Osgstu3 and osgstu4, encoding tau class glutathione S-transferases, are heavy metal- and hypoxic stress-induced and differentially salt stress-responsive in rice roots¹

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Abstract Glutathione S-transferases (GSTs) have poorly understood roles in plant responses to environmental stresses. A polyethylene glycol (PEG)-induced tau class GST was identified in rice roots by protein microsequencing. PEG and the heavy metals Cd (20 μM), Zn (30 μM), Co and Ni rapidly and markedly induced osgstu4 and osgstu3 in rice seedling roots. Osgstu4 and osgstu3 were also induced in roots by hypoxic stress but not by cold nor heat shock. Salt stress and abscisic acid (ABA) rapidly induced osgstu3 in rice roots, whereas osgstu4 exhibited a late salt stress and no ABA response. Salicylic acid, jasmonic acid and the auxin α-naphthalene acetic acid triggered osgstu4 and osgstu3 expression. Osgstu4 and osgstu3 were rapidly and markedly induced by the antioxidant dithiothreitol and the strong oxidant hydrogen peroxide, which suggested that redox perturbations and reactive oxygen species are involved in their stress response regulations.

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Key words: Glutathione S-transferase; Heavy metal stress; Hypoxic stress; Salt stress; Redox perturbations; Rice (Oryza sativa L.) roots

1. Introduction

Glutathione S-transferases (GSTs, EC 2.5.1.18) catalyze the conjugation of the tripeptide glutathione (GSH, γ-glutamyl-cysteinyl-glycine) to a wide range of xenobiotic or natural, often cytotoxic compounds with a reactive electrophilic center [1–5]. Plant GSTs are well known for their role in herbicide detoxification. GSTs ‘tag’ herbicides for vacuolar sequestration by ATP-binding cassette transporters that function as glutathione-conjugate Mg2+-ATPase pumps [1–3,6,7].

The ‘original’ functions of plant GSTs are poorly understood since few endogenous plant substrates for GSH conjugation have been identified [4]. GSTs can also function as non-catalytic carriers, e.g. of auxins and cytokinins [4,5,8] or anthocyanins [9,10], and thus contribute to hormone homeostasis or vascular anthocyanin sequestration respectively. Other GSTs catalyze alternative isomerization or peroxidase reactions and are involved in tyrosine metabolism or hydroxy-peroxide detoxification respectively [4,5,11]. GSTs may also function in cellular redox homeostasis [12], act as stress signaling proteins [13] or regulate apoptosis [14].

Plant gsts gene families are large and highly diverse: 48 members in Arabidopsis, over 25 in soybean, and 42 in maize [5,15]. Based on protein homology and gene organization, the plant GSTs have been subdivided in class Φ (F, previously type I), ζ (Z or type II), τ (U or type III), Θ (Τ or type IV) and λ (L) [3–5,12]. The plant-specific τ class gstu gene families of Arabidopsis, soybean and maize have 28, 20, and 28 members respectively [5,15].

Gst genes exhibit quite diverse regulations in response to biotic and abiotic stresses, but their presumably significant and versatile involvements in plant stress responses remain poorly understood. Gst responses to herbicides and herbicide safeners have been studied extensively [1,16] but give few clues to the natural functions of these GSTs. GSTs are involved in plant responses to pathogens and oxidative stress [1]. Twelve Arabidopsis gst genes exhibited a diverse range of responses to jasmonates, salicylic acid (SA), ethylene, pathogen infection and oxidative stress [17]. GSTs with peroxidase (POX) activity can catalyze the reduction of lipid hydroperoxides and alleviate oxidative stress. Overexpression of a tobacco POX-GST in Arabidopsis mediated protection against oxidative damage during various stresses [11].

GST responses to various other environmental stresses are less documented. Dehydration rapidly induced Arabidopsis Atgstf3 (erd11) and Atgstf4 (erd13), which were not abscisic acid (ABA)-responsive [18]. Dehydration caused a late induction of Atgstf8, which was attributed to secondary oxidative stress effects [19]. Redox perturbations were found to induce a number of λ class and DHAR gsts genes with potential roles in redox homeostasis [12]. Plant GSTs are potentially involved in heavy metal stress responses, but have received little attention in that context. Heavy metals were found to induce soybean Gmgst1 (Gh2/4), which was also polyethylene glycol (PEG)-
responsive [20], the wheat GST25 and GST26 proteins, which are also herbicide-induced [21] and maize bronze-2 or Zmsgstu4, involved in anthocyanin binding and sequestration [22]. Gst gene expression is often regulated by bZIP transcription factors, e.g. TGA1a, which exhibits a root-specific expression [23].

