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► **To cite this version:**

Cécile Herve, Pierre-Olivier de Franco, Agnès Groisillier, Thierry Tonon, Catherine Boyen. New members of the glutathione transferase family discovered in red and brown algae. *Biochemical Journal*, 2008, 412 (3), pp.535-544. 10.1042/BJ20071464 . hal-00478902

HAL Id: hal-00478902

<https://hal.science/hal-00478902v1>

Submitted on 30 Apr 2010

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1 **New members of the Glutathione transferase family discovered in Red**
2 **and Brown algae**

3
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12
13 Short title: Macroalgal GSTs

14
15 Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; EST, expressed sequence tag;
16 FDNB, 1-fluoro-2,4-dinitrobenzene; GSH, glutathione; GST, glutathione transferase; 4-
17 HNE, 4-hydroxynonenal; 12(+/-)-HpETE, 12-hydroperoxy-5Z,8Z,10E,14Z-
18 eicosatetraenoic acid; 13(S)-HpODE, 13S-hydroperoxy-9Z,11E-octadecadienoic acid;
19 13(S)-HpOTE, 13S-hydroperoxy-9Z,11E,15Z-octadecatrienoic acid; 15(S)-HETE, 15S-
20 hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid; MAPEG, membrane-associated proteins
21 involved in eicosanoid and glutathione metabolism; MeJA, methyl jasmonate; PUFA,
22 polyunsaturated fatty acids.

1 The GSTs (glutathione transferases) are involved in the detoxification of a wide variety
2 of hydrophobic substrates. These enzymes have been found in virtually all types of
3 organisms, including plants, animals, nematodes and bacteria. In the present study, we
4 report the molecular and biochemical characterization of algal GSTs. Phylogenetic
5 analysis showed that most of them were distinct from previously described GST classes
6 but were most closely related to the sigma class. Profiling of GST genes from the red
7 alga *Chondrus crispus* and brown alga *Laminaria digitata* was undertaken after
8 different chemical treatments and showed that they displayed contrasting patterns of
9 transcription. Recombinant algal GST from both species showed transferase activities
10 against the common substrates aryl halides, but also on the α,β -unsaturated carbonyl 4-
11 hydroxynonenal. As well, they exhibit significant peroxidation towards organic
12 hydroperoxides, including oxygenated derivatives of polyunsaturated fatty acids.
13 Among a range of compounds tested, cibacron blue was the most efficient inhibitor of
14 algal GSTs identified.

15

16 Key words: Glutathione transferase, *Chondrus crispus*, *Laminaria digitata*,
17 phylogenetic classification, recombinant proteins, methyl jasmonate.

1 INTRODUCTION

2

3 Among the cellular defense systems, GSTs (EC 2.5.1.18) form a highly diverse family
4 of proteins with functions ranging from detoxification to cell signaling. They are named
5 after their ability to catalyze the nucleophilic attack of GSH (glutathione) on
6 electrophilic substrates. In this manner, they contribute to the metabolism of drugs,
7 chemical carcinogens, pesticides and other xenobiotics [1]. Endogenous products
8 formed as secondary metabolites during oxidative stress are also substrates for GST
9 enzymes. In addition, several GSTs act as GSH-dependent peroxidases by catalyzing
10 the reduction of organic hydroperoxides to the less-toxic monohydroxy alcohols [2].
11 Beyond detoxification and involvement in various defense responses, there is increasing
12 evidence indicating that GSTs may be implicated in many other physiological processes
13 including isomerization of specific metabolites [3], transportation of endogenous
14 substrates [4], plant cell growth and development [5].

15 These proteins are found in almost all species and are divided into classes, based
16 primarily on sequence similarity. Currently recognized classes of cytosolic GSTs in
17 mammals include the Alpha, Mu, Omega, Pi, Sigma, Theta and Zeta classes [6]. GSTs
18 from the last two classes have also been identified in plants and other organisms. Other
19 distinct groups have been identified only in insects (Delta and Epsilon; [7]), plants
20 (Lambda, Phi, Tau; [1]), or prokaryotes (Beta; [8]). A Kappa class regroups
21 mitochondrial GSTs, and a family of microsomal GSTs comprises the membrane-
22 associated proteins designated MAPEG.

23 GST enzymes have been extensively purified from mammals, plants and
24 invertebrates [9]. In contrast, the occurrence of GSTs has not been explored in some

1 taxa, such as the marine algae. Algae currently encompass eight groups of
2 photosynthetic eukaryotes which have evolved independently in the early history of life.
3 These organisms have acquired their plastids through different endosymbiotic events. A
4 first event involving a cyanobacterial ancestor gave rise to the red algae (Rhodophytae)
5 and the green plants (green algae and land plants). Brown algae (Phaeophyceae) and
6 diatoms stem from a secondary endosymbiotic event, and together with the oomycetes,
7 belong to the Heterokonts [10].

8 So far, there has been virtually no report on the molecular characterization of
9 GSTs in algae, particularly seaweed, mostly due to a lack of genomic information in
10 these organisms. Recently, we analyzed cDNA catalogs from thallus and protoplasts of
11 the red alga *Chondrus crispus* and of the brown alga *Laminaria digitata*, and provided
12 the first genetic evidence of cytosolic GST sequences in both organisms [11, 12]. In the
13 present study, phylogenetic analyses demonstrate an unexpected link between
14 macroalgal GSTs and a large animal GST cluster including the Sigma class GSTs. We
15 thus propose that these red and brown algal GSTs, together with oomycetes
16 representatives, define a new group of GSTs closely-related to the Sigma class. In
17 addition expression profiling of these genes showed that red and brown algal GSTs
18 exhibit different patterns of transcription according to chemical treatments. Transferase
19 and peroxidase activities of the recombinant proteins from both algae were determined
20 in presence of different categories of substrates, and cibacronblue was determined as the
21 most effective inhibitor among the molecules tested.

1 EXPERIMENTAL

2

3 Plant material cultivation and chemical treatments

4

5 Gametophytic *Chondrus crispus* unialgal isolates, and sporophytic *Laminaria digitata*
6 unialgal isolates, were cultivated at 13°C in enriched seawater medium SFC [13], using
7 a photoperiod of 10 hours of light and 14 hours of darkness and a photon flux density of
8 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

9 CuSO_4 and ZnCl_2 were purchased from Prolabo, and other chemicals from
10 Sigma. The derivatives of polyunsaturated fatty acids 13(S)-HpODE (13S-hydroperoxy-
11 9Z,11E-octadecadienoic acid), 13(S)-HpOTE (13S-hydroperoxy-9Z,11E,15Z-
12 octadecatrienoic acid), 12(+/-)-HpETE (12-hydroperoxy-5Z,8Z,10E,14Z-
13 eicosatetraenoic acid), 15(S)-HETE (15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic
14 acid), and 4-HNE (4-hydroxynonenal) were obtained from Cayman Chemicals.
15 Oligogulonates were produced by enzyme degradation of sodium alginate [14].

