The still mysterious roles of cysteine-containing glutathione transferases in plants

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Glutathione transferases (GSTs) represent a widespread multigenic enzyme family able to modify a broad range of molecules. These notably include secondary metabolites and exogenous substrates often referred to as xenobiotics, usually for their detoxification, subsequent transport or export. To achieve this, these enzymes can bind non-substrate ligands (ligandin function) and/or catalyze the conjugation of glutathione onto the targeted molecules, the latter activity being exhibited by GSTs having a serine or a tyrosine as catalytic residues. Besides, other GST members possess a catalytic cysteine residue, a substitution that radically changes enzyme properties. Instead of promoting GSH-conjugation reactions, cysteine-containing GSTs (Cys-GSTs) are able to perform deglutathionylation reactions similarly to glutaredoxins but the targets are usually different since glutaredoxin substrates are mostly oxidized proteins and Cys-GST substrates are metabolites. The Cys-GSTs are found in most organisms and form several classes. While Beta and Omega GSTs and chloride intracellular channel proteins (CLICs) are not found in plants, these organisms possess microsomal ProstaGlandin E-Synthase type 2, glutathionyl hydroquinone reductases, Lambda, Iota and Hemerythrin GSTs and dehydroascorbate reductases (DHARs); the four last classes being restricted to the green lineage. In plants, whereas the role of DHARs is clearly associated to the reduction of dehydroascorbate to ascorbate, the physiological roles of other Cys-GSTs remain largely unknown. In this context, a genomic and phylogenetic analysis of Cys-GSTs in photosynthetic organisms provides an updated classification that is discussed in the light of the recent literature about the functional and structural properties of Cys-GSTs. Considering the antioxidant potencies of phenolic compounds and more generally of secondary metabolites, the connection of GSTs with secondary metabolism may be interesting from a pharmacological perspective.

Keywords: cysteines, deglutathionylation, glutathione transferases, photosynthetic organisms, phylogeny

INTRODUCTION

Glutathione is a tripeptide with the sequence γGlu-Cys-Gly that is mostly present in reduced (GSH) or disulfide (GSSG) forms, even though nitrosogluthathione (GSNO) may represent another important source. Under physiological conditions, free glutathione is present in concentrations ranging from 1 to 10 mM with the reduced form largely predominating over the oxidized form (Gutscher et al., 2008; Pallardo et al., 2009). As such, glutathione is the major non-protein thiol source in eukaryote cells, likely constituting a crucial redox buffer (Rouhier et al., 2008). Glutathione can also fulfill additional roles. In eukaryotes, glutathione is essential for a proper development, controlling in particular cell-cycle progression. Apart from development, glutathione is crucial for stress response by (i) neutralizing radicals, (ii) participating in heavy metal tolerance, either directly or as a constitutive element of phytochelatins, (iii) contributing to the regeneration of antioxidant molecules such as ascorbate and α-tocopherol, and (iv) providing electrons and protons to glutathione transferases (GSTs) or to peroxiredoxins, both with and without the involvement of glutaredoxins (Grxs) for peroxide removal (Rouhier et al., 2008). While the exact role of glutathione has not been completely defined, it is clear that, depending on its redox state, glutathione can react with various intracellular molecules and that glutathionylation/deglutathionylation reactions of both proteins and smaller compounds are central to GSH functions. Protein glutathionylation is a reversible post-translational modification that is now recognized as a major signaling or protective mechanism. It occurs under basal non-stress conditions but has mostly been documented in response to oxidative stress conditions (Zaffagnini et al., 2012). The reversible reaction i.e., deglutathionylation, occurs either by the intervention of Grxs or by direct thiol/disulfide exchange reactions with GSH once an appropriate GSH/GSSG ratio has been restored.
In addition to proteins, glutathionylation of metabolites has also attracted a lot of attention as it constitutes an intermediate step in a number of metabolic processes and detoxification pathways. It has been well-established that most organisms possess a three-step detoxification system to eliminate endogenous and exogenous toxic compounds (Coleman et al., 1997; Morel et al., 2013). In the first step, enzymes such as cytochrome P450 monooxygenases catalyze various reactions (oxidation, reduction or hydrolysis) to expose or introduce a functional moiety on hydrophobic substrates. In the second step, conjugating enzymes perform addition reactions (e.g., acetyl, methyl, glucuronic acid) on these newly modified, electrophilic substrates. The glutathione addition onto electrophilic molecules is well-recognized and is mediated by specific classes of GSTs having usually a serine or a tyrosine as a catalytic residue. Finally, glutathionylated products are either exported from the cells or sequestered in vacuoles. In plants, GSTs have been identified by showing glutathionylation of the herbicide atrazine (Lamoureux et al., 1970). Most subsequent studies have focused on these GST types that are here referred to as glutathionylating GSTs, although other biochemical activities have been described for some GST classes. To cite a few, numerous GSTs exhibit GSH-dependent peroxidase activities reducing simple peroxides but also organic hydroperoxides (Tang and Tu, 1994; Marrs, 1996; Hurst et al., 1998). Theta GSTs were shown to catalyze the isomerization of maleylactoacetate into fumarylactoacetate, a key component of the catabolism of tyrosine and phenylalanine (Thom et al., 2001; Fernandez-Canon et al., 2002). Besides these catalytic functions, GSTs could also exhibit ligandin functions, binding hydrophobic substrates in a so-called L-site for transport and storage purposes. In plants, it has been documented that GSTs with ligandin properties are not only implicated in the transport of anthocyanins and flavonoids but also of hormones such as auxin and cytokinin, which suggests a possible role in cell signaling (Smith et al., 2003; Kitamura et al., 2004; Moons, 2005).

With the increasing number of biochemical studies, it became clear that several GSTs do not have a glutathionylation activity but instead catalyze the opposite reaction; deglutathionylation (Dixon and Edwards, 2010a,b; Xun et al., 2010; Board, 2011; Meux et al., 2011). This capacity usually originates from the replacement of the catalytic serine or tyrosine residues in the active site motif by a cysteinyl residue as demonstrated for marmalain, insect and fungal Omega GSTs (GSTOs) (Board et al., 2000; Kim et al., 2006; Yamamoto et al., 2009; Meux et al., 2013), for plant Lambda GSTs (GSTLs) (Dixon and Edwards, 2010b; Lallement et al., 2014) and for bacterial and fungal glutathionyl hydroquinone reductases (GHRs) (Xun et al., 2010; Meux et al., 2011). However, the physiological functions of these enzymes have rarely been elucidated. Human GSTOs may be involved in arsenic biotransformation, reducing methyl and dimethyl arsenate (Zakharyan et al., 2001; Burmeister et al., 2008), whereas plant GSTLs may be involved in flavonoid metabolism and/or trafficking (Dixon and Edwards, 2010b). Interestingly, while the role of GSTs has classically been associated to the modification of small molecules and the role of glutaredoxins to the deglutathionylation of proteins, it has recently been shown that human GSTO1-1 can de glutathionylate β-actin, which should prompt us to consider proteins as GST substrates (Menon and Board, 2013). Concerning GHRs, the bacterial and fungal members characterized so far are involved in the catabolism of chlorinated quinones and in lignin degradation through the deglutathionylation of glutathionylated intermediates (Reddy and Gold, 2001; Masai et al., 2003; Huang et al., 2008; Meux et al., 2011). While these glutathionylated compounds constitute intermediates in catabolism pathways, they may also constitute intermediates for biosynthetic pathways as shown for sulfur-containing defense molecules such as camalexins or glucosinolates (Su et al., 2011). However, the roles of GSTs in the secondary metabolism are less documented compared to the ones of cytochrome P450 monooxygenases and to their involvement in xenobiotic detoxification. A plausible explanation is that intracellular GSH-conjugated compounds have rarely been successfully identified from plant extracts, possibly due to their transient nature or to the difficulty to isolate them. As examples, glutathionylated compounds have been identified as precursors of aromas in fruits (Fedrizzi et al., 2012; Peña-Gallego et al., 2012) or as conjugated oxylipins upon leaf infiltration of keto-fatty acids (Davoine et al., 2005).

Based on the biochemical properties of GSTs and their functional association with cytochrome P450 monooxygenases, acquiring fundamental knowledge about GST functions, regulation and substrates may be beneficial for diverse pharmaceutical and biotechnological applications. In biotechnology, the ability of some GSTs to catalyze GSH-conjugation reactions has been exploited not only for the development of sensitive biosensors or enzyme assays for the determination of the concentration of various pesticides and herbicides (Chronopoulou and Labrou, 2009) but also for the development of herbicide and stress-tolerant plants. Moreover, among the thousands of natural plant products, including polyphenols, flavonoids, alkaloids, and quinones, several molecules possess antimicrobial, anticarcinogenic, anti-inflammatory, or antioxidant properties (Lewis and Ausubel, 2006; Saleem et al., 2010) not to speak about unidentified or untested molecules. Although they are often relatively low abundant molecules, they also constitute a recognized source of molecules important for the cosmetic industry (fragrance) or for nutrition (gustatory perception/dietary complements) contributing to extend the color or aroma palette. For instance, GSTs, notably those from the Pi and Alpha classes, are known to be present in the olfactory epithelium and particularly in the covering mucus layer, where they would serve for metabolizing odorant molecules. For example, Canfosfamide (TLK286, TELCYTA®), a cancer cell-activated prodrug, was designed to exploit the elevated levels and the activity of glutathione S-transferase P1-1 (GSTP1-1) that is overexpressed in many human cancer cells (Tew, 2005). Hence, GSTs could be useful for product transformation but also...
for synthetic biology or metabolic engineering approaches, with the aim of generating new chemical entities.

Over the past years, the GST classification has constantly evolved, notably due to the increase of genomic data and to the presence of particular isoforms in a specific subset of organisms. The objective of this review is to present an overview of cysteine-containing GST (Cys-GST) classes in photosynthetic organisms by describing known data concerning the gene expression, the protein subcellular localization and their biochemical and structural properties.

**THE GST FAMILY IN PHOTOSYNTHETIC ORGANISMS**

The present phylogenetic analysis focuses on photosynthetic organisms and as a basis uses the Cd00570 Sequence Cluster of the “conserved domains” tool in NCBI, which includes the GST classes that contain the typical N-terminal thioredoxin (Trx) domain found in GSTs. This cluster is part of the thioredoxin superfamily, among other well-known clusters such as thioredoxin, glutaredoxin, peroxiredoxin, protein disulfide isomerase (PDI), and disulfide bond A (DbA) oxidoreductase to name a few. The main criterion used is a minima the presence of the two classical GST domains, the N-terminal thioredoxin-like domain with a β1α1β2α2β3β4α3 topology and a C-terminal all-helical domain, that together form a typical GST fold. For this reason, Kappa GSTs and mPGES-1 (mesosomal ProstaGlandin E-Synthase type 1), one subclass of MAPEGs (Membrane Associated Proteins in Eicosanoid and Glutathione metabolism) (Bresell et al., 2005), enzymes often integrated into the GST superfamily, are not considered here, even though some terrestrial plants and algae possess at least one mPGES-1 representative. The phylogenetic analysis of all GSTs found in euakaryote photosynthetic organisms has been fitted to the aforementioned criterion, which allows identification of 14 classes (Figure 1). The sequences used were those present in model organisms including a gymnosperm: *Pinus tabulaeformis*, several angiosperms: *Arabidopsis thaliana*, *Populus trichocarpa*, *Oryza sativa*, *Solanum lycopersicum*, and *Hordeum vulgare*, a lycophyte: *Selaginella moellendorffii* and a moss: *Physcomitrella patens*.

