutamate, α-aminobutyrate, ATP, or enzyme extract. γ-ECS activity was detected in extracts of JTG10 expressing the pM2A open reading

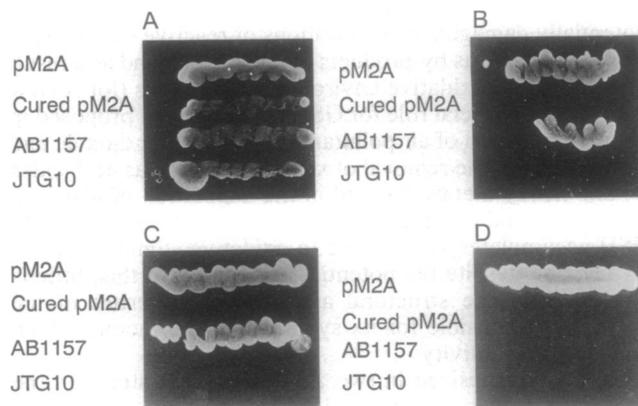

FIG. 1. Complementation of 8-HQ sensitivity in *E. coli* mutant JTG10 by pM2A. (*A*) Growth on minimal medium. (*B*) Growth on minimal medium supplemented with 8-HQ at 10 $\mu g \cdot ml^{-1}$. (*C*) Growth on minimal medium supplemented with 8-HQ and 1 mM IPTG. (*D*) Growth on minimal medium supplemented with 8-HQ, IPTG, kanamycin at 50 $\mu g \cdot ml^{-1}$, and ampicillin at 100 $\mu g \cdot ml^{-1}$.

Table 1. γ-ECS activity measured in *E. coli* extracts

Treatment of extract	γ-ECS activity, nmol P_i per min per mg protein	
	JTG10/pM2A	AB1157
None (− IPTG)	3.45 ± 0.04	25.66 ± 0.06
None	11.54 ± 0.16	26.57 ± 0.23
GSH (1 mM)	3.85 ± 0.04	23.42 ± 0.56
GSH (5 mM)	ND	18.23 ± 0.27
DTT (1 mM)	10.33 ± 0.11	25.52 ± 0.08
DTT (5 mM)	9.47 ± 0.22	25.44 ± 0.21
BSO (100 mM)	5.67 ± 0.12	14.35 ± 0.24
BSO (1 mM)	ND	ND

Except where indicated, all extracts were prepared from *E. coli* grown in the presence of 1 mM IPTG. Results are presented as mean ± SEM. DTT, dithiothreitol; BSO, buthionine sulfoximine; ND, not detected.

frame (Table 1) but not in untransformed controls (data not shown). Enzyme activity was stable for up to 2 days at 4°C and extracts could be stored frozen at −70°C and thawed with no detectable loss of activity. Unlike observations of the tobacco enzyme (23), addition of the reducing agents dithiothreitol or dithioerythritol to the lysis buffer or to the crude extract had no significant effect on enzyme activity, indicating that the differences in primary structure as revealed by Southern hybridization (see below) are reflected in functional differences. Incubation of the extracts for 20 min with 100 μM or 1 mM BSO resulted in a 48.5% or 100% decrease in activity, respectively (Table 1). Thus, in common with γ-ECS from all sources studied to date (23, 24), recombinant *Arabidopsis* γ-ECS is inhibited by γ-glutamyl phosphate analogs such as BSO *in vitro* (Table 1) and *in vivo* (11). Similarly, the enzyme from all sources is sensitive to feedback inhibition by GSH (23, 25). Taken together, these lines of evidence prove unambiguously that the cDNA insert contained in pM2A encoded an *Arabidopsis* γ-ECS. To the best of our knowledge, the cloning of a cDNA encoding an enzyme responsible for the biosynthesis of GSH from higher plants has not been reported previously.

Sequence Analysis. The nucleotide sequence of the 1975-bp DNA insert contained on pM2B was determined (Fig. 2). Partial sequences of pM1B, pM8B, and pM10B were also determined and were found to be identical to the corresponding regions on pM2B (data not shown). Therefore, all the cDNAs isolated belong to a single class corresponding to the locus we have designated *GSHA*. The 1975-nt sequence consists of a 114-nt 5′ untranslated region that precedes the first ATG, a 1620-nt open reading frame encoding 571 amino acids between nucleotides 115 and 1716, and a 268-nt 3′ untranslated region containing a 27-nt inverted repeat. While the identity of the initiator methionine is not known, the sequence surrounding the first ATG codon, starting at nt 115, conforms to the consensus sequence (AACAAUGGC) for the initiator methionine in plants. The derived polypeptide has a predicted molecular mass of 59.9 kDa. Computer-aided comparison of the amino acid sequence of *Arabidopsis* γ-ECS to the γ-ECS sequences from rat (26), human (27), *E. coli* (28), and yeast (29) revealed only 15–18% identity and 43–48% similarity. In only two regions was any extended amino acid sequence identity observed. The first region, amino acids 116–131, contains 10 of 16 amino acids which are identical to a region in pea glutathione reductase, amino acids 455–468, thought to be involved in binding of oxidized GSH (30) (Fig. 3*A*). This is of potential significance, since γ-ECS from all sources binds GSH. Thus, given the common element in all of the proteins and since they all interact with GSH, it is tempting to suggest that this region of homology reflects shared function. A further region of interest, amino acids