16 To perform chemical treatments, algae were first maintained in Petri dishes with
17 seawater for at least 18 hours. Final concentrations of chemicals were as follows: 10
18 mM H_2O_2 ; 500 μM each tested pesticide; 100 or 200 μM each heavy metal tested in *L.*
19 *digitata* and *C. crispus* respectively; 100 μM MeJA (methyl jasmonate); 25 or 50 μM
20 each PUFA (polyunsaturated fatty acids) derivative in *L. digitata* and *C. crispus*
21 respectively; and 15 $\mu\text{g mL}^{-1}$ oligogulonates. An equal volume of solvent was used in
22 each corresponding control treatment. After 3 and 6 hours of incubation for *C. crispus*
23 and *L. digitata* respectively, tissues were harvested, frozen in liquid nitrogen and stored
24 at -80°C until use.

1

2 **DNA and RNA isolation**

3

4 Extractions of genomic DNA and total RNA from gametophyte tissues of *C. crispus*
5 and sporophyte tissues of *L. digitata* were performed using the two-phase partitioning
6 protocol described in Hervé et al. [15]. To improve final yields with *L. digitata* tissues,
7 an additional step of polysaccharide precipitation, consisting of slow addition of 0.3 V
8 of absolute ethanol to the extraction mixture, was included in the protocol before RNA
9 precipitation by LiCl.

10

11 **Identification of cDNA and corresponding genes encoding GST**

12

13 EST encoding GSTs were identified in *C. crispus* and *L. digitata* through analysis of
14 corresponding cDNA libraries [11, 16]. To obtain the genomic sequence of *CcGST1*,
15 *CcGST2*, *CcGST3*, *LdGST1*, *LdGST2* and *LdGST3* genes, PCR amplifications were
16 performed on genomic DNA using specific primers flanking the coding region of each
17 gene. The genomic sequence of *LdGST4* could not be amplified in a single PCR, due to
18 the presence of large introns. All the primers used in this study are reported in
19 Supplementary Table S1. PCR conditions consisted of an initial denaturation of 5 min at
20 95°C, followed by 35 cycles of 45 s at 95°C, 60 s at 58°C and 100 s at 72°C, and a final
21 extension of 10 min at 72°C. PCR products were recovered from agarose gels, purified
22 using the Mini Elute Purification kit (Qiagen), and sequenced in both directions.

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24 **Bioinformatic analysis**

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Protein alignment was initially performed using the Mafft program at <http://bioinformatics.uams.edu/mafft/> and improved manually by comparison with a structural alignment of GST proteins with resolved crystal structures from the Sigma, Pi and Alpha classes. The alignment obtained was edited with ESPript at <http://esprict.ibcp.fr/ESPript/ESPript/>.

For phylogenetic analyses, full-length deduced amino acid sequences were aligned using the Muscle 3.6 software [17]. For the most exhaustive analysis, 130 sequences were selected from public databases and used as representative members of each of the known GST classes, excluding the microsomal MAPEG and mitochondrial Kappa classes due to their extreme divergence. Oomycete (*Phytophthora ramorum*, *Phytophthora sojae*) and algal sequences (*Phaeodactylum tricornutum*, *Thalassiosira pseudonana*, *Ostreococcus tauri*, *Ostreococcus lucimarinus*, *Cyanidioschyzon merolae*, *Chlamydomonas reinhardtii*) were searched from genome projects or from public- and restricted-access EST collections (*Chondrus crispus*, *Gracilaria tenuistipitata*, *Gracilaria gracilis*, *Gracilaria lemaneiformis*, *Porphyra yezoensis*, *Laminaria digitata*, *Laminaria japonica*, *Ectocarpus siliculosus*, *Fucus serratus*, *Fucus vesiculosus*, *Ulva linza*, *Fragilariopsis cylindrus*). The multiple-protein alignments were analyzed using the neighbor-joining and maximum likelihood methods, based on distances derived from the PAM matrix, with Mega 3.1 [18] and Phyml 2.4.4 [19] programs, respectively. The robustness of the branches was evaluated using bootstrap replicates.

Sequences selected from public databases and used for the phylogenetic analysis are listed in Supplementary Table S3.

1 *Real-time PCR*

2

3 Total RNA were reverse transcribed using the Superscript II RT kit (Invitrogen), and
4 real-time PCR was performed in a GeneAmp 5700 sequence detection system using the
5 SYBR Green PCR master kit (Applied Biosystems) according to the manufacturer's
6 instructions. The primers used in the reaction were recommended by Primers Express
7 1.0 software (Applied Biosystems) and are listed in Supplementary Table S1. All 40
8 PCR cycles were performed according to the following temperature regimen: 95 °C for
9 15 s, 60 °C for 60 s.

10 To generate a standard curve, genomic DNA of *C. crispus* and *L. digitata* were
11 used as reference matrixes. Knowing the genome mass of the *C. crispus* gametophyte
12 (0.16 pg) and the *L. digitata* sporophyte (1.4 pg), it was possible to determine the
13 number of cDNA molecules present in each sample tested as described in Hervé et al.
14 [15]. The quantity in unknown samples was expressed as a number of *C. crispus* or *L.*
15 *digitata* genomes per nanogram of total RNA. Actin and tubulin genes were used as
16 internal controls for *C. crispus* and *L. digitata* assays respectively, and corresponding
17 primers were designed based on sequences deposited in Genbank (accession numbers
18 U03676 and AW400737, respectively).

19 The relative changes in the GST mRNA expression were calculated as x -fold
20 changes relative to the appropriate control experiment for the different chemical
21 treatments.

22

23 **Heterologous expression and protein purification**

24

1 The open reading frames of the GST transcripts from *C. crispus* and *L. digitata* were
2 amplified by PCR using the primers listed in Supplementary Table S1. These primers
3 were designed to incorporate *Bam*HI and *Eco*RI restriction sites into the 5' and 3' ends,
4 respectively. PCR conditions consisted of an initial denaturation of 5 min at 94°C,
5 followed by 30 cycles of 30 s at 94°C, 30 s at 50°C and 2 min at 72°C, and a final
6 extension step of 10 min at 72°C. The resulting PCR products were purified, digested
7 with the appropriate restriction enzymes and subcloned into the pFO4 expression vector,
8 a vector modified from pET15 (Novagen) to be compatible with the *Bam*HI/*Eco*RI and
9 *Bam*HI/*Mfe*I ligation strategies.