Among these 14 classes, Tau, Phi, Zeta, Theta, and tetrachlorohydroquinone dehalogenase (TCHQD) classes clearly contain GSTs with a catalytic serine. The nature of the catalytic residue in the EF1By and Ure2p classes is less clear, but RIRT and APNG motifs are found at a position similar to the active site signature in other GSTs. Finally, the seven other classes (Iota GSTs, Hemerythin GSTs (GSTHs), Dehydroascorbate (DHA) reductases (DHARs), GSTLs, GHRs, mPGES-2s, and metaxins) contain members that clearly display a very conserved cysteine in the active site motif, hence suggesting that they belong to Cys-GSTs. Metaxins are part of the mitochondrial translocation system of the mitochondrial outer membrane, being anchored through their C-terminal region whereas the rest of the protein is oriented to the cytosol (Lister et al., 2007). However, they have not been integrated in this study. Indeed, although having the typical GST fold, none of the cysteine of the CPxC signature found in plant sequences is conserved in other organisms notably mammals, and there is no evidence for a cysteine involvement or for a requirement of GSH for their function. Concerning mPGES-2s, they were initially not considered as GSTs because GSH was not absolutely required for the detected activity e.g., the isomerization of ProstaGlandin H2 (PGH2) (Tanikawa et al., 2002) and because they exhibited a low similarity with GSTs identified at that time. However, based on (i) its typical GST structure, (ii) the identification of another activity strictly requiring GSH, and (iii) the identification of additional more closely related Cys-GSTs (Yamada et al., 2005; Takusagawa, 2013), mPGES-2s do in fact belong to the GST family. It is worth mentioning that Beta GSTs (GSTBs) and GSTOs as well as chloride intracellular channel proteins (CLICs), which belong to Cys-GSTs, are not found in plants. Moreover, particular proteins that are listed as putative GST members under the name “2-GST_N” have not been considered here. Although they have two repeated N-terminal Trx domains and a quite conserved CPFC motif in one of them, they lack the C-terminal domain. Since the GST1 and GSTH isoforms have not yet been characterized, we have essentially focused the following parts on the four remaining Cys-GST classes, describing in detail the current knowledge on DHAR, GHR, GSTL and mPGES-2 enzymes.

**FIGURE 1 | Rooted phylogenetic tree of plant GSTs.** The sequences used are those identified in *Arabidopsis thaliana* (Lan et al., 2009), *Hordeum vulgare* (Rezaei et al., 2013), *Oryza sativa* (Lan et al., 2009), *Physcomitrella patens* (Li et al., 2013), *Pinus tabulaeformis* (Lan et al., 2019), *Populus trichocarpa* (Lan et al., 2009), and *Solanum lycopersicum* (Caiszar et al., 2014). Sequences were aligned with PROMALS3D and alignment manually adjusted with Seaview software (Gouy et al., 2010). The phylogenetic tree was constructed with BioNJ (Gascuel, 1997) in Seaview, rooted with *E. coli* glutaredoxin 2 and edited with Figtree software (http://tree.bio.ed.ac.uk/software/figtree/). The robustness of the branches was assessed by the bootstrap method with 500 replications. Various classes can be distinguished: Dehydroascorbate reductase (DHAR), Elongation factor 1B (EF1B), Glutathionyl hydroquinone reductase (GHR), PHI (GSTF), Hemerythin (GSTH), IOTA (GSHL), Lambda (GSHL), TAU (GSTU), ZETA (GSTZ), Microsomal prostaglandin E synthase type 2 (mPGES-2), Tetrachloro-hydroquinone dehalogenase (TCHQD), and URE2p. The scale marker represents 0.1 substitutions per residue. For clarity, the names of the sequences have not been indicated but all sequences are available in the Supplementary Material.
DISTRIBUTION OF CYSTEINYL GSTs IN PHOTOSYNTHETIC ORGANISMS: GENE CONTENT, STRUCTURE, AND ORGANIZATION

After retrieving all Cys-GST sequences present in representative cyanobacterial and algal genomes as well as in all completely sequenced terrestrial plant genomes, a comparative genomic analysis was performed to get an accurate classification of Cys-GSTs in the green lineage. The resulting phylogenetic tree confirmed six major clades corresponding to the 6 earlier defined classes (Figure 2). In previous phylogenetic analyses conducted with other gene families of the thioredoxin superfamily, e.g., thioredoxins, glutaredoxins, peroxiredoxins and PDIs, the gene structure (number of exons in eukaryotic genes) was conserved and coherent with the classes (Meyer et al., 2002; Rouhier and Jacquot, 2005; Rouhier et al., 2006; Selles et al., 2011). Here, the gene structure was not informative as it was not at all conserved among species, even the phylogenetically close ones.

The DHAR class is essentially present in terrestrial plants. Indeed, this class is absent in cyanobacteria and a single gene, that likely represents the ancestor DHAR gene, is found in a few algae of the chlorophyceae and trebouxiophyceae classes but not in prasinophyceae. The number of DHAR genes in a given species usually lies between 2 and 3 (Table 1). For species possessing a higher number of genes, the increase is not due to tandem duplication as the genes are neither found at adjacent positions nor on the same chromosome in most cases. In our chosen well-annotated reference genomes, there are two genes in O. sativa and S. moellendorffii and three in A. thaliana, P. trichocarpa, and P. patens. DHARs are split in two well-differentiated subgroups, the genes coding for chloroplastic proteins (CPFC active site motif) and those coding for proteins that are likely all cytosolic (CPFS active site motif) (Figure 2) with algal sequences being dispersed but on the same branch. Since algal genes code for proteins that are devoid of targeting sequences, the ancestral gene might be the one coding for the cytosolic members. Among terrestrial plants, all organisms have at least one member in each clade.

GSTLs appear unique to terrestrial plants, the number of genes ranging generally from 2 to 4 with the exception of S. moellendorffii, where the gene seems absent (Table 1). In A. thaliana, two genes (AtGSTL1 and AtGSTL2) are repeated in tandem on the chromosome 5, likely indicating a recent duplication event. On the other hand, specific expansions have arisen in some species such as Aquilegia coerulea, Malus domestica, Eucalyptus grandis, and Panicum virgatum which have 5–8 genes. In this case, some events of tandem duplication have largely contributed to this increase. This is particularly true in Eucalyptus grandis, a species in which there are two gene clusters, one having a series of four

FIGURE 2 | Unrooted phylogenetic tree of Cys-GSTs present in the green lineage. Sequences were aligned with PROMAL3D using 1Z9H, 3PPU, and 4PQH PDB structures as templates. Then the alignment has been manually adjusted with Seaview software. The phylogenetic tree was constructed with BioNJ and edited with Figtree software (http://tree.bio.ed.ac.uk/software/figtree/). The robustness of the branches was assessed by the bootstrap method with 500 replications. The scale marker represents 0.1 substitutions per residue. For clarity, the names of the sequences have not been indicated but all sequences are available in the Supplementary Material.
### Table 1 | Cys-GST gene content in sequenced chlorophytes and embryophytes.

| Family                | Species                                      | DHAR | GHR | GSTL | mPGES-2 | GSTI | GSTH | Total |
|-----------------------|----------------------------------------------|------|-----|------|---------|------|------|-------|
| **VIRIDIPLANTAE**     |                                              |      |     |      |         |      |      |       |
| Chlorophyte           | *Chlamydomonas reinhardtii* v5.5              | 1    | 3   | 0    | 1       | 1    | 0    | 6     |
|                       | *Chlorella sp. NC64A*                        | 1    | 2   | 0    | 1       | 1    | 0    | 5     |
|                       | *Coccomyxa subellipsioidea C-169 v2.0*       | 2    | 2   | 0    | 1       | 1    | 0    | 6     |
|                       | *Micromonas pusilla CCMP1545 v3.0*           | 0    | 2   | 0    | 1       | 1    | 0    | 4     |
|                       | *Micromonas pusilla RCC298 v3.0*             | 0    | 2   | 0    | 1       | 1    | 0    | 4     |
|                       | *Ostreococcus lucimarinus v2.0*              | 0    | 1   | 0    | 1       | 1    | 0    | 3     |
|                       | *Volvox carteri v2.0*                       | 1    | 0   | 0    | 1       | 1    | 0    | 3     |
| **Embryophyte**       |                                              |      |     |      |         |      |      |       |
| Physcomitrella patens | v3.0                                         | 3    | 2   | 1    | 2       | 1    | 8    | 17    |
| **Tracheophyta**      |                                              |      |     |      |         |      |      |       |
|                        |                                              | 2    | 5   | 0    | 1       | 1    | 2    | 11    |
| **Angiosperm**        |                                              |      |     |      |         |      |      |       |
| Grass                 |                                              | 2    | 2   | 2    | 1       | 0    | 0    | 7     |
|                       | *Brachypodium distachyon* v1.2               | 2    | 2   | 3    | 1       | 0    | 0    | 8     |
|                       | *Oryza sativa* v70                          | 3    | 3   | 6    | 1       | 0    | 0    | 13    |
|                       | *Panicum virgatum* v1.1                     | 2    | 2   | 4    | 1       | 0    | 0    | 9     |
|                       | *Setaria italica* v2.1                      | 3    | 2   | 4    | 1       | 0    | 0    | 10    |
|                       | *Sorghum bicolor* v2.1                      | 4    | 7   | 4    | 1       | 0    | 0    | 16    |
| Eudicot               |                                              | 2    | 2   | 6    | 1       | 0    | 0    | 11    |
|                       | *Aquilegia coerulea* v1.1                   |      |     |      |         |      |      |       |
| Pentapetalae          |                                              | 2    | 2   | 3    | 2       | 0    | 0    | 9     |
|                       | *Mimulus guttatus* v2.0                     | 2    | 2   | 5    | 1       | 0    | 0    | 10    |
|                       | *Solanum lycopersicum* iTAG2.3              |      |     |      |         |      |      |       |
|                       | *Solanum tuberosum* v3.4                    |      |     |      |         |      |      |       |
|                       | *Vitis vinifera Genoscope.12X*              | 2    | 1   | 4    | 2       | 0    | 0    | 9     |
| Rosid                 |                                              |      |     |      |         |      |      |       |
|                       |                                              |      |     |      |         |      |      |       |
| Poplar-Malvidae       |                                              |      |     |      |         |      |      |       |
| Eucalyptus grandis v1.1 |                                            | 3    | 3   | 8    | 3       | 0    | 0    | 17    |
|                       | *Populus trichocarpa* v3.0                  | 3    | 2   | 3    | 3       | 0    | 0    | 11    |
| Brassicales-Malvales  |                                              |      |     |      |         |      |      |       |
| Carica papaya ASGPBv0.4 |                                            | 2    | 0   | 2    | 1       | 0    | 0    | 5     |
| Gossypium raimondii v2.1 |                                        | 3    | 2   | 3    | 2       | 0    | 0    | 10    |
|                       | *Theobroma cacao* v1.1                      | 2    | 3   | 2    | 2       | 0    | 0    | 9     |
| Brassicaceae          |                                              |      |     |      |         |      |      |       |
| Arabidopsis lyrata v1.0 |                                            | 3    | 4   | 2    | 1       | 0    | 0    | 10    |
| Arabidopsis thaliana TAIR10 |                                      | 3    | 4   | 3    | 1       | 0    | 0    | 11    |
| Bochea stricta v1.2   |                                            | 3    | 4   | 3    | 1       | 0    | 0    | 11    |
| Brassica rapa FPac v1.3 |                                            | 5    | 4   | 3    | 1       | 0    | 0    | 13    |
| Capsella grandiflora v1.1 |                                        | 3    | 4   | 2    | 1       | 0    | 0    | 10    |
| Capsella rubella v1.0  |                                            | 3    | 2   | 1    | 1       | 0    | 0    | 7     |
| Eutrema salsugineum v1.0 |                                        | 3    | 3   | 3    | 1       | 0    | 0    | 10    |
| Citrus                |                                              |      |     |      |         |      |      |       |
| Citrus sinensis v1.1  |                                            | 2    | 2   | 3    | 2       | 0    | 0    | 9     |
| Citrus clementina v1.0 |                                            | 2    | 2   | 3    | 2       | 0    | 0    | 9     |
| Fabidae               |                                              |      |     |      |         |      |      |       |
| Linum usitatissimum v1.0 |                                        | 5    | 4   | 4    | 2       | 0    | 0    | 15    |
| Manihot esculenta v4.1 |                                            | 2    | 1   | 2    | 2       | 0    | 0    | 7     |
| Ricinus communis v0.1  |                                            | 3    | 2   | 3    | 1       | 0    | 0    | 9     |
| Nitrogen-fixing       |                                              |      |     |      |         |      |      |       |
| Cucumis sativus v1.0  |                                            | 2    | 2   | 3    | 2       | 0    | 0    | 9     |
| Fragaria vesca v1.1   |                                            | 2    | 2   | 3    | 2       | 0    | 0    | 9     |
| Glycine max Wm82.a2 v1 |                                            | 4    | 2   | 5    | 3       | 0    | 0    | 14    |
| Malus domestica v1.0   |                                            | 8    | 5   | 6    | 4       | 0    | 0    | 23    |
| Medicago truncatula Mt4.0v1 |                                      | 2    | 1   | 4    | 2       | 0    | 0    | 9     |
| Phaseolus vulgaris v1.0 |                                            | 2    | 2   | 4    | 2       | 0    | 0    | 10    |
| Prunus persica v1.0    |                                            | 2    | 2   | 2    | 2       | 0    | 0    | 8     |