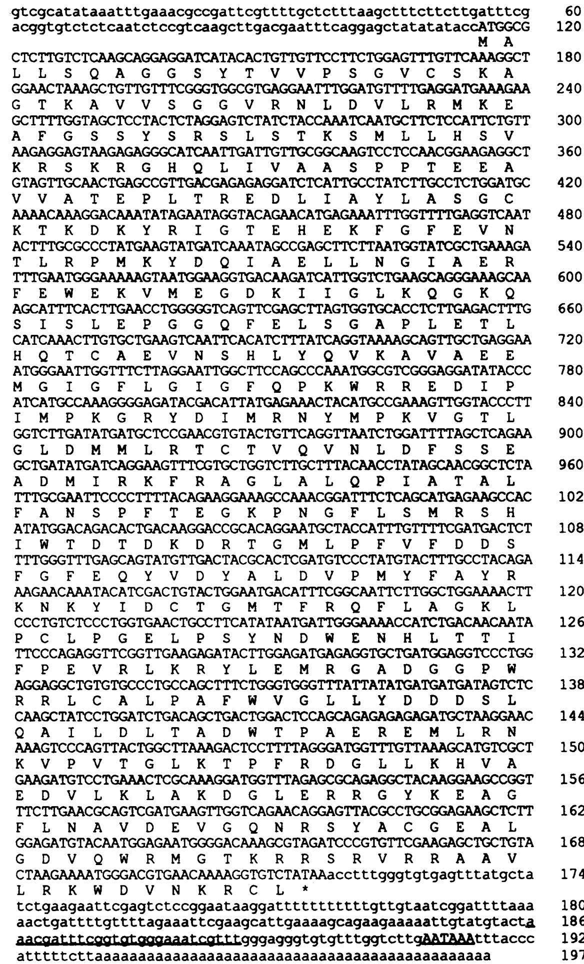

FIG. 2. Nucleotide and deduced amino acid sequences of pM2B encoding *Arabidopsis* γ-ECS. The nucleic acid sequence is presented on the top line with the derived amino acid sequence below. Amino acid residues are numbered on the left; nucleotide positions are numbered on the right. The 5′ and 3′ untranslated regions are shown in lowercase type. Uppercase type indicates the sequence from the first ATG, to the stop codon TAA. An inverted repeat is in lowercase underlined type and the putative poly(A) addition signal is in uppercase underlined type.

409–431, was identified which shared sequence homology with motifs of presumed functional importance in the amino acid sequence of glutathione *S*-transferases (Fig. 3*B*; refs. 31–33).

Genomic Southern Blot Analysis. Genomic Southern blot analysis was performed to estimate the copy number of *GSHA* in the *Arabidopsis* genome and to make a preliminary comparison of the structural relationships of *GSHA* between various plant species. Hybridization of the entire cDNA (pM2B) to a single DNA fragment in the *Bam*HI digest and to three fragments in a *Hin*cII or *Hin*dIII digest of *Arabidopsis* genomic DNA (Fig. 4*A*) indicates that the corresponding gene is represented by only a few copies in the *Arabidopsis* genome, given the known restriction map of the cDNA clone pM2A (Fig. 4*B*). Remarkably, under low-stringency conditions, only weak hybridization of the *Xho* I coding sequence fragment to maize DNA was seen and no hybridization to tobacco DNA was seen, and the hybridization to maize DNA was lost after stringent washing (data not shown). Thus the γ-ECS of other plant species analyzed here appears to be unrelated at the level of primary structure.

Northern Blot Analysis. Northern blot analysis of total RNA isolated from *Arabidopsis* root and leaves was used to determine the pattern of expression of the gene correspond-