10 Recombinant plasmids were used to transform *Escherichia coli* strain
11 BL21(DE3). Transformed colonies were grown at 37°C overnight in a Luria-Bertani
12 medium containing ampicillin. The culture was diluted 1:100 with auto-inducible ZYP
13 medium [20] containing ampicillin and subjected to further incubation at 20°C until the
14 culture density reached saturation. After centrifugation, pelleted bacteria were
15 resuspended in Tris-HCl pH7.5 buffer containing 300 mM NaCl and 60 mM imidazole
16 and disrupted using a French press prior to centrifugation. The resulting supernatant was
17 applied onto a Chelating Fast Flow Sepharose column charged with 100 mM NiSO₄
18 (Amersham Biosciences). After washing, the bound proteins were eluted with a linear
19 gradient of imidazole ranging from 60 mM to 300 mM. The protein eluted was collected
20 and dialyzed against a buffer containing 20 mM Tris-HCl pH7.5, 100 mM NaCl, 1 mM
21 EDTA and 1 mM DTT. Glycerol was added to dialyzed proteins to a final concentration
22 of 20 % (v/v) before storage at -80°C. Final protein concentrations were estimated by
23 the Bradford method with reagents purchased from Bio-Rad. The apparent molecular
24 mass of the purified recombinant proteins was evaluated by size-exclusion

1 chromatography using a 1.6 cm × 60 cm Superdex 200 column (Amersham Pharmacia
2 Biotech). The column was equilibrated in 50 mM Tris-HCl pH 7.5 buffer containing
3 100 mM NaCl. Molecular masses of standards used for calibration are: carbonic
4 anhydrase, 29 kDa; serum albumine bovine, 66 kDa; alcohol dehydrogenase, 150 kDa;
5 β-amylase, 200 kDa; apoferritin, 443 kDa; thyroglobulin, 669 kDa; blue dextran 2,000
6 kDa.

7

8 **Enzyme assays**

9

10 GST enzyme activity assays were carried out in a microplate Safire² UV
11 spectrophotometer reader (Tecan), except for 4-HNE and trans-2-nonenal that used a
12 UV-2401-PC spectrophotometer (Shimadzu). In all instances, the non-enzymatic
13 reaction was measured and subtracted from the overall reaction rate. Assays were
14 conducted at 30°C and measures were performed during 10 min after addition of second
15 substrate. Ethacrynic acid, *trans*-4-phenyl-3-buten-2-one, *para*-nitrophenyl acetate and
16 cumene hydroperoxide were generously provided by Fabrice Morel (U620 Inserm,
17 Rennes, France). Except the PUFA hydroperoxides and the 4-HNE obtained from
18 Cayman chemicals, all chemicals were purchased from Sigma.

19 Glutathione transferase and glutathione peroxidase activities were measured as
20 reported by Thomson et al. [21]. Enzyme activity with aldehydes 4-HNE and trans-2-
21 nonenal was assessed as reported by Alin et al. [22]. Substrate concentrations for each
22 test are given in the Supplementary Table S2. A unit of enzyme activity is defined as the
23 amount of enzyme that catalyses the turnover of 1 μmol substrate per min. Specific
24 activity is expressed in μmol min⁻¹ per mg of enzyme. The apparent K_m value for GSH

1 was determined using GSH concentration ranging from 250 μ M to 2 mM, and a fixed
2 CDNB concentration of 2 mM. Kinetic parameters were calculated by the method of
3 Lineweaver-Burk.

4 Inhibitory effects on enzyme activity were measured by pre-incubating the
5 enzymes 5 min in presence of GSH (1 mM final concentration) and several quantities of
6 inhibitor (from 0.05 μ M to 2 mM final concentration). Reactions were initiated by
7 addition of 1-chloro-2,4-dinitrobenzene (CDNB; final concentration of 1 mM). The
8 concentration of each inhibitor giving 50 % inhibition (IC_{50}) was determined
9 graphically.
10

1 RESULTS

2

3 Sequence analysis of cDNA-encoding algal GSTs

4

5 Seven putative cytosolic GSTs were identified through analysis of algal EST libraries:
6 three from the rhodophyte *C. crispus* (CcGST1, CcGST2, CcGST3) and four from the
7 phaeophyte *L. digitata* (LdGST1, LdGST2, LdGST3, LdGST4). Their length ranged
8 from 201 to 230 amino acids. In both protoplast libraries, these GST genes were among
9 the most represented sequences. The cDNA sequences corresponding to the complete
10 coding regions of these GSTs have been deposited in GenBank under the following
11 accession numbers: EF423557 for *CcGST1*, EF423558 for *CcGST2*, EF423559 for
12 *CcGST3*, EF422836 for *LdGST1*, EF424837 for *LdGST2*, EF422838 for *LdGST3* and
13 EF422839 for *LdGST4*.

14 The GenBank database was searched using the protein blast program and
15 revealed that CcGST1, CcGST2, LdGST1, LdGST2, LdGST3 and LdGST4 were most
16 similar to Sigma-class enzymes, with a maximum of 33 % identity. Conserved residues
17 usually encountered in GST classes from mammals were also found in the algal
18 sequences. Of significance was the highly conserved catalytic tyrosine found in the
19 early N-terminal portion of most mammalian GSTs (Figure 1; [21]). In the mammalian
20 Pi, Mu and Sigma classes, the glutamine and serine residues just prior to the α 3-helice
21 are also involved in the interaction with GSH [9]. Compared to other Sigma-class GST
22 members, the C-terminal domain of the algal sequences were highly divergent, which
23 may indicate different substrate selectivities and specificities of the algal GSTs. In
24 contrast to the other GSTs identified in this study, CcGST3 shared higher identities (25-

1 26 %) with sequences from the Zeta, Beta, Delta and Epsilon classes. Two tyrosine
2 residues are found in the early N-terminal portion of the CcGST3 sequence and may be
3 involved in the GSH binding (see Supplementary Figure S1).

4 The similarity of amino acids between the six most-related algal sequences was
5 28-63 %, indicating a high degree of heterogeneity. The highest identities were
6 observed between CcGST1 and CcGST2 (51 %), and between LdGST1, LdGST2 and
7 LdGST3 (50-63 %). LdGST4 was the most divergent among the *L. digitata* sequences,
8 as CcGST3 was among the *C. crispus* sequences.

9 While classification in different GST families can be based on sequence
10 similarities alone, distinct gene structure provides additional supporting evidence [1,
11 23]. Therefore, the cDNA sequences of *C. crispus* and *L. digitata* GSTs were compared
12 with their corresponding genomic DNA sequences. The genes encoding CcGST3,
13 LdGST1, LdGST2 and LdGST3 were intronless. *CcGST1* holds an intron of 93 bp,
14 whose size and position coincides exactly with those of the unique intron of 100 bp
15 encountered in *CcGST2* (Figure 2). The *LdGST4* genomic sequence contains four exons
16 and three introns. Their length could not be determined accurately due to the low PCR-
17 product yield for this gene. This could be attributed to the presence of large introns in
18 the sequence.