Sequences have been retrieved from Phytozome 10 and Joint Genome Institute databases. They are provided as Supplementary Material.
genes in a row. In the phylogenetic tree, the genes coding for the chloroplastic and cytosolic isoforms clearly separate into two groups. Since the single GSTL gene found in *P. patens* encodes a chloroplastic protein, the ancestral version in the green lineage should be the chloroplastic-encoding gene. On the other hand, the absence of GSTL genes in cyanobacteria and algae raises the question of the appearance of these isoforms. In a few cyanobacteria and algae, there are orphan, non-annotated sequences sharing similar active site motifs (CPYA). For this reason, it is tempting to speculate that these sequences might correspond to the ancestral gene and that it has been lost in most organisms. The fact that the overall similarity of these orphan sequences with GSTLs is low and that they do not necessarily form a single clade with GSTLs could come from their rapid and independent evolution. This will have to be further explored when additional genomes and sequences will be available.

The GHR class is widespread, with at least one gene present in almost all analyzed photosynthetic organisms and in most species. There are between 2 and 4 GHR genes (Table 1). The absence of gene in some species might be due to either annotation problems or gene loss. Furthermore, the gene family expansion found in some species, e.g., *Zea mays*, *S. moellendorfii*, and *M. domestica* cannot be explained by tandem duplication in this case. For *M. domestica*, which has by far the highest number of Cys-GST genes (23 genes) and exhibits gene expansion in all classes, this can be explained by a recent genome-wide duplication (Velasco et al., 2010). The widespread nature of GHRs is also true outside photosynthetic organisms since they are present almost everywhere including archaea of the halobacteriaceae order, but excluding mammals (Table 2). Overall, this suggests that GHRs have crucial functions, or at least functions that cannot be ensured by other GSTs.

Regarding the mPGES-2 class, its genes are absent in cyanobacteria whereas at least one gene is present in algae and terrestrial plants. This suggests that mPGES-2 proteins may have a widespread and essential function. Since most organisms retained only one gene, the duplication observed in some specific organisms probably derives from isolated events. Additionally, these GSTs are also largely distributed among kingdoms since they are found in mammals, nematodes, insects, and trypanosomatids but not fungi (Table 2).

The last two classes, GSTI and GSTH, are restricted to specific organisms. The GSTIs are found as a single gene in some cyanobacteria, algae, and in non-vascular plants (*S. moellendorfii*, and *P. patens*). In the phylogenetic tree, they form a single clade that is close to DHAR, possibly indicating that DHARs derive from GSTIs. The fact that GSTIs have been lost at some steps in the green lineage evolution and are no longer present in most terrestrial plants may also indicate that the associated function(s) disappeared or that other GSTs fulfill similar roles. The distribution of GSTHs is even more puzzling as from current available genomes, they are only found in *S. moellendorfii* and *P. patens*. The presence of 8 genes in *P. patens* is particularly striking, taking into account that, from the analysis of EST sequences, there is evidence for the expression of six genes. In *P. patens*, all these genes form a single gene cluster that likely originates from several duplication events.

### Table 2 | Characteristics and distribution of Cys-GSTs.

| Class    | Origin                  | Typical catalytic motif | Average amino acid length | Oligomerization state |
|----------|-------------------------|-------------------------|---------------------------|-----------------------|
| GSTB     | Bacteria                | GA12CS                  | 210                       | Dimer                 |
| GSTO     | Mammals, insects, fungi | 35CPFA                  | 250                       | Dimer                 |
| CLIC     | Animals                 | 35CPFS                  | 250                       | Monomer               |
| GSTL     | Terrestrial plants      | 40CPF/YA                | 230                       | Monomer               |
| DHAR     | Algae, terrestrial plants | 20CPFC/S             | 220                       | Monomer               |
| GHR      | Some metazoan but animals, algae, terrestrial plants, fungi, cyanobacteria, bacteria, archaea | 50CPWA | 330 | Dimer |
| mPGES-2  | Animals, protists, algae, terrestrial plants | 110CPFC | 310 | Dimer |
| GSTH     | Bryophyta, lycophyta   | 50CPF/YT                | 510                       | ?                     |
| GSTI     | Algae, bryophyta, lycophyta | 120CPYCY            | 490                       | ?                     |

The case of CLIC proteins is particular since it exists under both a monomeric soluble and an oligomeric transmembrane form. Moreover, the formation of an intramolecular disulfide bond promotes a structural transition that exposes a large hydrophobic surface changing the monomer into a non-covalent dimer (Littler et al., 2004).

### SEQUENCE CHARACTERISTICS AND DOMAIN ORGANIZATION OF CYSTEINYL GSTs

From the phylogenetic tree and the amino acid sequence alignments, there are key sequence differences that allow differentiation of each class. In addition to describing conserved motifs typical of each class, we have paid attention to the presence of N- and C-terminal extensions or of sequence insertions. Some differences are also reflected at the structural level since DHARs and GSTIs are monomeric enzymes, whereas GHRs and mPGES-2s are dimeric enzymes (Table 2). Thus, the residues forming the dimeric interface should also constitute a good criterion for distinguishing monomeric from dimeric proteins. This will be discussed further when relevant, either in this section or in the section dealing with the structural characteristics. It is worth noting that N-terminal extensions corresponding to predicted targeting sequences have been excluded when describing the size of the proteins and the percentage identity among isoforms.

From previous phylogenetic analyses conducted with Trx superfamily members, important features to consider have been defined. First, the nature and the position of the active site motif is an essential element. It should be recurrently situated at the beginning of the first α-helix of the thioredoxin fold, which does not mean that extra α-helices cannot be found before. For this reason, the position of the catalytic cysteine can vary although it is generally found around position 20 to 50 (Table 2). The
second major characteristic used for comparison is the nature of the residue found before a very conserved cis-proline that is typical of proteins of the Trx superfamily. This cis-proline is generally positioned 30 to 40 amino acids after the active site signature. According to this feature, an alignment of three representative sequences from each class allowed determining the residues that are conserved among Cys-GSTs (Figure 3A). The numbering used is the one corresponding to poplar GSTL1. There are six residues that are mostly conserved in all Cys-GST classes, or at least in the sequences used for the alignment. As expected, the catalytic Cys, found at position 36, and the cis-Pro discussed above at position 80, are conserved. Interestingly the Pro37 adjacent to the catalytic Cys is also found in the vast majority of sequences. Hence, the differences between GST classes will be essentially visible by checking the two other positions of the CPx active site motif. Besides, three other residues are also commonly found in all GSTs. As expected for enzymes that share the same co-substrate, some of the residues contributing to the recognition of GSH, Ser92 and Asp172, are conserved. The last conserved residue is a Gly at position 166 for which function is unknown.

With these features in mind, the difference between classes has been simply analyzed by looking at some specific criteria: (i) the percentage identity, (ii) the size of the proteins, (iii) the presence of extra domains, and (iv) the presence of three specific signatures, i.e. the active site sequence motif and the residues immediately before the cis-Pro and before the serine involved in GSH binding. Concerning the first criterion, the percentage identity between members of a given class is usually above 50%, whereas it is usually below 20% between classes.