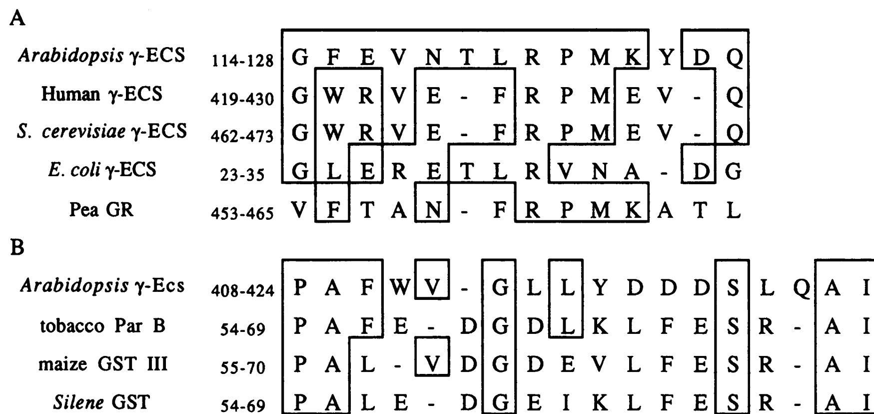

FIG. 3. (*A*) Comparison of the derived amino acid sequences of pM2B with the putative oxidized GSH binding site conserved between glutathione reductase (GR) from a number of sources and γ-ECS from rat (27), human (28), *E. coli* (29), and *Saccharomyces cerevisiae* yeast (30). (*B*) Partial amino acid sequence comparison of γ-ECS with glutathione *S*-transferases (GST) from *Silene* (31), tobacco (32), and maize (33). The sequences are aligned for maximal homology; dashes indicate gaps.

ing to the cDNA isolated. An *Xho* I fragment containing the entire cDNA hybridized to an abundant mRNA of ≈2 kb; equal levels of expression were seen in roots and leaves (Fig. 5).

DISCUSSION

Details of the structural and kinetic properties of plant γ-ECS and its precise subcellular location are presently lacking. Consequently the factors regulating the synthesis of GSH in plants cannot be fully evaluated. This report, which describes the isolation of a cDNA encoding an *Arabidopsis* γ-ECS by functional complementation of an *E. coli* mutant deficient in this enzyme, is therefore of primary importance in our current efforts to understand the details of GSH metabolism in higher plants.

Chloroplastic and cytosolic forms of γ-ECS exist in plants (4). The N-terminal residue of the mature protein is unknown, and we therefore cannot predict the subcellular location of the polypeptide encoded by the cDNA isolated. However, two lines of evidence suggest that the cDNA described in this paper corresponds to the chloroplastic form of the enzyme. First, the N-terminal region of the derived amino acid sequence of the clone described here is rich in hydroxylated and hydrophobic amino acids, a feature typical of chloroplast transit peptides. Second, preliminary experiments demonstrated proteolytic cleavage of *in vitro* translated pM2B by a partially purified pea signal peptide protease and uptake and processing of the protein into isolated chloroplasts *in vitro* (data not shown). Presumably in the roots, where high levels of *GSHA* mRNA were detected, the encoded polypeptide is targeted to the proplastids, where, in maize roots at least, high levels of γ-ECS activity can be measured (34). DNA gel blot analysis demonstrated that the gene corresponding to the cDNA described, *GSHA*, is represented by only one or two copies in the *Arabidopsis* genome. It is likely that in plants, as in other eukaryotes, mitochondrial forms of the enzyme do not exist (4). To determine the extent of sequence conservation of γ-ECS among higher plants, DNA gel blot analysis was carried out, using the *Arabidopsis* γ-ECS cDNA as a probe to genomic DNA from maize and tobacco. Surprisingly, this probe did not hybridize to tobacco DNA and only a weak signal was detected against maize DNA. This suggests that plant γ-ECS molecules are structurally highly divergent, and this is reflected in differences in the kinetic properties of the enzyme measured in extracts from *Arabidopsis* (M.J.M., unpublished data), recombinant *Arabidopsis* enzyme (this paper), maize (34), and tobacco (23). In direct contrast to tobacco, *Arabidopsis* γ-ECS activity is stable upon storage and is not inactivated by reductants. The tobacco enzyme is dimeric, the two subunits being held in an active conformation by disulfide bonds, and its activity cannot be detected in crude leaf extracts (23). Indeed, we were unable to detect γ-ECS activity in crude extracts of tobacco leaf, although this activity could be detected in crude extracts of *Arabidopsis* leaf tissue (M.J.M., unpublished data). Measurement of recombinant *Arabidopsis* γ-ECS activity suggests the enzyme is monomeric, since it is not susceptible to dissociation and subsequent inactivation by reductants. The maize enzyme is active in the presence of dithiothreitol, can be measured in crude extracts, and thus appears to be more similar to the *Arabidopsis* enzyme—a proposition supported by weak, yet significant, hybridization of the cDNA to maize genomic DNA at low stringency.