19

20 **Phylogenetic position of algal GSTs**

21

22 In order to elucidate phylogenetic relationships between the identified algal GSTs with
23 sequences from other organisms, we searched for additional GST genes using genomic
24 resources available for micro- and macroalgae. Among the sequences identified, 7 full-

1 length putative cytosolic GST genes that are well supported by ESTs were considered
2 for further analyses. These sequences were as follow: two from *Gracilaria tenuistipitata*
3 (Rhodophyta), one from the green macroalga *Ulva linza* (Chlorophyta) and four for the
4 brown macroalga *Ectocarpus siliculosus* (Phaeophyceae). They will be made available
5 upon request to the authors. Sequences from the oomycetes *Phytophthora sojae* and
6 *Phytophthora ramorum* were also taken into account. Two other previously identified
7 GST sequences were also included: CspGST1 from the green unicellular alga
8 *Coocomyxa sp.* [24] and PiGST1 from the oomycete *Phytophthora infestans* [25]. The
9 red alga CcGST3 sequence was not included due to its highly divergent pattern. In total,
10 155 sequences were integrated in a multiple protein alignment. Several optimal trees
11 were then generated from both distance and maximum likelihood methods, and their
12 topologies were essentially the same, with slight differences observed in the bootstrap
13 values supporting the nodes. The most reliable tree is presented in Figure 3. Two major
14 groups of GST proteins can be established, in agreement with previous studies [26, 27].
15 The first group contains proteins specific of different eukaryotic organisms, as plants
16 (Tau, Lambda and Phi classes), animals (Omega class) and insects (Delta and Epsilon
17 classes). The Zeta and Theta classes have counterparts in plants and animals, and
18 several sequences from the oomycetes *P. sojae* and *P. ramorum* can also be found close
19 to these classes. This phylogeny confirms that the recently described Rho class, thought
20 to be fish-specific, also comprises green algal sequences [24]. The second major group
21 includes the Alpha, Pi, Mu and Sigma classes, primarily encountered in mammals. Most
22 of the newly isolated sequences from this study fall into this group and are closely
23 related to the Sigma GST members. Because of this unexpected distribution, we
24 undertook a more focused phylogenetic analysis, restricted to GSTs of the Alpha, Mu,

1 Pi and Sigma classes and including the “algae-oomycetes” sequences (see
2 Supplementary Figure S2). The overall topology already observed in the previous
3 phylogenetic tree was confirmed and a more detailed analyze is discussed on line.

4 5 **Different transcription patterns for brown and red algal GSTs**

6
7 In both *C. crispus* and *L. digitata*, GST sequences were identified through analysis of
8 protoplast cDNA libraries and were among the most represented genes, suggesting a
9 possible role of these enzymes in detoxification processes during protoplast generation.
10 Changes in expression of these genes were further investigated under different chemical
11 treatments and after short exposure times (Figure 4).

12 In the red alga *C. crispus*, treatments with H₂O₂, the auxin analogue 2,4-D and
13 the herbicide glyphosate showed a slight increase of accumulation in GST transcripts
14 (Figure 4A). Incubation in presence of the herbicides metolachlor and atrazine, and the
15 insecticide dichlorvos strongly induced expression of the three GST genes. The major
16 effects were observed for *CcGST1* with 23-fold induction by metolachlor and 18-fold
17 induction by dichlorvos. Heavy metals caused a modest repression effect on GST gene
18 transcription, with a maximum of a 2-fold decrease. The most marked effects were
19 observed after incubation in the presence of fatty acid derivatives. MeJA treatment
20 induced a 6- to 43-fold change in transcript accumulation. Similar ranges of induction
21 were observed with oxylipins, stronger effects being obtained with 13(S)-HpODE on
22 *CcGST2* (12-fold) for the C18 derivatives, and with 15(S)-HETE on *CcGST3* (80-fold)
23 for the C20 derivatives.

1 In the brown alga *L. digitata*, elicitors released after wounding and degradation
2 of the cell wall such as oligoguluronates are recognized as defense signals and induce an
3 oxidative burst in the alga [28]. *LdGST* genes were all induced by the oligoguluronates
4 (4 to 24-fold), *LdGST4* being the most up-regulated gene (Figure 4B). A similar pattern
5 of variation was obtained in presence of copper, with *LdGST2* being the most up-
6 regulated (30-fold). In presence of fatty acid derivatives, slight changes of transcript
7 levels were observed with 15(S)-HETE, while MeJA caused a repression with a
8 maximum of a 6-fold decrease for *LdGST1*.

10 **Biochemical studies of algal GST**

11 In order to investigate the biochemical features and to determine the catalytic properties
12 of the GST enzymes from both algae, the GST genes presently described were assayed
13 for heterologous expression in *Escherichia coli*. Only CcGST2 and LdGST3 were
14 selected for further investigation since they were the most highly expressed in the
15 soluble fraction. Migration on SDS-PAGE gels of these enzymes showed that the
16 purified proteins gave single bands of 26 kDa. Gel-filtration chromatography analysis
17 showed a single peak with molecular mass of 52 kDa for both GSTs, strongly
18 suggesting that the active form of these enzymes exhibits homodimeric structures (see
19 Supplementary Figure S3).

20 The catalytic specificities of CcGST2 and LdGST3 were explored using a range
21 of GST substrates. In presence of the commonly used CDNB, the two algal GSTs
22 exhibited moderate transferase activity (Table 1). Higher transferase activities were
23 measured towards several model aryl halide substrates, as illustrated by the results
24 obtained with CcGST2 in presence of 1-fluoro-2,4-dinitrobenzene (FDNB; 20.34 μ mol

1 min⁻¹ mg⁻¹). Slight transferase activities were also observed with one aldehyde of the
2 α,β -unsaturated carbonyls, the 4-HNE. No activity was detected with the *para*-
3 nitrophenyl acetate substrate. The algal GSTs were also incubated in presence of the
4 synthetic cumene hydroperoxide and *t*-butyl hydroperoxide substrates to ascertain their
5 glutathione peroxidase activity. Results show that the recombinant enzymes exhibited
6 similar moderate activity to reduce cumene hydroperoxide (Table 1). In addition, both
7 proteins had activity towards hydroperoxides of C18 polyunsaturated fatty acids (13-
8 HpODE and 13-HpOTE).

9 The K_m values of CcGST2 and LdGST3 for GSH in presence of CDNB were
10 calculated to be 0.9 and 1.1 mM respectively (see Supplementary Table S4).
11 Measurement of GST activity through a wide range of pH for the two purified
12 recombinant enzymes indicated that optimal pH was 8 and 10 for CcGST2 and LdGST3
13 respectively (see Supplementary Figure S4).

14 To complete these data, inhibiting assays were also performed and results are
15 presented in Table 2. Cibacron blue, a typical ligandin-type inhibitor, showed the most
16 potent inhibition effect (IC_{50} of 0.5 and 0.1 μ M for CcGST2 and LdGST3 respectively),
17 whereas chenodeoxycholic acid, lithocholic acid, indomethacin and acetyl salicylic acid
18 did not show any marked inhibition effect on the activity of either enzyme.