Protein sizes vary slightly within classes, but they vary more significantly between classes. DHARs and GSTLs are the shorter Cys-GSTs, since they have about the same size ranging from 210 to 220 amino acids for the former and from 230 and 240 amino acids for the latter (Table 2). Nevertheless, compared to all other Cys-GSTs, DHARs have a nine amino acid insertion before the α1 helix and thus the active site motif (Figure 3). GHRs and mPGES-2s also have approximately the same size, ca 330 residues, but the sequence insertions explaining the difference with DHARs or GSTLs are not found at the same position. In the case of mPGES-2, the difference comes from the presence of an N-terminal membrane-anchoring region and of an insertion of about 40 residues between the α4 and α5 helices (Figure 3B). This insertion is different from the 20 amino acid insertion found between α3 and α4 helices in vertebrate isoforms which corresponds to two α-helices and two β-sheets (this is further discussed in Section 3D Structures). For GHRs, the size difference is essentially linked to insertions in the Trx domain (ca 35 amino acids between the active site motif and the cis-Pro, i.e. between the β2 strand and α2 helix) and to a final extension of 20 to 25 amino acids. The latter contains most of the residues responsible for the atypical GHR dimerization (see Section 3D Structures). Finally, GSTIs and GSTHs contain about 500 residues. GSTIs are slightly extended in the N-terminal part, but it is not yet clear whether this is a targeting sequence. Most of the additional sequence (around 120–140 residues) is present at the C-terminus and could correspond to a phycoerythrin α-subunit domain found in phy-cobilisome proteins (Figure 3B). GSTHs are also extended at the C-terminal end but this is due to the presence of an hemerythrin domain of ca 150–200 residues as its name suggests, followed by about 100 additional amino acids with no domain annotation (Figure 3B).

In the next part, we focused on the conservation of the sequence signatures mentioned above. If we consider the CPYC or CPFC active sites found in glutaredoxins as a reference, all plant Cys-GSTs display a reminiscent catalytic motif that differs by only one residue, with the exception of most GHRs which have two variations in their CPWA motif (Table 2). In these proteins, the catalytic cysteine is usually found at position 50. It is interesting to note that most algae have two GHR members including one isoform with an atypical CPWC motif (Figure 2). Except two algal sequences having a CPYC active site, mPGES-2s have usually a very conserved CPFC motif found around the position 110 owing to the presence of the N-terminal membrane-anchoring region. For DHARs, except a few sequences where the catalytic cysteine seems to be replaced by a glycine, the active site motif, found around the position 20, is usually quite conserved being of the CPFC or CPFS form. Among GSTLs, the active site motif is found around position 40 and it is mostly of the CP[F/Y]A form. The similarity with GSTOs (active site sequence and size of the proteins) might suggest a common origin. This is further supported by the fact that organisms having GSTOs do not have GSTLs and vice versa. Another extremely interesting observation is that GSTLs with SPFA motifs can be found in a few analyzed species as E. grandis, Linum usitatissimum, M. domestica, and Ricinus communis. This is also true for some fungal GSTOs found in particular in Phanerochaete chrysosporium or carnosa and Trametes versicolor where the classical CPY/FA motif is replaced by a SPY[C/S] motif (Morel et al., 2009). Although it should confer opposite properties (glutathionylating vs. de-glutathionylating activities) to the proteins, this suggests that GST genes can be maintained in genomes as long as the replaced amino acid conserves catalytic functions. Concerning GSTIs and GSTHs, the fact that these sequences are restricted to a few species and that the number of sequences available is low makes the analysis of amino acid conservation less robust. Nevertheless, it appears that most GSTI sequences exhibit a conserved WCPYC motif, except one representative from C. reinhardtii that has a RCPYC sequence. If it turns out that the N-terminal extension is indeed a targeting sequence, this motif is located around the position 60, otherwise it is located around the position 120. In GSTHs, the active sequence is CP[F/Y]T and depending on the isoform considered, it is usually found around position 50 or 70.

Finally, the residues associated to the cis-Pro80 and to Ser92 may help to definitely discriminate GST classes. The classes that cannot be differentiated using these signatures are GSTLs and GHRs which usually exhibit VP and ES motives, and DHARs and mPGES-2 which have VP and DS motives. However, as explained above, the other factors will allow distinguishing them. The last two classes, GSTIs and GSTHs, have specific recognizable sequences, LP and ES or [F/Y]P and GS, respectively.

**GENE EXPRESSION**

To date, there is not much data available on plant Cys-GSTs, both at genetic and physiological levels. Nonetheless, the physiological
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FIGURE 3 | Amino acid alignment and protein architecture of plant Cys-GSTs. (A) Amino acid sequence alignment of three representative members from each Cys-GST class. The sequences were structurally aligned using PROMALS3D server using as references the solved structures of PtGSTL1 [PDB code 4PQH (Lallement et al., 2014)], PtGSTL3 [PDB code 4PQI (Lallement et al., 2014)], Phanerochaete chrysosporium GHR1 [PDB code 3PPU (Meux et al., 2011)], and Macaca fascicularis mPGES-2 [PDB code 1Z9H (Yamada et al., 2005)] since there is no structure available for DHARs, GSTIs, and GSTHs. Since the structure of poplar GSTL1 has been solved, its secondary structures have been indicated as reference using ESPript 3.0 (http://espript.ibcp.fr/ESPript/ESPript/index.php), with the helices and the arrows corresponding respectively to α-helices and to β-strands. Strictly conserved residues are marked in white characters on a red background, whereas residues with similar functional groups are in red characters on white background. The indicated numbering corresponds to that of PtGSTL1 which has been used as a whole. For clarity, N- and C-terminal extensions present in Cys-GSTs have been removed from the alignment to keep only the sequences corresponding to secondary structures forming the GST fold. At is for Arabidopsis thaliana, Pp for Populus trichocarpa, Os for Oryza sativa, Pp for Physcomitrella patens, Sm for Selaginella moellendorffii, and Vc for Volvox carteri. The catalytic cysteine (*), cis-proline (cis-Pro), residues stabilizing the γ-glutamate residue of glutathione (##) and N-cap residue are shown. The N-capping box is surrounded in green.

(Continued)
roles of these enzymes can certainly be better understood by delineating the gene expression in plant organs or in response to environmental constraints. Since only partial information is available for each class, analyzing microarray experiments represents a valuable approach ahead of targeted expression studies. Hereto, *A. thaliana* microarray experiments were analyzed to assess both the developmental expression patterns of each GST using the EFP browser (Winter et al., 2007) and the transcriptional regulation occurring in response to environmental stimuli using Genevestigator (3072 perturbations were analyzed) (Hruz et al., 2008). Among the 14 genes coding for Cys-GSTs in Arabidopsis, four groups can be distinguished based on the absolute levels of expression, ranging from high to low: (i) DHAR1, DHAR3 and GSTL3, (ii) GSTL2, DHAR2 and GHR1, (iii) mPGES-2, GHR2 and GHR4, and (iv) GSTL1 and GHR3.

The expression of mPGES-2 genes has never been studied in plants. In mammals, however, the genes and corresponding enzymes have been shown to be constitutively expressed and involved in prostaglandin E metabolism, respectively (Murakami et al., 2003). In Arabidopsis, *mPGES-2* transcripts are found in all analyzed organs and at quite constant level (Figure 4). Based on the data available in Genevestigator, *A. thaliana* *mPGES-2* is the most significantly regulated gene in response to environmental stresses among Cys-GSTs. It is overexpressed in response to cold, drought, hypoxia, increases in light, chemical and biotic stresses, and shows its strongest up-regulation under heat stress. Altogether, this suggests that this gene may be involved in general tolerance mechanisms to biotic and abiotic stresses.

Regarding GSTL genes, *AtGSTL2* and *AtGSTL3* show similar expression patterns, particularly in green tissues, such as leaves, flower sepals, siliques, and developing seeds, whereas *AtGSTL1* transcripts are almost exclusively found in roots, senescing leaves, and pollen (Figure 4). In terms of stress responses, *AtGSTL1* is strongly up-regulated in several conditions including biotic interactions, treatment with chemicals, salt and iron-starvation stresses and, to a lesser extent, in response to hypoxia. This is consistent with a study showing that the *AtGSTL1* gene is induced in root cell cultures in response to buthionine sulfoximine (BSO), tert-butyl hydroperoxide, dichloromid, and 2,4 dichlorophenoxy acetic acid (Dixon et al., 2002). This also corroborates the observation that expression of tomato GSTL3 is induced by salt treatments in both roots and leaves (Csizsar et al., 2014). Interestingly, in contrast to *AtGSTL1*, *AtGSTL2*, and *AtGSTL3* are less responsive to environmental stress factors, but respond specifically to conditions involving increases in light, such as germination and light-shifts. These differences in expression patterns may help identifying the function of the three *AtGSTL* genes. Additionally, a number of studies has explored the expression and tissue distribution of GSTLs in other plant species. For instance, the three rice GSTL genes are all differentially expressed in response to arsenic treatments (Kumar et al., 2013a). Moreover, *OsGSTL1* and *OsGSTL2* are both constitutively expressed and involved in xenobiotic and oxidative stress tolerance in rice, whereas *OsGSTL2* is also specifically up-regulated in roots after herbicide (chlorosulfuron and glyphosate) and hormone treatments (salicylic acid and naphthalene acetic acid) (Hu et al., 2009, 2011a,b). Consistently, Arabidopsis transgenic lines expressing *OsGSTL2* are more tolerant to abiotic stresses such as heavy metals, cold, drought, and salt stress (Kumar et al., 2013a,b). Recently, the expression of the three poplar GSTL genes was studied in a naturally growing *Populus trichocarpa* adult tree (Lallement et al., 2014). One of these genes, *PigGSTL3*, generates two transcripts by alternative splicing, *PigGSTL3A* and *PigGSTL3B*, the latter being very weakly expressed. While *PigGSTL2* and *PigGSTL3A* seem to be constitutively expressed, all *PigGSTL* genes are preferentially expressed in the reproductive organs (flowers, fruits, buds) (Lallement et al., 2014). Nevertheless, poplar GSTLs have also been detected in leaves and roots (Lan et al., 2009). Altogether these results suggest that GSTLs are mainly expressed in organs that have a more intense secondary metabolism, which is consistent with the proposal that GSTLs are involved in the biosynthesis and/or maintenance of the flavonoid pool (see the Section Enzymatic Properties and Physiological Roles).

The three *A. thaliana* DHAR genes are expressed in most organs tested. Although *AtDHAR1* and *AtDHAR3* are either weakly or not expressed in pollen, this may be compensated by *AtDHAR2* which has its highest expression in this organ (Figure 4). Aside from this, the only notable difference is that *AtDHAR3* is relatively strongly expressed in leaves, which is consistent with its predicted localization to plastids (Figure 4). In other organisms where DHAR expression was studied, the genes were shown to be expressed in most tissues/organs. This is the case for the three poplar DHARs in roots, shoots, leaves, phloem, and buds (Lan et al., 2009; Tang and Yang, 2013) and for one DHAR from *Pinus bungeana* in buds, needles, phloem from stems, roots, and seedlings (Yang et al., 2009). However, in the moss *P. patens*, one of the three genes does not seem to be expressed at all (Liu et al., 2013). In response to environmental variations, the microarray data of *A. thaliana* indicates that *AtDHAR2* is the most responsive gene being up-regulated during germination and in response to biotic stress, chemicals, hypoxia, and drought. In contrast, *AtDHAR1* and *AtDHAR3* are up-regulated in only two conditions, excess light or drought and excess light or germination, respectively. Surprisingly, both genes are down-regulated in response to chemicals, a condition where GSTs are usually over-expressed. In plants, several independent studies have been performed with the aim of addressing the role of DHARs during stress response. For instance, *AtDHAR1* is...
up-regulated in response to norflurazon, menadione, paraquat, and antimycin A, treatments known to produce reactive oxygen species (Chew et al., 2003). On the other hand, AtDHAR2 expression is induced in root cell cultures in response to BSO and chloro-dinitrobenzene (CDNB) (Dixon et al., 2002). However, in other organisms, DHARs are not always regulated in the same manner in response to stress conditions. For example, DHAR2 from P. patens is strongly down-regulated by the addition of H₂O₂, salt, salicylic acid, and atrazine (Liu et al., 2013), whereas poplar DHAR2, in contrast to poplar DHAR3, is up-regulated in shoots but not in roots in response to H₂O₂, atrazine and to a lesser extent CDNB (Lan et al., 2009). Altogether these data...
point to a crucial function of DHARs and consequently ascorbic acid for stress responses, although the pattern of expression and regulation of DHAR genes differ from one organism to another.