It has previously been proposed that γ-ECS has evolved independently in different organisms and yet functionally similar active centers have been achieved. Functionally,

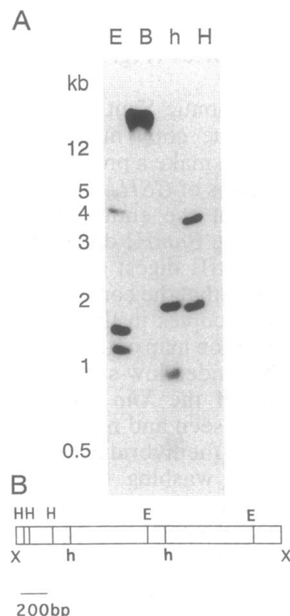

FIG. 4. (*A*) Genomic Southern analysis of *Arabidopsis* γ-ECS. Total genomic DNA (5 μg) was digested with *Eco*RI (lane E), *Hin*dIII (lane H), *Bam*HI (lane B), or *Hin*cII (lane h), separated by electrophoresis on a 0.8% agarose gel, transferred to a Hybond-N membrane, and hybridized with a 1975-bp coding region fragment of pM2A. Size markers (1-kb ladder; BRL) are indicated to the left. (*B*) Restriction map of the pM2A cDNA. H, *Hin*dIII; E, *Eco*RI; h, *Hin*cII; X, *Xho* I.

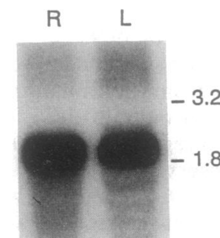

FIG. 5. Expression of γ-ECS in *Arabidopsis* leaf (lane L) and root (lane R). RNA (10 μg) was separated on formaldehyde/agarose gels, transferred to Hybond-N, and hybridized with a ^{32}P-labeled γ-ECS coding region probe. Ribosomal RNA was used as size markers (right, in kb).

enzymes from all organisms catalyze the same reaction and exhibit the same substrate specificity and apparent K_m values; all enzymes are feedback inhibited in a nonallosteric manner by GSH (Table 1; refs. 1, 23, and 25), and all are inhibited by sulfoximine analogs of γ-glutamyl phosphate (23, 24). However, sequence analysis suggests that the five γ-ECS sequenced to date are structurally dissimilar. The rat (26) and human (27) enzymes are composed of a large (73-kDa) subunit responsible for enzyme activity and a small (27.7-kDa) subunit (35). The derived amino acid sequences of rat and human large subunits share an 89% sequence identity. In contrast, the structurally dissimilar *E. coli* enzyme (28) is a single 60-kDa polypeptide and shows only 8% amino acid sequence identity to the rat and human sequences; furthermore, the two classes of enzyme have no antigenic determinants in common (25). The yeast enzyme falls into a third class, sharing higher sequence similarity with the rat and human enzymes than that of *E. coli* (29). Computer-aided comparison of the *Arabidopsis* γ-ECS to all of the available γ-ECS sequences revealed an extremely low level of identity (15–19%) and similarity (44–48%). The strongest overall similarity was found with rat, human, and yeast enzymes, although the predicted size of the polypeptide was closest to that of *E. coli*. It is possible that the *E. coli* enzyme represents the ancestral enzyme from which evolution occurred independently in eukaryotes to achieve a structure which permitted optimal activity in each of the respective organisms. This is perhaps reflected in the low GSH levels measured in *E. coli* extracts as a result of plant γ-ECS activity compared to the level in *E. coli* controls even when substrates were not limiting. This may be because the intracellular conditions within *E. coli* are not optimal for plant enzyme activity or that the presence of a putative chloroplast transit peptide or improper folding results in only a small proportion of the expressed protein being active. Alternatively, the low accumulation of GSH in the complemented mutant may be a result of the extreme sensitivity of the recombinant plant enzyme to feedback inhibition by GSH compared with the activity measured in crude extracts of *E. coli* (Table 1). Clearly, certain motifs which determine the activity of the enzyme have been retained and remain largely unaltered (Fig. 3A). We believe of particular relevance is the fact that in all of the regions of identity, the residues Lys and Arg appear to be involved. This is in agreement with recent proposals of a role for these residues in the binding of GSH to a broad class of enzymes which use GSH as a substrate (36, 37). Given the common element in all of the proteins and since they all interact with GSH, it is possible that this region may be responsible for GSH binding. However, we cannot, without further study, draw definite conclusions about functional significance of these sequences.

In conclusion, these findings are of importance, since they provide further evidence that γ-ECS has evolved independently in different organisms. They suggest too that details of GSH synthesis arising from study of the process in *Arabidopsis* will not necessarily be reflected in other plant systems. Furthermore, the simplicity of the *Arabidopsis* gene family and the stability of the *Arabidopsis* γ-ECS should greatly facilitate determination of the kinetic properties and physiological requirements of γ-ECS in higher plants. Such information will provide a significant contribution to closing the present gap in our knowledge as to how apparently structurally dissimilar enzymes have evolved to have similar catalytic activities. The availability of γ-ECS cDNA is of further practical importance in the construction of transgenic plants with a potentially altered capacity for GSH synthesis. These will be invaluable in detailed analyses of the role of GSH in plants.

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