1 DISCUSSION

2

3 The soluble glutathione transferases constitute a widespread enzyme superfamily that
4 has been subdivided into an ever-increasing number of classes [29]. While there are no
5 clearly established criteria concerning the extent of sequence similarity required for
6 placing a GST in a particular class, it is generally accepted that GSTs of the same class
7 share more than ca. 50% sequence identity, while GSTs of different classes share less
8 than ca. 30% identity [23, 30]. Some of these classes are ubiquitous all the way up to the
9 kingdom level, whereas others are exclusively found in particular groups of organisms
10 such as mammals, plants or insects. As large-scale EST and genomic approaches are
11 being applied to previously uninvestigated phyla, new GST genes and even new GST
12 classes are being discovered. Here we identified and characterized GST genes from two
13 independent eukaryotic lineages, the Rhodophyta (red algae) and the Heterokonta
14 (brown algae and oomycetes).

15 The phylogenetic analyses showed that the GST sequences from the red and
16 brown algae, along with some oomycetes, fall into a distinctive, newly identified group.
17 Within this new cluster, sequences may be assigned to several sub-groups: GtGST
18 sequences are closely related to CcGST sequences, while EsGST3, EsGST4 and
19 LdGST4 group together; LdGST1, LdGST2, LdGST3 and EsGST1 appear to form
20 another group. This grouping is further supported by evidence for distinct gene
21 structure. The *LdGST1*, *LdGST2* and *LdGST3* genes are intronless, while *CcGST1* and
22 *CcGST2* genes contain a single intron, and the *LdGST4* gene has three introns. In
23 addition, it is interesting to note that the brown algal sequences appear to be more
24 closely related to the red algal enzymes than to the *P. sojae* sequences. Considering the

1 common evolutionary history of oomycetes and brown algae in the heterokont lineage,
2 their respective GST members were expected to group in the same cluster. The
3 divergence among the sequences identified in this work is also illustrated by long
4 branch lengths, of great magnitude compared to the Alpha-, Mu- and Pi-class clusters
5 whose members are much more closely related to each other. This higher evolutionary
6 rate may also reflect an adaptive response to habitat. It is tempting to suggest that this
7 unexpected topology results from convergent evolution of algal enzymes in response to
8 a specific adaptation to the marine environment.

9 This new set of GSTs are closely related to the Sigma GST enzymes which is
10 encountered in both vertebrates and invertebrates, while the Alpha, Pi and Mu members
11 are only found in vertebrates [31]. It was initially suggested that the Alpha-class
12 enzymes were the oldest members of this clade and that they had been subsequently lost
13 in invertebrates [9, 29, 31]. In contrast, Frova [23] recently proposed that the Sigma-
14 class GSTs diverged before the Alpha, Mu and Pi group and has evolved distinct
15 functional properties in the diverse lineages. The occurrence of sequences closely linked
16 to the Sigma enzymes in algae and oomycetes supports this last hypothesis suggesting
17 that the entire Sigma class emerged before the eukaryotes split and diverged extensively
18 later in independent lineages.

19 Among the GST genes isolated from the red algae, CcGST3 represents an
20 intriguing sequence. It exhibits only low similarity with other GSTs from an algal
21 origin. No intron was found in its gene sequence. Despite the fact that we cannot rule
22 out a bacterial origin for this GST, several observations point towards CcGST3 being an
23 endogenous algal sequence: a polyA tail is present at the end of the corresponding
24 mRNA and the likely active sites residues of the encoded protein may be different from

1 the ones encountered in prokaryotic GSTs. Indeed, GSH-binding is known to involve a
2 conserved cystein residue in the N-terminal portion of the bacterial GSTs. While no
3 such residue is found in the putative GSH-binding domain of CcGST3 (see
4 Supplementary Figure S1), two tyrosine residues are encountered in its N-terminal
5 portion which may play this role. In addition, and as discussed below, *CcGST3* follow a
6 transcription pattern similar to the others *CcGSTs* and sometimes being the most
7 affected during the treatments.

8 Biotic stress and chemicals are known to affect the expression of GSTs at the
9 transcriptional level [1], transcript accumulations for *C. crispus* and *L. digitata* genes
10 were determined in response to various treatments. The seven identified genes were
11 constitutively expressed in the two algae. The basal levels of expression were lower for
12 the *LdGST* genes than for the *CcGST* genes. *CcGST2* was expressed at the same level as
13 the housekeeping gene actin, indicating that this GST protein plays a major role in the
14 basic metabolism of *C. crispus*. *CcGST* genes were highly induced by the herbicides
15 atrazine and metolachlor and the insecticide dichlorvos. This increase in gene
16 expression was correlated with a higher GST activity in total extracts of the red alga
17 treated during 24 hours with metolachlor and dichlorvos (data not shown). The red
18 macroalgae may therefore have the potential to remove xenobiotic pollutants from their
19 environment.

20 In contrast to these results and considering what is known in higher plants [32]
21 heavy metals such as cadmium, copper, zinc and aluminium caused a decrease in
22 expression of the three *CcGST* genes. Opposite results were observed for the *LdGST*
23 genes during treatment with CuSO₄. Incubation of *L. digitata* sporophytes with
24 oligogulonates resulted in a strong induction of its GST genes. These oligosaccharides

1 are recognized as endogenous elicitors by the alga, inducing oxidative burst production
2 as part of the active defense system [28]. Copper has also been shown to activate
3 transient massive release of reactive oxygen species in this alga (data not shown).
4 Therefore the accumulation of *LdGST* transcripts may be mediated through induced
5 H₂O₂ production. This hypothesis is not relevant for *C. crispus* since application of an
6 oxidative stress seems to have almost no effect on the accumulation of *CcGST*
7 transcripts.

8 Interestingly, contrasting results were also obtained for GST from both algae in
9 presence of oxylipins derived from C18 and C20 polyunsaturated fatty acids. Several
10 oxylipin structures have been described so far in marine algae, some of them being
11 closely related, and even identical, to those identified in terrestrial plants (C18-
12 derivatives; [33]) and mammals (C20-derivatives; [34]). Their biological functions
13 remain elusive, despite the fact that some of them have been demonstrated to act as
14 potential defense and signaling mediators in red marine alga [35]. The present study
15 demonstrated that C18 and C20 oxylipins greatly increased levels of *CcGST* transcripts,
16 confirming previous results obtained after treatment of *C. crispus* with MeJA . The
17 precise roles of *CcGST* during these treatments are unknown. They may be expressed as
18 active participants in a more global defense response to detoxify by-products released
19 during biotic stresses. However, they could also have a more dedicated function, such as
20 a potential involvement in fatty acid and oxylipin metabolism. Since the tested oxylipins
21 had the opposite effect on *LdGST* genes, these molecules may have different roles in the
22 two algae, and/or GSTs may not drive similar processes in the two lineages.