Since GHRs were only recently identified in plants, they have not been studied in detail and very little is known about their expression and regulation. In yeast, GHR genes were formerly referred to as GSTO1, GSTO2, and GSTO3. The GSTO1 gene, which encodes a peroxisomal protein involved in sulfur metabolism, was shown to be induced by oxidative stress conditions (Barreto et al., 2006). Based on the analysis of *A. thaliana* microarrays, it is clear that GHRs are among the Cys-GSTs that are the least expressed, even though they are expressed in all plant organs analyzed. Apart from GHR2, which shows no preferential expression, the genes are each predominantly expressed in a particular organ, roots for GHR1, senescent leaves for GHR3 and mature seeds for GHR4 (Figure 4). In response to stress conditions, *A. thaliana* GHR genes are differentially regulated as well. AtGHR4 appears only to be down-regulated during germination. While AtGHR1 is down-regulated during germination and in response to cold, it is up-regulated in response to chemicals and biotic stress, together with GHR3. However, the expression of AtGHR1 is also increased by hypoxia, while AtGHR3 is increased in response to salt treatments and increases in light. Finally, in contrast to AtGHR1 and AtGHR4, AtGHR2 is up-regulated during germination and also shows up-regulation in response to iron starvation.

**SUBCELLULAR LOCALIZATION**

Deciphering the subcellular localization of all these proteins should also contribute to the understanding of their biological role. The data present in the literature for *A. thaliana* and poplar Cys-GSTs, originating from proteomic studies or from GFP fusion experiments have been compiled together with bioinformatic predictions for the presence of targeting sequences and summarized in Table 3. First, while not much is known about plant mPGES-2s, mammalian mPGES-2s exhibit a dual subcellular localization associated to both the Golgi membrane through their N-terminal part and the cytoplasm after proteolytic cleavage of the N-terminal hydrophobic domain (Tanikawa et al., 2002; Murakami et al., 2003). Similarly, plant mPGES-2 proteins possess an N-terminal extension, but it is predicted to correspond to a mitochondrial or plastid targeting sequence. This might be supported by the identification of this protein in two proteomic studies of mitochondrial protein fractions (Table 3) (Heazlewood et al., 2004; Klodmann et al., 2011). However, a careful inspection of the nature of the amino acids present in this region is rather consistent with a membrane-anchoring tail. Accordingly, the Arabidopsis ortholog was also identified in a proteomic study of plasma membrane proteins and an N-terminal transmembrane domain is indeed predicted by some prediction programs devoted to their identification (Table 3) (Mitra et al., 2009). Altogether, these data will have to be firmly established by complementary experiments, especially if a cleavage could also generate soluble isoforms. The mPGES-2s could be the only membrane associated Cys-GSTs since no other protein was predicted to possess a membrane-anchoring region.

Among DHARs, based on its occurrence in chloroplast proteome analyses and the presence of an N-terminal extension in the protein sequence, *A. thaliana* DHAR3 should be plastidic (Table 3). Poplar and *P. patens* orthologs have a similar localization (Liu et al., 2013; Tang and Yang, 2013). The two other proteins from Arabidopsis are predicted to be cytosolic as they do not exhibit visible targeting sequences. However, proteome analyses and GFP fusion proteins indicate that AtDHAR1 is also present in peroxisomes (Reumann et al., 2009; Grefen et al., 2010).

Concerning GSTLs, based on proteome analyses and on the presence of an N-terminal predicted targeting sequence, AtGSTL2 is clearly a plastidial protein and it may also be present in peroxisomes as shown by GFP fusion experiments as well as in the cytosol (Dixon et al., 2009). The poplar ortholog, PtGSTL1, is also present in plastids but a priori not in peroxisomes (Lallement et al., 2014) and the *P. patens* isoform is also plastidial (Liu et al., 2013). The two other GSTLs found in *A. thaliana* and *P. trichocarpa* should be cytosolic proteins, although a nuclear localization was observed when poplar proteins were fused to GFP (Lallement et al., 2014). Considering the absence of a clear NLS (nuclear localization signal), this nuclear localization is more likely due to a passive diffusion through nuclear pore rather than to a specific targeting.

Concerning GHRs, there is no information available yet. Among the four isoforms found in *A. thaliana*, two of them are predicted to be chloroplastic proteins and have additionally been identified from high-throughput proteomic analyses (Table 3). Concerning Hemerythrin GSTs, the proteins do not exhibit clear targeting sequences suggesting cytosolic localization. Accordingly, four GSTs from *P. patens*, PgGSTH1, 2, 3, and 7, presented a nucleo-cytoplasmic localization in GFP fusion experiments (Liu et al., 2013). Concerning GSTIs, according to the existence of clearly visible N-terminal extensions in some representative members, several prediction programs indicate that they could be targeted either to the chloroplasts or to mitochondria, although this remains to be demonstrated experimentally.

**3D STRUCTURES**

At the structural level, GSTs consist of an N-terminal domain adopting a thioredoxin fold and an all-helical C-terminal domain (Atkinson and Babbitt, 2009). The GSH binding site, or G site, is located in a cleft formed between the two domains and most of the residues contacting GSH are provided by the N-terminal domain. The binding site for the hydrophobic electrophiles, or H site, is located immediately adjacent to the G site and forms part of the solvent-exposed cleft between both domains. For the H site, most of the residues contacting the electrophiles are provided by the C-terminal domain. Both sites form the protein active site. Moreover, non-catalytic ligandin sites (L site) were defined in GSTs. Two types of L site may be roughly distinguished: those overlapping partially with the H site and those located at the dimer interface straddling the two fold axis (Littwack et al., 1971; McTigue et al., 1995; Rossjohn et al., 1998; Smith et al., 2003; Axarli et al., 2004; Dixon et al., 2008; Brock et al., 2013).

The N-terminal thioredoxin domain is often described as two distinct motifs: an N-terminal motif (β1α1β2) and a C-terminal...
The catalytic signature is located at the beginning of the conserved N-capping box (S/TXXD) including the S/T N-cap GlyGS (glycine residue of glutathione). In addition to these usual interactions with GSH, other less conserved residues can also contribute to the stabilization of GSH in specific cases. The C-terminal domain exhibits a bundle of helices whose number varies between each class. This less conserved domain, compared to the N-terminal domain, notably contains a well-conserved N-capping box (S/TXXD) including the S/T N-cap residue (Figure 3A) and a hydrophobic staple motif located at the

Table 3 | Subcellular localization of Arabidopsis thaliana Cys-GST members and of poplar orthologs.

| Gene name | Accession number | Predicted subcellular localization | Confirmed localization and other proteomic evidence | Amino acid length | Orthologs in poplar | References |
|-----------|------------------|-----------------------------------|-----------------------------------------------------|------------------|---------------------|------------|
| DHAR1     | At1g19570        | Elsewhere                         | - FP fusions: cytosol, peroxisome                  | 213              | - FP fusions:      | ^aGrefen et al., 2010 |
|           |                  |                                    | - High-throughput proteomic: mitochondrial, cytosol, plasma membrane, chloroplast |                  | DHAR2: cytosol, | ^bReumann et al., 2009 |
|           |                  |                                    |                                                     |                  | DHAR3: cytosol, | ^cChew et al., 2003 |
| DHAR2     | At1g75270        | Elsewhere                         | - High-throughput proteomic: cytosol, plasma membrane | 213              | - FP fusions:      | ^dIto et al., 2011 |
|           |                  |                                    |                                                     |                  | DHAR1: cytosol, | ^eMarmagne et al., 2007 |
|           |                  |                                    |                                                     |                  |                    | ^fPelletier et al., 2006 |
| DHAR3     | At5g16710        | Chloroplast                       | - High-throughput proteomic: chloroplast           | 258              | - FP fusions:      | ^gIto et al., 2011 |
|           |                  |                                    |                                                     |                  | GSTL2, GSTL3A & B: nucloeytoplasmic | ^hMarmagne et al., 2007 |
|           |                  |                                    |                                                     |                  |                    | ^iTang and Yang, 2013 |
| GSTL1     | At5g02780        | Elsewhere                         | None                                                | 237              | - FP fusions:      | ^jLallement et al., 2014 |
|           |                  |                                    |                                                     |                  | GSTL2, GSTL3A & B: nucloeytoplasmic |                |
| GSTL2     | At3g55040        | Chloroplast                       | - FP fusions: peroxisome                           | 292              | - FP fusions:      | ^kDixon et al., 2009 |
|           |                  |                                    | - High-throughput proteomic: chloroplast           |                  | GSTL1: chloroplast | ^lZybailov et al., 2008 |
|           |                  |                                    |                                                     |                  |                    | ^mPelletier et al., 2006 |
| GSTL3     | At5g02790        | Elsewhere                         | - High-throughput proteomic: cytosol               | 235              | - FP fusions:      | ^nIto et al., 2011 |
|           |                  |                                    |                                                     |                  | GSTL2, GSTL3A & B: nucloeytoplasmic | ^oLallement et al., 2014 |
| GHR1      | At4g19880        | Chloroplast                       | - High-throughput proteomic: cytosol, chloroplast  | 356              |                    | ^pIto et al., 2011 |
| GHR2      | At5g45020        | Elsewhere                         | None                                                | 325              |                    | ^qKlodmann et al., 2011 |
| GHR3      | At5g44990        | Elsewhere                         | None                                                | 350              |                    |                        |
| GHR4      | At5g44000        | Chloroplast                       | - High-throughput proteomic: chloroplast           | 399              |                    | ^rFerro et al., 2010 |
| mPGES-2   | At5g42150        | Mitochondria or chloroplast       | - High-throughput proteomic: mitochondrial, plasma membrane | 315              |                    | ^sHeazlewood et al., 2004 |
|           |                  |                                    |                                                     |                  |                    | ^tKlodmann et al., 2011 |
|           |                  |                                    |                                                     |                  |                    | ^uMitra et al., 2009 |

The prediction of subcellular localization was performed by compiling results obtained from various softwares as Predotar, TargetP and Wolfsort. Experimental confirmation consisting of high-throughput proteome analyses and fusions with fluorescent proteins (FP fusions) and associated references are indicated when available.

The β3β4α3 motif (β3β4α3) linked by helix α2 and which together form a four β-sheet in the order 2134 with β3 anti-parallel to the others (Figure 5A). Despite the low primary sequence conservation between GST classes, the position of the key residues is maintained. As mentioned above, the cysteine or serine of the catalytic signature is located at the beginning of α1 helix which also contains charged residues involved in the proton transfer reaction. For GSTs having a catalytic tyrosine residue, it is positioned at the end of the β1 strand. The invariant cis-Pro residue is located in the loop region before β3 and is thought to be implicated in the maintenance of the enzyme fold rather than playing a role in the enzymatic reaction (Figure 3A) (Allocati et al., 1999). The residues responsible for the non-covalent anchoring of GSH are well-conserved in most known GSTs. Glutamate/aspartate/glutamine residues and the adjacent serine residue in the loop β4-α3, stabilize the charged group of GluGS (γ-glutamate residue of glutathione). The main chain of a valine/leucine/isoleucine/threonine residue that precedes the conserved cis-proline is hydrogen-bonded to the backbone of CysGS (cysteine residue of glutathione) (Figure 3A). In the loop β2-α2, a charged residue (lysine, arginine), not present in all GSTs, makes a salt bridge with the carboxyl group of GlyGS (glycine residue of glutathione). In addition to these usual interactions with GSH, other less conserved residues can also contribute to the stabilization of GSH in specific cases.

The C-terminal domain exhibits a bundle of helices whose number varies between each class. This less conserved domain, compared to the N-terminal domain, notably contains a well-conserved N-capping box (S/TXXD) including the S/T N-cap residue (Figure 3A) and a hydrophobic staple motif located at the
FIGURE 5 | Structural organization of Cys-GSTs. All structures are shown as cartoon with the N- and C-terminal domains colored in cyan and in purple, respectively. Glutathione (GSH) or glutathione adducts (GS) are represented as sticks. In F, glutathione is only present in monomer A. All figures have been prepared with Pymol software. (A,B) Monomeric organization of (A) GSTL3 from Populus trichocarpa (PDB code 4PQI) and (B) CLIC1 from Homo sapiens (PDB code 1K0M). These monomeric enzymes illustrate the classical GST fold which consists of an N-terminal domain adopting a thioredoxin fold ($\beta_1\alpha_1\beta_2\alpha_2\beta_3\beta_4\alpha_3$) and an all helical C-terminal domain. Human CLIC1 (B) harbors a long negatively charged loop also referred as “foot loop” (colored in red) inserted between helices 5 and 6. This loop is characteristic of CLICs and might be responsible for interaction with other proteins. The glutathione adduct (GS) has been modeled based on the superimposition with a glutathionylated version of Homo sapiens CLIC1 (PDB code 1K0N). (C,D) Classical dimerization mode of GSTs as shown using (C) Ochrobactrum anthrapi GSTB (PDB code 2NTO) and (D) Homo sapiens GSTO1-1 (PDB code 1EEM). The monomers associate along a structural C2 axis. The N-terminal domain loop $a_2\beta_3$, strand $\beta_4$ and helix $a_3$ of one subunit interacts with the C-terminal domain (helices $a_4$ and $a_5$) of the other monomer. The dimer interface is either hydrophilic (C) or hydrophobic (D). The hydrophobic interaction is characterized by the insertion of a phenylalanine (or a tyrosine) residue belonging to the $a_2\beta_3$ loop into a hydrophobic pocket located between helices $a_4$ and $a_5$ of the C-terminal domain of the other subunit ("lock-and-key" motif). (E) Macaca fascicularis mPGES-2 (PDB code 2PBJ). The dimerization occurs via a $a_3\beta_4^\prime\beta_4^\prime\beta_4^\prime\alpha_3^\prime$ structure (colored in red) inserted between $a_3$ and $a_4$ that interacts with those of the other monomer (colored inruby). Note that this insertion is not found in plant sequences. (F) Phanerochaete chrysosporium GHR1 (3PPU). The two monomers interact via their C-terminal domain (in red) and are related to each other by a 2-fold symmetry axis.
N-terminal part of α6 helix. The N-capping box motif has been proposed to participate to the nucleation of helices as well as their folding and stabilization by forming reciprocal main chain–side chain hydrogen bonds between the N-cap (Ser/Thr) and the N3 (Glu/Asp) residues. The hydrophobic staple motif consists of a specific i,i+5 hydrophobic interaction between a residue (N’) that precedes the N-cap residue and a residue (N4) located within the α6-helix. The nomenclature commonly used is as follows: N-N’-Ncap-N1-N2-N3-N4 (Xhs/TXXDh, with h: hydrophobic residue and X: non conserved residue) (Richardson and Richardson, 1988). These two local structural motifs have a critical role in protein folding and stability of α-helices. The substitution of the capping residue greatly destabilizes the structure of GSTs, as well as their folding. It has also been proposed that the hydrophobic staple motif represents an evolutionarily conserved determinant for rapid folding of the enzyme. In addition, a glycine residue located four amino acids upstream (three residues in GSTLs) the N-cap residue (S/T) is also well-conserved in GSTs and is likely essential for folding by stabilizing the GXXhs(S/T)XXDh conserved loop-helix substructure (Kong et al., 2003) demonstrating the importance of these motifs both for protein folding and stability. Concerning the residues involved in the H site, they are generally hydrophobic and are located in a crevice between the N- and C-terminal domains at the vicinity of the G site. The nature of the amino acids contributing to the substrate recognition in this H-site has not been identified so often since it generally requires the crystal structure of complexes. Moreover, from known examples, they are quite variable among GST classes which likely explain the diversity of substrates accommodated by the different GSTs but may at the same time also explain the lack of specificity among certain classes for some substrates (Wilce and Parker, 1994; Armstrong, 1997). For these reasons, we will not discuss in detail the structure and residues forming the H site in each GST class.

With the exception of a few classes such as GSTLs, DHARs and soluble CLICs that exist as monomeric enzymes (Figures 5A, B) (Dixon et al., 2002), other GSTs are mostly dimeric proteins and very often adopt the same dimerization mode. Both subunits are connected along a structural C2 axis roughly parallel to helix bundle axis (binary axial symmetry). The main interactions between the two subunits are held between the N-terminal domain of one subunit and the C-terminal domain of the other. Thus, the loop α2-β3, the strand β4 and the helix α3 of one subunit interacts with the helices α4 and α5 of the other subunit as in GSTBs (Figure 5C). This dimer is considered as the classical dimerization mode in GSTs. In Theta, Sigma and Beta members, the interaction surface is rather hydrophilic whereas in Alpha, Phi, Mu, Omega, Pi, Tau, Zeta, and FuA GSTs, the surface is more hydrophobic (Frova, 2006). The hydrophobic interaction is characterized by a hydrophobic “lock-and-key” (or “ball and socket”) motif which holds the two protomers together and which is established due to the side chain of a phenylalanine (or a tyrosine) residue (key) belonging to the α2-β3 loop (Dirr et al., 1994). This residue is inserted into a hydrophobic pocket (lock) located between helices α4 and α5 of the C-terminal domain of the other subunit as shown for human GSTO1-1 (Figure 5D). This particular interaction is absent in Theta, Sigma, Beta, and Tau members and is replaced by an extensive network of polar interactions (Figure 5C) (Armstrong, 1997; Stevens et al., 2000). Beyond the canonical dimer, other dimerization modes have been described for GSTs. For example, FuA GST dimeric arrangement is close to the one observed in the canonical dimer in that their C2 axis is along the same direction (Figure S1A). In FuA GSTs, the two protomers are translated in the interface plane bringing the α-helical domains closer to each other. An additional β-hairpin (β2’-β2”) inserted between α2 and β3 inhibits the formation of the regular GST dimer and acts as a lid over the G site (Mathieu et al., 2012). A GST from the soil bacterium Ralstonia solanacearum (PDB code 4KT9) exhibits a similar dimerization mode as GSTFuA. In this case, the β-hairpin (β2’-β2”) is absent but a long C-terminal extension, which extends the β-sheet structure, prevents the formation of the classical dimer (Figure S1B). In Macaca fascicularis mPGES-2, the dimerization remains similar to the canonical assembly and occurs through an insertion of two α-helices and two β-strands (α3’β4’β4”α3’”) between α3 and α4 that interacts with those of the other monomer (Figure 5E) (Yamada et al., 2005; Yamada and Takusagawa, 2007). However, this insertion seems to be specific to vertebrates and is absent in photosynthetic organisms, which suggests a different organization. In GHRs, the mode of dimerization is completely different, the monomers associate exclusively via their C-terminal domain and notably via a coil of about 20 residues that follows the helix α9 (Meux et al., 2011). Helix α9 is a structural characteristic that is also found in GSTOs (Board et al., 2000), Tau GSTs (Thom et al., 2002), Delta GSTs (Oakley et al., 2001) and GSTLs (Lallement et al., 2014). The 20 C-terminal residues of one monomer mainly interact with the N-terminal end of helix α5 and with the C-terminal end of helix α6 of the other monomer, allowing the formation of a dimer that completely differs from the usual GST dimer (Figure 5F) (Meux et al., 2011). In addition, a recently characterized GST from Leishmania infantum (TDR1 protein) does not exhibit the canonical dimerization mode but consists of a unique trimer of subunits each containing two glutathione S-transferase domains (Figure S1C) (Fyfe et al., 2012). While the diversity of GST quaternary structures might still grow with the release and accumulation of structural data, the majority of GSTs adopts the canonical dimeric quaternary structure.