23 It is also worth noting that, despite structural differences in gene organization
24 among GST genes within a species, all GSTs from the same alga exhibited a similar

1 transcription profile. This probably suggests functional convergence of these enzymes
2 within a species, and their importance in specific metabolic pathways. Further
3 experiments are required to identify the biological significance of this result.
4 Expression and purification of recombinant CcGST and LdGST enzymes in *E. coli*
5 made it possible to characterize the properties of these proteins. The catalytic profile of
6 CcGST2 was similar to that of LdGST3, but the red alga GST showed greater activity
7 towards FDNB substrates. In contrast, LdGST3 exhibited a higher transferase activity in
8 presence of 4-HNE, an electrophilic end-product of lipid peroxidation. Both enzymes
9 have no activity on the related compound *trans*-2-nonenal. In addition to their
10 glutathione transferase activity, both enzymes are able to reduce organic hydroperoxides
11 of fatty acids. This peroxidase activity might play a role in the algae during oxidative
12 stress, in preventing the production of cytotoxic aldehydes from the organic
13 hydroperoxides. Algal GST K_m values illustrate a moderate affinity substrate to GSH.
14 These results are however in agreement with published K_m^{GSH} values for other GST
15 (Konishi *et al.*, 2005, Park *et al.*, 2005, Urade *et al.*, 1987, Zeng *et al.*, 2005).

16 In addition to detoxifying electrophilic compounds, there is growing evidence
17 that GST members carry out additional functions. The GST activities are quite diverse,
18 not only between, but also within classes, as demonstrated for members of the Sigma
19 class. For example, in cephalopods, S-crystallin proteins are highly abundant in eye
20 lenses, and have lost their GST activity due to insertion of 11 residues between the
21 conserved $\alpha 4$ and $\alpha 5$ helices [36]. In *Drosophila melanogaster*, Sigma-type GSTs have
22 considerable activities towards α, β -unsaturated carbonyl compounds derived from a
23 secondary lipid-peroxidation [37]. In vertebrates, they are known as glutathione-
24 dependent prostaglandin D-synthases (PGDS), catalysing the isomerisation of

1 prostaglandin H2 to prostaglandin D2 [21]. This activity is also conserved in the Sigma
2 GST from the parasitic flatworms *Schistosoma* [38], despite their distinct phylogenetic
3 position.

4 We previously described the production of cyclopentenonic prostaglandins of
5 the A2, B1, B2 and E2 series after various treatments in *C. crispus* [35]. In mammals,
6 these molecules are produced by cyclooxygenases [39], and pharmacological studies
7 have shown that acetyl salicylic acid and indomethacin inhibits specifically these
8 enzymes [40]. In the red alga, the enzymatic machinery involved in the production of
9 prostaglandins remains elusive. However, synthesis of these cyclopentenones in *C.*
10 *crispus* is strongly affected in presence of cibacronblue, while no alteration was
11 monitored in presence of either indomethacin or acetyl salicylic acid (data not shown).
12 Interestingly, similar inhibitor action has been observed on CcGST2 activity. Therefore,
13 we are currently undertaking further experiments to determine if this GST might play a
14 role in prostaglandin synthesis in *C. crispus*.

15 To conclude, the increasing number of genomic projects dealing with previously
16 uninvestigated lineages will probably greatly modify our current knowledge on the
17 evolution diversity and function of GSTs in the near future. As illustrated in the present
18 study with GSTs characterized from *C. crispus* and *L. digitata*, the challenge lies in
19 identifying their functions, not necessarily associated with detoxification. Further
20 investigations on these proteins, including structural biology approaches, may provide
21 some clues on the catalytic specificities and physiological functions of these enzymes in
22 macroalgae.

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C. Hervé was supported by a BDI fellowship from the Centre National de la Recherche Scientifique. PO de Franco was supported by the French Ministère de l'Enseignement Supérieur et de la Recherche. We thank G. Michel for technical support and helpful advice on structural alignment analyses. We are grateful to F. Morel for supplying GST substrates as well as for valuable discussion and to M. Cock for providing access to the *E. siliculosus* EST database. Part of this work was performed in the framework of the "Marine Genomics Europe" NoE (EC contract N° GOCE-CT-2004-505403).

SUPPLEMENTARY DATA

The following supplementary data is available for this article:

Figure S1 Alignment of the predicted amino acid sequence of CcGST3 with sequences from the Beta and Delta classes

Figure S2 Maximum likelihood phylogenetic tree showing the relationship between selected GSTs from algae and oomycetes and the Alpha, Pi, Mu and Sigma GST classes

Figure S3 Purification of recombinant CcGST2 and LdGST3 enzymes

Figure S4 Effect of pH on CcGST2 and LdGST3 glutathione transferase activity

Table S1 List of oligonucleotide primers used in this study

Table S2 Concentrations of substrates used for the determination of specific transferase and peroxidase activities

- 1 **Table S3 List of sequences used for the phylogenetic analysis**
- 2 **Table S4 Kinetic constant values for the recombinant CcGST2 and LdGST3**
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1 **Table 1 Substrate specificities of the recombinant CcGST2 and LdGST3**

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Substrate	Specific activity ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ of protein)	
	CcGST2	LdGST3
Aryl halides		
1-chloro-2,4-dinitrobenzene (CDNB)	1.56 ± 0.08	0.98 ± 0.10
1-bromo-2,4-dinitrobenzene (BDNB)	1.56 ± 0.08	3.34 ± 0.04
1-fluoro-2,4-dinitrobenzene (FDNB)	20.34 ± 0.59	7.44 ± 0.66
1-iodo-2,4-dinitrobenzene (IDNB)	3.84 ± 0.12	1.29 ± 0.11
<i>para</i> -nitrobenzoyl chloride	not detected	not detected
α,β -unsaturated carbonyls		
Ethacrynic acid	not detected	not detected
<i>trans</i> -4-phenyl-3-buten-2-one	not detected	not detected
4-hydroxynonenal (4-HNE)	0.20 ± 0.02	0.69 ± 0.01
<i>trans</i> -2-nonenal	not detected	not detected
Miscellaneous		
<i>para</i> -nitrophenyl acetate	not detected	not detected
Organic hydroperoxides		
Cumene hydroperoxide	0.15 ± 0.03	0.12 ± 0.01
<i>t</i> -butyl hydroperoxide	0.04 ± 0.01	0.61 ± 0.12
13-HpODE	0.23 ± 0.01	0.06 ± 0.01
13-HpOTE	0.05 ± 0.01	0.08 ± 0.01

4

5 Results are expressed as means \pm SD of four determinations.

6

1 **Table 2 Inhibitor sensitivities of recombinant CcGST2 and LdGST3**

Compound	Inhibitor concentration range (μM)	IC ₅₀ (μM)	
		CcGST2	LdGST3
Cibacron Blue	0-10	0.5	0.1
Chenodeoxycholic acid	0-1000	564	93
Lithocholic acid	0-1000	119	83
Indomethacin	0-1000	623	71
Acetyl salicylic acid	0-1000	> 1000	> 1000

2

3 Results are expressed as the mean of four determinations.

1 **FIGURE LEGENDS**

2

3 **Figure 1 Alignment of GST amino-acid sequences from *C. crispus* and *L. digitata***
4 **with mammalian sequences from the Sigma class**

5 Conserved amino acids highlighted in black are identical and residues boxed in grey are
6 similar. Known secondary structure of the human sequence HsGSTS1 is shown above
7 the alignment. Alpha helixes and beta strands are represented as helixes and arrows
8 respectively, and beta turns are marked with TT. Potential catalytically important
9 residues involved in GSH-binding are shown as filled arrowheads. This alignment was
10 created using the following sequences (organism, Genbank/PDB accession numbers):
11 HsGSTS1 (*Homo sapiens*, AAH20734, 1IYI), RnGSTS1 (*Rattus norvegicus*,
12 NP8113832).