To date, there are 10 structures of bacterial GSTBs (Table 4). A B. xenovorans GSTB structure has been obtained in complex with GSH in the G site and the physiological product, 2-hydroxy-6-oxo-6-phenyl-2,4-dione, in the H site (Toscheva et al., 2006). Concerning CLICs, structures from three organisms, Caenorhabditis elegans, Drosophila melanogaster, and Homo sapiens are available (Harrop et al., 2001; Littler et al., 2008). Besides, a few structures have been obtained for the other cysteinyl-GSTs but not for DHAR. For mPGES-2s, only the structure of the M. fasciculartis isomorph has been solved (Yamada et al., 2005). Concerning GSTOs, in addition to one structure from Bombyx mori GSTO3 (Chen et al., 2011), several structures are known for human GSTO1 and GSTO2, alone or in complex with GSH or some substrates (Table 4). Recently, the first 3D structures of GSTLs (poplar GLST1 and L3) in complex with glutathione have been solved (Lallement et al., 2014). Finally, a few GHR/xi GST structures have been solved from various
Table 4 | Tridimensional structures of Cys-GSTs from all kingdoms.

| Class | Name | Organism | Ligand 1 | Ligand 2 | PDB | References |
|-------|------|----------|----------|----------|-----|------------|
| ?     | TDR1 | L. infantum | GSH | 1,2-Ethanediol | 4AGS | Fyle et al., 2012 |
| ?     | LigG | S. paucimobilis | GSH | SO₃⁺; Acetate | 4G10 | Meux et al., 2012 |
| GSTB  | BphK | B. xenovorans | GSH | 2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid | 2DSA | Tocheva et al., 2006 |
| GSTB  | E. coli | GTS | – | – | 1A0F | Nishida et al., 1998 |
| GSTB  | M. haemolytica | GSH | Triethylene glycol; Cl⁻; Acetate | 4IV9 | Unpublished |
| GSTB  | M. capsulatus | GSH | Glycerol | 3UAR | Unpublished |
| GSTB  | O. anthropi | GSH | SO₄²⁻ | 2NTO | Federici et al., 2007 |
| GSTB  | P. mirabilis | GSH | – | 1PMT | Rossjohn et al., 1998 |
| GSTB  | S. flexneri | GSH | – | 4KGI | Unpublished |
| GSTB  | S. paucimobilis | GSH | – | 1F2E | Unpublished |
| GSTB  | X. fastidiosa | GSH | Cl⁻ | 2X64 | Unpublished |
| GSTB  | Y. pestis | GSH | Glycerol | 4G9H | Unpublished |
| CLIC  | EXC-4 | C. elegans | – | Ca²⁺; I⁻ | 2IV9 | Littler et al., 2008 |
| CLIC  | CLIC1 | H. sapiens | GSH | – | 1K0N | Harrop et al., 2001 |
| CLIC  | CLIC4 | H. sapiens | – | – | 2AHE | Littler et al., 2004 |
| CLIC  | CLIC2 | H. sapiens | GSH | – | 2RAV | Cromer et al., 2007 |
| CLIC  | CLIC3 | H. sapiens | – | SO₄²⁻ | 3PY7 | Littler et al., 2010 |
| GRX   | Gn2 | E. coli | – | – | 1G7O | Xia et al., 2001 |
| GRX   | Gn2 | S. enterica | GSH | SO₂⁻; Cl⁻ | 3R4 | Unpublished |
| GSTO* | GSTO3-3 | B. mori | – | Glycerol | 3RBT | Chen et al., 2011 |
| GSTO  | GSTO1-1 | H. sapiens | GSH | SO₂⁻ | 1EEM | Board et al., 2000 |
| GSTO  | GSTO2-2 | H. sapiens | Cl⁻ | 3O19 | Zhou et al., 2012 |
| mPGES-2 | mPGES-2 | M. fascicularis | – | Indomethacin; Cl⁻; Acetate | 129H | Yamada et al., 2005 |
| GHR   | YqG | E. coli | – | GS-menadione | 4G0K | Green et al., 2012 |
| GHR   | C. glutamicum | – | 1,2-Ethanediol; Glycerol | 3M1G | Unpublished |
| GHR   | CphP | P. chrysosporium | GSH | – | 3PPU | Meux et al., 2011 |
| GHR   | PcpF | P. chrysosporium | – | – | 4FQU | Green et al., 2012 |
| GHR   | G. bronchialis | – | – | 4PTS | Unpublished |
| GSTL  | GSTL3 | P. trichocarpa | GSH | Ca²⁺ | 4PO1 | Lallement et al., 2014 |
| GSTL  | GSTL1 | P. trichocarpa | GSH | Na⁺ | 4POH | Lallement et al., 2014 |

Available Cys-GST structures have been retrieved from the RCSB Protein data bank (http://www.rcsb.org/pdb/home/home.do). Only the first solved structures of wild-type isoforms have been listed, but for several proteins as human CLIC or GSTOs or E. coli YqjG, other structures have been obtained either for mutated proteins or for wild-type proteins in complex with GSH or another second ligand. For instance, in the case of E. coli YqjG, there are 3 structures described in the same study, one in apoform, one with GSH and one with GS-menadione. Beyond GSH, structures of Cys-GSTs with physiological substrates have been obtained in rare cases. The compounds indicated in the ligand 2 column essentially come from crystallization solutions.

*GSTO3-3 from B. mori is phylogenetically related to and classified as GSTOs although it displays an asparagine instead of the catalytic cysteine.

ENZYMATIC PROPERTIES AND PHYSIOLOGICAL ROLES

As already mentioned, owing to the presence of a catalytic cysteine residue, Cys-GSTs have particular enzymatic properties since they should in principle catalyzedeglutathionylation reactions by performing nucleophilic attacks on various GSH-conjugated substrates (Board et al., 2000; Dixon et al., 2002; Meux et al., 2011). Accordingly, most if not all GSTLs, GHRs, GSTOs and DHARs characterized so far exhibit thiol-transferase and DHAR activities but no transference, peroxidase or isomerase activities except for a Beta GST from Proteus mirabilis, which possesses a slight peroxidase activity on cumene hydroperoxide (k_cat around 0.01 s⁻¹) and a non-negligible GSH transference activity on CDNB (k_cat around 2 s⁻¹) (Table 5) (Federici et al., 2010). This was surprising since the transference, peroxidase or isomerase activities are usually specific to Ser- or Tyr-containing GSTs as Phi, Tau, and Zeta GSTs. Indeed, it necessitates the activation of thiolate form of glutathione for direct glutathionylation reaction toward non-conjugated substrates (Dirr et al., 1994; Armstrong, 1997; Roxas et al., 1997). In the absence of known physiological substrates, hydroxyl-ethyl disulfide (HED), and DHA are often used to characterize the activity of recombinant GSTs as well as glutaredoxins, but it turns out that most glutathione-dependent oxidoreductases display such activities with very similar kinetic parameters (Table 5). The only notable exception is DHARs, for which DHA reduction is truly relevant. Consistently, they reduce DHA into ascorbate with a better efficiency (k_cat around 10⁴ s⁻¹, k_cat/k_m around 10⁷ M⁻¹s⁻¹) compared to the other enzymes (k_cat around 10² s⁻¹) and to the reduction of glutathionylated-mercaptoprotoehanol, the product formed upon incubation between GSH and HED (k_cat around 10⁸ s⁻¹) (Table 5) (Dixon et al., 2002). The DHAR-mediated DHA...
any Cys-GST is presented in

...that should apply for any glutathionylated substrate and have carbon-sulfur bonds (Meux et al., 2011, 2013; Lam et al., sulfur-sulfur bond, other glutathionylated substrates used so far...  

...and enzymes that have CPFC active sites. For instance, which have CPFC active sites. For instance, DHAR isoforms...  

...on GSSG treat-

ment by mass spectrometry (Dixon et al., 2002). Hence, it is...  

...to form mixed disulfides with GSH, there is little doubt that the catalytic cysteine performs a nucleophilic attack on GSH-conjugated substrates. The catalytic cysteine of Cys-GSTs becomes glutathionylated while the product of the reaction is released. The regeneration of these glutathionylated GST forms requires a GSH molecule, forming GSSG as another end product. While reduced Cys-GSTs are ready for another catalytic cycle, GSSG will be reduced back to GSH by glutathione reductase at the expense of NADPH. Since most Cys-GSTs have a single cysteine in the active site motif, they should follow this reaction mechanism. However, a few isoforms have an additional cysteine in the active site. This is the case of some DHAR isoforms which have CPFC active sites. For instance, A. thaliana DHAR3 was shown to form an intramolecular disulfide upon GSSG treatment by mass spectrometry (Dixon et al., 2002). Hence, it is possible that it constitutes either an intermediate step of the catalytic mechanism or possibly in other circumstances a protective mechanism that prevents over-oxidation of the catalytic cysteine into sulfenic, sulfinic, or sulfonic acid forms. Whatever the explanation is, the reduction of this disulfide would require a dithiol-disulfide exchange reaction. The most likely possibility is that it involves the successive intervention of two glutathione molecules, but another possibility is that a thioredoxin participates to this reduction step. Indeed, A. thaliana DHAR3 was isolated at least in two previous studies aiming at identifying thioredoxin targets (Marchand et al., 2004, 2006).

Besides glutathionylated-mercaptoethanol which contains a sulfur-sulfur bond, other glutathionylated substrates used so far have carbon-sulfur bonds (Meux et al., 2011, 2013; Lam et al., 2012; Lallemand et al., 2014). For instance, beyond their DHAR activity, fungal and bacterial GHRs characterized so far efficiently reduce glutathionylated (chlorinated) (hydro)quinones with $k_{\text{cat}}$ around $10^3 \text{ s}^{-1}$ and $k_{\text{cat}}/K_m$ up to $10^6 \text{ M}^{-1}\text{ s}^{-1}$ (Table 5) (Huang et al., 2008; Xun et al., 2010; Lam et al., 2012). However, there are some contrasting data in the literature. Some GHRs seem unable to catalyze the deglutathionylation of GSH conjugated-oxidized quinones and would be specific of glutathionylated reduced forms (Lam et al., 2012). On the other hand, using menadione as a substrate, a fungal GHR proved to deglutathionylate both forms with similar rates, but it is more efficient with the reduced forms because of a much better affinity (Meux et al., 2011). The latter observation points to the importance of the alcohol function for GHR recognition. Despite the above-mentioned discrepancy, it appears that GHRs are central to the regulation of the quinone redox state, likely preventing toxicity of quinones, either naturally present or found as environmental pollutants. Indeed, benzoquinones can covalently react with diverse macromolecules whereas hydroquinones, conjugated or not with glutathione, are prone to auto-oxidation forming reactive oxygen species. Since the major quinone forms found in the cells, ubiquinone and plastoquinone, are located into membranes and do not have electrophilic carbon groups that could be substituted by GSH, the question of the GHR physiological substrates is still open. Several other compounds often derived from lipids or fatty acids have alcohol functions and reactive electrophilic groups that might constitute possible substrates. As explained below, strategies aiming at identifying physiological substrates/ligands have been recently developed for other GSTs and they should be applied to GHRs. It is also possible that GHRs have protein substrates. For instance, it was proposed that the role of S. cerevisiae GTO1 could be related to the redox regulation of a Str3 cystathionine beta-lyase (Barreto et al., 2006). To date, the activity assays clearly separate GHRs from GSTLs and GSTOs which often catalyze the reduction of

### Table 5 | Enzymatic and ligandin activities detected for Cys-GSTs.

| GSTL | DHAR | GHR | mPGES-2 | GSTB | GSTO |
|------|------|-----|---------|------|------|
| Thiol-transferase | $10^{-10^{2}} \text{abc}$ | $10^{-10^{2}} \text{a}$ | $10^{-10^{2}} \text{abc}$ | ? | ? |
| DHA reductase | $10^{-10^{2}} \text{abc}$ | $10^{-10^{4}} \text{abhd}$ | $10^{-10^{2}} \text{a}$ | ? | ? |
| Glutathionylation | nd | nd | nd | 10^{-10^{3}} | ? |
| Deglutathionylation | PAP-SG | $10^{-10^{2}} \text{c}$ | ? | ? | ? |
| | (CII)Qu-SG | nd | nd | ? | ? |
| | TET-SG | nd | nd | ? | ? |
| | Q-SG | 1-10^{2bc} | ? | ? | ? |
| PGH$_2$ isomerization | ? | ? | ? | 10^{-2} | ? |
| PGH$_2$ degradation | ? | ? | ? | $10^{-10^{3}} \text{ef}$ | ? |
| Peroxidase | nd | nd | nd | 0.1 | ? |
| Esterase | 0.01-0.1 | ? | ? | ? | ? |

The data representing turnover numbers in min$^{-1}$ have been extracted from the following references: a (Dixon et al., 2002); b (Dixon and Edwards, 2010b); c (Lallemand et al., 2014); d (Tang and Yang, 2013); e (Meux et al., 2011); f (Lam et al., 2012); g (Meux et al., 2013); h (Yamada and Takusagawa, 2007); i (Allocati et al., 2000); j (Allocati et al., 2008); k (Perito et al., 1996); l (Allocati et al., 2009); m (Board et al., 2000); n (Board and Anders, 2007); o (Brock et al., 2013); p (Sampayo-Reyes and Zakharyan, 2006).

Nd, not detected; ? not examined; *GSH-independent activity, **GSH-dependent activity, ***a slightly different substrate, acetophenone, was used (Board and Anders, 2007), ****$k_{\text{cat}}/K_m$ in mM.
activity on the fluorescent probe chloromethyl fluorescein diacetylcellular extracts by competition assays using fluorescent probes. The possibility to achieve this goal is to screen chemical libraries or other GSTs is to identify relevant physiological substrates. One tetralone (Meux et al., 2013; Lallement et al., 2014).

the ability to remove the bound GSH molecule on glutathionyl difference between GSTLs and GSTOs is that only GSTOs have by competition experiments. Incidentally, one of the reported probe was initially used to identify tetralone as a GSTO substrate with other Phi and Tau GSTs, the latter binding porphyrin intermediates and fatty acids (Dixon et al., 2008, 2011; Dixon and Edwards, 2009). It was then confirmed by enzymatic analyses that GSTLs from Arabidopsis, wheat and poplar can perform deglutathionylation of glutathionylated quercetin (Dixon and Edwards, 2010b; Lallement et al., 2014). However, the fact that the turnover numbers are quite low and similar to those obtained with other oxidoreductases (Grxs, Trxs, GSTOs, GHRs, and DHARs) from various organisms (Lallement et al., 2014) and that a quercetin derivative was also isolated from a ligand fishing experiment performed with a Phi GST (Dixon et al., 2011) raises the question of a specific role of GSTLs in quercetin recycling and in the maintenance of a reduced flavonoids pool. Overall, this may indicate that flavonoids are GST substrates, but it does not tell exactly which enzyme(s) is (are) really efficient in vivo.

From a biochemical point of view, mPGES-2 can hardly be compared with other Cys-GSTs. Indeed, although it has been shown that they do not display GSH transferase activity, none of the usual activities of Cys-GSTs were assayed (Watanabe et al., 1999). Since mammalian mPGES-2s have a defined role in prostanoid metabolism, all studies primarily investigated the PGH2 conversion into PGE2 (Watanabe et al., 1997). However, an issue was the observation that mPGES-2 activity was partially independent from glutathione and that DTT induced a 4-fold better efficiency of the proteins (Tanikawa et al., 2002). A recently solved structure of a heme-bound mPGES-2 allowed solving this discrepancy. Indeed, it seems that the isomerization activity is catalyzed by a heme-free enzyme, whereas heme-bound mPGES-2s can degrade PGH2 into hydroxyl heptadecatrienoic acid and malondialdehyde, instead of converting it to PGE2 (Jania et al., 2009; Takusagawa, 2013). This activity relies to the binding of a heme, which is stabilized by hydrogen bonds when a glutathione is present in the active site (Yamada and Takusagawa, 2007; Takusagawa, 2013). Hence, this may help explaining that the activity of isomerization is increased by adding DTT as it contributed to remove both GSH and heme from the active site. Overall, the current view in animals is that the physiological role of mPGES-2s is related to the degradation of PGH2 rather than to its isomerization into PGE2. This is also consistent with the fact that mPGES-1s also catalyze the GSH-dependent PGH2 isomerization into PGE2 with good efficiencies (Thoren et al., 2003). Since there is no PGH2 and PGE2 in plants, the physiological roles and substrates of mPGES-2 are unclear. Looking for

the same glutathionyl derivatives. GSTLs and GSTOs do not catalyze the deglutathionylation of glutathionylated quinones (Meux et al., 2013; Lallement et al., 2014). However, contrary to GHRs, GSTOs and GSTLs perform deglutathionylation of glutathionyl tetralone and/or acetophenone-derivatives with relatively good catalytic constants (kcat around 104 s−1) (Table 5) (Meux et al., 2013; Lallement et al., 2014) and they exhibit a weak esterase activity on the fluorescent probe chloromethyl fluorescein diacetate (CMFDA) (Meux et al., 2013; Lallement et al., 2014). This probe was initially used to identify tetralone as a GSTO substrate by competition experiments. Incidentally, one of the reported difference between GSTLs and GSTOs is that only GSTOs have the ability to remove the bound GSH molecule on glutathionyl tetralone (Meux et al., 2013; Lallement et al., 2014).

One of the major current challenge concerning Cys-GSTs and other GSTs is to identify relevant physiological substrates. One possibility to achieve this goal is to screen chemical libraries or cellular extracts by competition assays using fluorescent probes such as CFMDA or 8-anilino-1-naphthalenesulfonic acid (ANS). This has been successful in several cases both for Cys-GSTs (Son et al., 2010; Meux et al., 2013) and for those having a catalytic serine (Mathieu et al., 2012, 2013). Besides, Dixon and co-workers have identified several flavonoids derived from kaempferol which can bind tightly to GSTLs from Arabidopsis and wheat by ligand and fishing approaches (Dixon and Edwards, 2010a). These approaches consist in isolating by affinity chromatographies and identifying natural physiological substrates from plants using in vitro and in vivo approaches. Both methods rely on the use of tagged proteins either by mixing them with crude or fractionated extracts or to secondary metabolite enriched-extracts, or by expressing them in planta in order to really trap physiological protein-substrate complexes. They proved to work also with Phi and Tau GSTs, the latter binding porphyrin intermediates and fatty acids (Dixon et al., 2008, 2011; Dixon and Edwards, 2009). It was then confirmed by enzymatic analyses that GSTLs from Arabidopsis, wheat and poplar can perform deglutathionylation of glutathionylated quercetin (Dixon and Edwards, 2010b; Lallement et al., 2014).

![FIGURE 6 | Catalytic mechanisms of Cys-GSTs. The deglutathionylation of GSH-conjugated substrates occurs via the nucleophilic attack of the catalytic cysteine which is assumed to be at least partially under the thiolate form at physiological pH, owing to a decreased pKa value. Consequently, the catalytic cysteine is itself glutathionylated and it is regenerated using a glutathione molecule. For Cys-GSTs having another cysteine either in the active site (some DHAR isoforms) or at proximity (some GSTL isoforms), the identification of proteins with an intramolecular disulfide suggests that this might constitute either an intermediate step of the catalytic mechanism or more likely a protective mechanism that prevents oxidation of the catalytic cysteine into sulfenic acid forms or eventually higher oxidized forms as sulfinic or sulfonic acid forms. In the case of the formation of a disulfide an additional glutathione molecule would be required. It may be that thioredoxin participate to this reduction step as DHAR was isolated as a thioredoxin target.](image-url)
possible related candidate molecules in plants, oxylipins might constitute such substrates. Indeed, these molecules, derived from the enzymatic and non-enzymatic peroxidation of fatty acids, exhibit a reactive carbonyl structure, which makes them highly reactive electrophilic species and they are formed at proximity of mPGES-2 localization (Farmer and Mueller, 2013). These compounds participate to numerous developmental processes and to stress response. It is for instance documented that the expression of some GSTs is induced by 12-oxo-phytidieneic acid (OPDA), a phytohormone precursor, and phytoprostane A1 (PPA1) (Mueller et al., 2008). Consequently, it was hypothesized that GSTs might reduce the reactive cyclopentenone ring to an unreactive ring. Hence, by glutathionylating or deglutathionylating these molecules, GSTs and possibly mPGES-2s could modulate the concerned signaling pathways. To conclude, although Cys-GSTs are encoded by multigenic families, there is a pressing need to perform reverse genetics by systematically generating single or multiple mutant lines or overexpressing lines to delineate the exact function of these proteins.

MATERIALS AND METHODS

SEQUENCE RETRIEVAL, STRUCTURAL ALIGNMENT AND PHYLOGENETIC ANALYSES

Sequences have been retrieved by iterative blastp analyses using a set of as variable GST sequences as possible either from the cyanobase (http://genome.microbeld.jp/cyanobase/) for cyanobacteria, from the jgi genome portal (http://genom.jgi.doe.gov/) for most algae and from the version 10 of the phytozome portal (http://phytozome.jgi.doe.gov/pz/portal.html) for terrestrial plants. When needed and possible, sequences have been completed and validated by analyzing the presence of ESTs using tblastn analyses against the NCBI protein databank. Sequences were then aligned with PRIMAL3D (http://prodata.swmed.edu/promals3d/promals3d.php) (Pei et al., 2008) and alignment manually adjusted with Seaview software (Gouy et al., 2010). The phylogenetic tree was constructed with BioNJ (Gascuel, 1997) in Seaview and edited with Figtree software (http://tree.bio.ed.ac.uk/software/figtree/).

SUBCELLULAR LOCALIZATION

The GST subcellular localization was defined based on the available literature, as well as from database mining using TAIR v10, and from the following prediction softwares, Predotar, TargetP, and Wolfpsort.

EXPRESSION ANALYSES

For developmental conditions, expression data from Gene Expression Map of Arabidopsis Development (Schmid et al., 2005) were retrieved using the eFP browser (Winter et al., 2007). Each gene was normalized to its maximum expression within the selected dataset. Further, the number of developmental conditions was reduced to eight classes in order to gain a better overview of the overall expression profile of each GST during Arabidopsis development. These classes were grouped together and their relative expression averaged as follows: Mature seeds (stages 9, 10/11, 12, and 15), Senescing leaves, Leaves (rosette leaves 4, 6, 8, and 10), Shoot Apices (vegetative, transition, and inflorescence), and Roots (from seedlings and mature rosettes).

For perturbation conditions, expression data for each GST were obtained from Genevestigator V3 (Hruz et al., 2008). Only data with a p-value < 0.001 were included in the analysis. Perturbations in the resulting lists were grouped into 11 classes: Biotic stress (B), Chemicals (Che), Germination (Ge), Light (Li), N-starvation (-N), Fe-deficiency (-Fe), Salt stress (Sa), Hypoxia (Hy), Drought (Dr), Heat (He), and Cold (Co).

Data were analyzed using Open Office Calc (Apache), graphed using PRISM (GraphPad) and clustered using Multi-experiment Viewer (MeV).

AUTHOR CONTRIBUTIONS

Pierre-Alexandre Lallement, Arnaud Hecker, and Nicolas Rouhier carried out the in silico genome analyses. Bastiaan Brouwer and Olivier Keetch performed the transcriptome and subcellular prediction analyses. All authors participated to the writing of the manuscript, have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fphar.2014.00192/abstract

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