13

14 **Figure 2 Exon/intron structure of genes encoding GSTs in *C. crispus* and *L. digitata***

15 The genomic DNA sequences of coding regions are represented; solid rectangles denote
16 exons. A scale bar is given.

17

18 **Figure 3 Unrooted neighbor-joining phylogenetic tree showing the relationship**
19 **between GSTs from algae, oomycetes and the major GST classes.**

20 Clustering of proteins into GST classes is indicated. Numbers in brackets refer to the
21 number of sequences included for each class that have been collapsed in the figure. The
22 numbers on the branches indicate the percentage of node support from 1,000 bootstrap
23 replicates.

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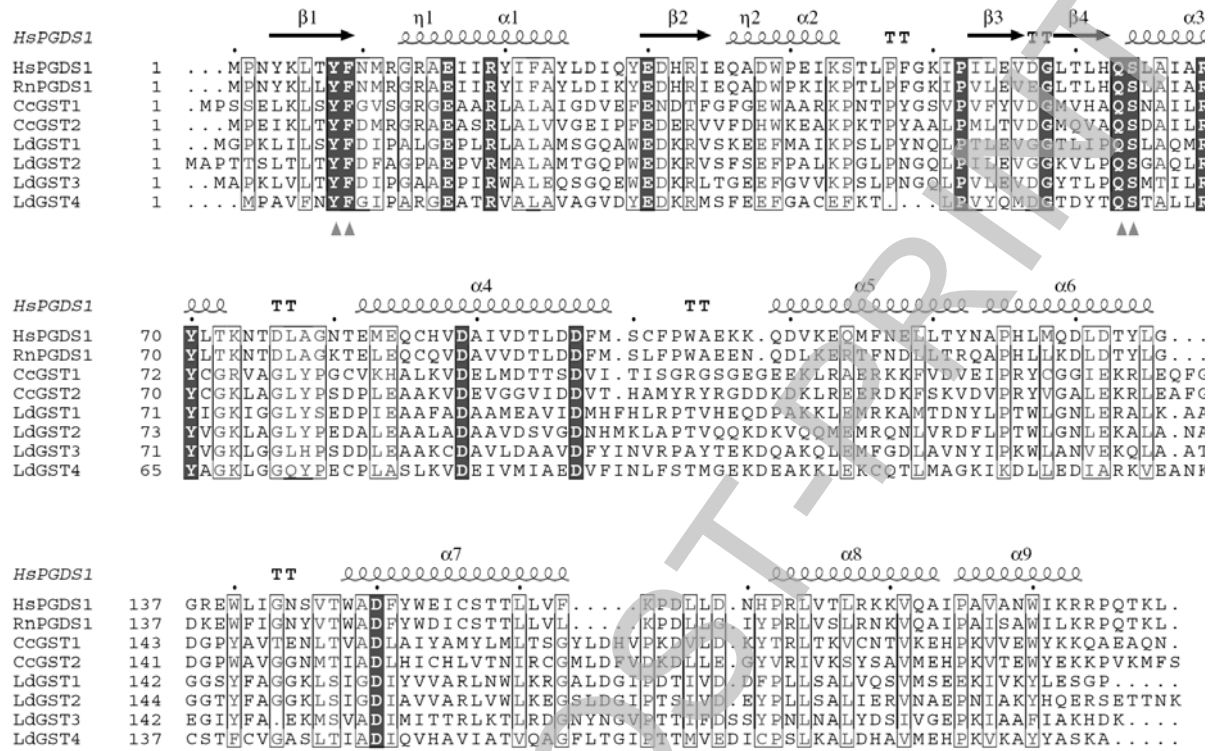
1 **Figure 4 Real-time PCR analysis of *C. crispus* and *L. digitata* GST genes after**
2 **different chemical treatments**

3 The relative gene expression ratios for the GST transcripts were calculated as described
4 in the Experimental procedures. **(a)** Expression profile of GST transcripts from the red
5 alga *C. crispus*. A set of 16 products was tested, including six pesticides, four heavy
6 metals and five fatty acid derivatives. **(b)** Expression profile of GST transcripts from the
7 brown alga *L. digitata*. Oligoguluronates, known as endogenous oligosaccharide
8 elicitors, were tested, as one heavy metal and two fatty acid derivatives.

9 Bars: standard errors. Values represent the mean of two determinations from three
10 independent biological samples.

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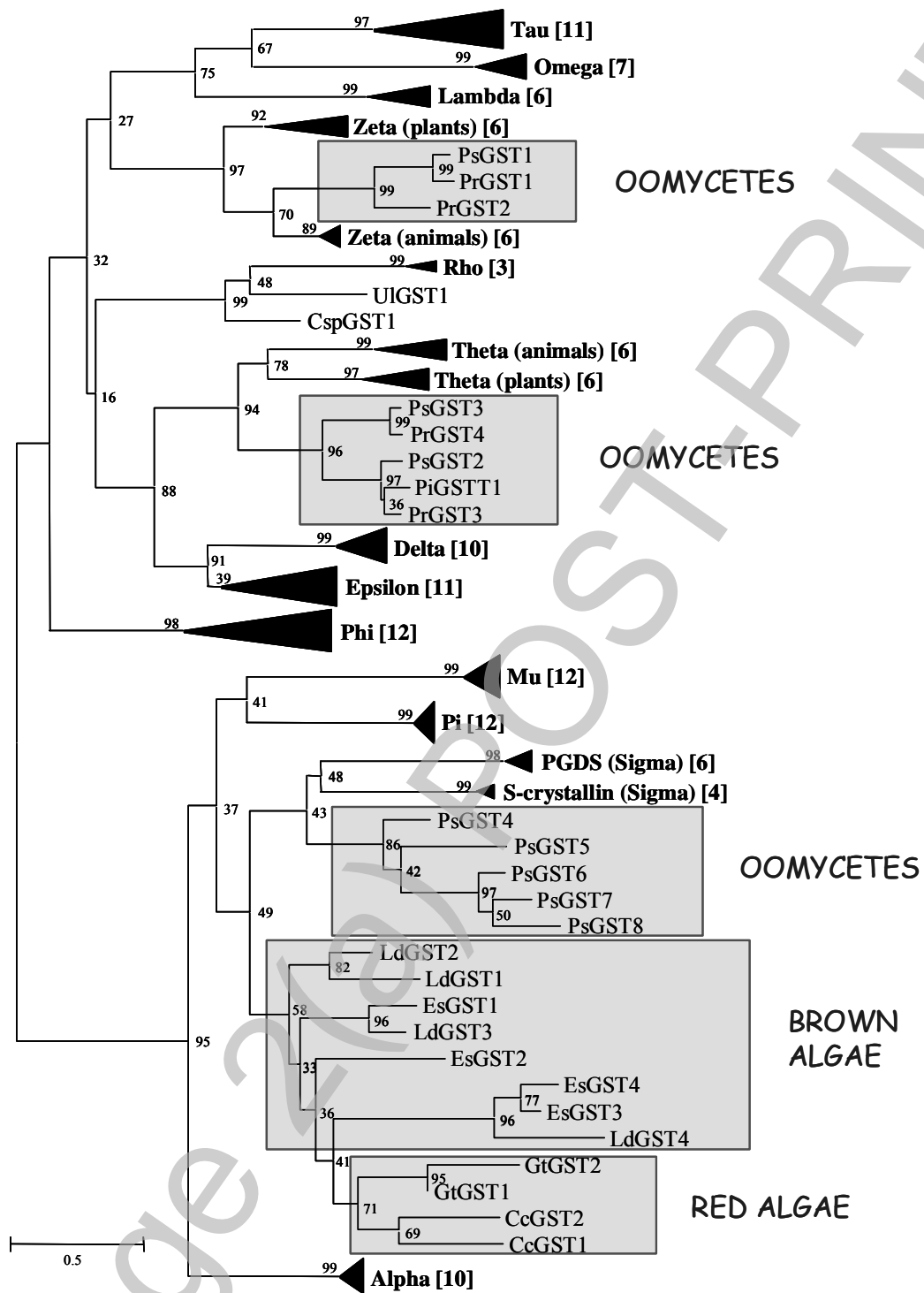
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5 **Figure 1**

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Stage 2(a) PROOF

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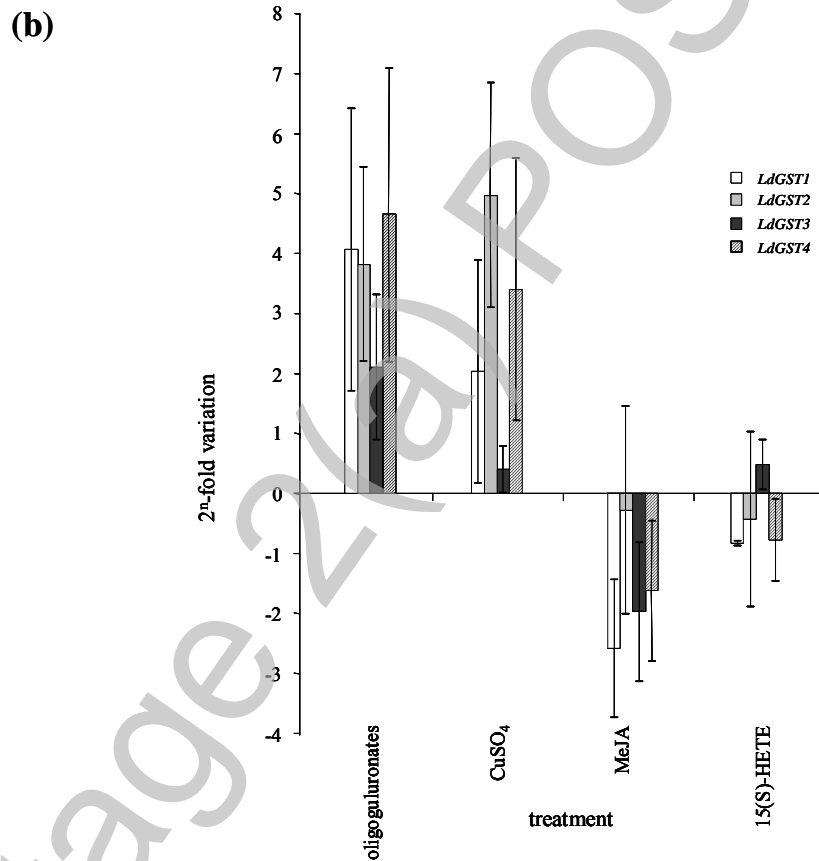
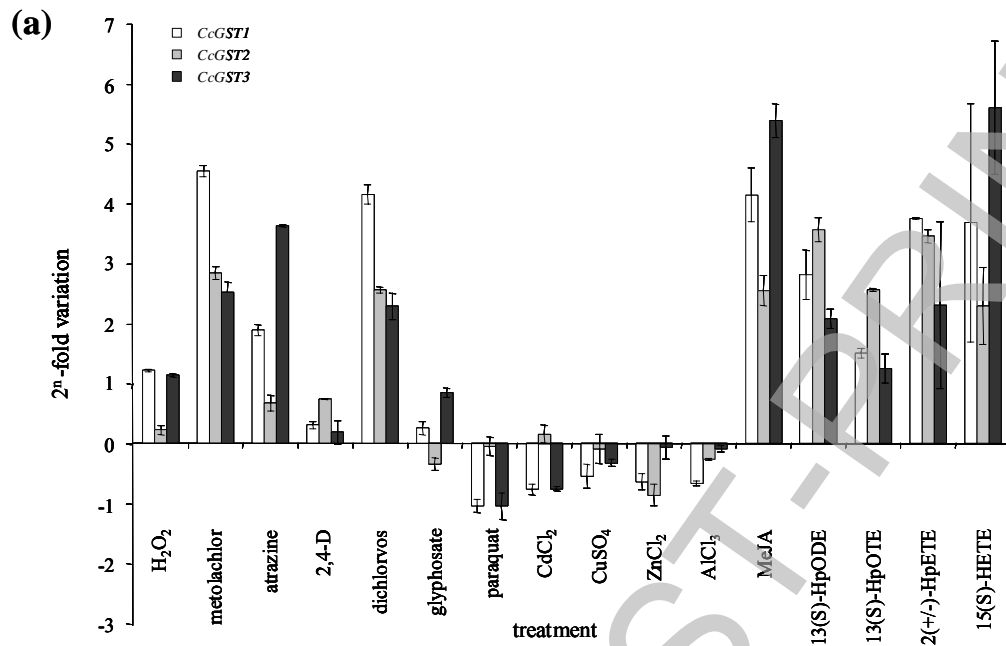


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3 **Figure 3**

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4 **Figure 4**

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