In this study, we present a characterization of osgstu3 and osgstu4, encoding GSTUs of rice. OsGSTU4 was identified by protein microsequencing in roots of rice. We compare osgstu3 and osgstu4 expression in response to various environmental stresses while focusing on roots, which have important roles in plant responses to soil stresses. We furthermore investigate the effect of plant growth regulators and cellular conditions that might signal stress-responsive gene expression in rice roots.

2. Materials and methods

2.1. Plant material and growth conditions

Rice seeds (Oryza sativa ssp. Indica var. Pokkali) were kindly supplied by the International Rice Research Institute (Manilla, Philippines). Approximately 40 seeds were germinated on grids, placed above pots containing N-enriched half-strength Hoagland solution (pH 5.6) and grown at 27°C, 16 h of light, 8 h of dark for 15 days. The growth solutions were autoclaved or filter-sterilized.

2.2. Stress, plant hormone and heavy metal treatments

Grids holding 15-day-old seedlings were placed on top of pots containing growth solution supplemented with the indicated amounts of PEG 20000 (Fisher Scientific, Ottawa, ON, Canada), ZnSO4 containing growth solution supplemented with the indicated amounts of (pH 5.6) and grown at 27°C, 16 h of light, 8 h of dark for 15 days. The growth solutions were autoclaved or filter-sterilized.

Two-dimensional protein gel analysis, in situ trypsin digestion and microsequencing of the 29-kDa protein, immobilized on protein blots, was performed as described before [24,25].

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR) cloning of osgstu4 and osgstu3

The 3’ RACE osgstu4 primer 5’-CGTGTTTACATCCAATCATTCTC-3’ (IGA) was based on D41737, a 386-bp expressed sequence tag (EST) of the Japanese rice variety Nipponbare that correctly encoded three peptides of the 29-kDa rice protein. The 3’ osgstu4 cDNAs were 1165 bp, 1000 bp and 884 bp long and included 3’ untranslated regions (UTRs) of 377 bp, 194 bp and 103 bp respectively with varying poly-A tails. The 5’ RACE osgstu4 RT primer and nested primer were 5’-CGTGTTTACATCCAATCATTCTC-3’ and 5’-GGTGTTCATCCAAATCATTCTC-3’ respectively. The overlapping 5’ osgstu4 cDNA was 300 bp long, including a 5’ UTR of 81 bp. The 5’ RACE osgstu3 primer 5’-TCTGGGATGTCCAGTCACT-3’ was based on D41737, a 305-bp EST from the Japanese rice variety Nipponbare that was homologous to osgstu3. The 3’ osgstu3 cDNAs were typically 688 bp long and all included a 188-bp 3’ UTR. RT-PCR using the primes 5’-AGCTGACGAAAGAACAATCGAAG-3’ (1GB) and 5’-GGATCCAGTCAGGCTCCAT-3’ (2GB) yielded an overlapping 5’ osgstu3 cDNA of 617 bp. All PCR products were cloned in pGEM-T (Promega, Madison, WI, USA).

2.5. DNA sequencing

Sequencing reactions were performed in both directions with fluorescent dye primers (LI-COR, Lincoln, NE, USA) using the 77-Sequencing Kit of Amersham Pharmacia Biotech (Piscataway, NJ, USA) and analyzed by the Canadian Molecular Research Services (CMRS, Ottawa, ON, Canada). Sequences were confirmed twice with fragments obtained from independent PCR amplifications to discern possible mutations due to reading mistakes of the Taq polymerase.

2.6. RT-PCR expression analysis

Total RNA was prepared from rice roots by phenol extraction, quantified spectrophotometrically, carefully diluted to equal concentrations and quality checked by agarose gel electrophoresis. 1 μg of each RNA sample was used for first strand cDNA synthesis with a 17-mer oligo-dT primer and Superscript II RNase H- Reverse Transcriptase (Invitrogen). Reactions without RT were performed in parallel for each sample. One-thirtieth of the first strand cDNA products was used for amplification. Twenty-eight amplification cycles of 94°C (30 s), 60°C (30 s) and 72°C (1 min) were performed in a thermal cycler with Peltier-effect heat pump (MJ Research, Incline Village, NV, USA). Linearity between the amount of input RNA and the final RT-PCR products was verified and confirmed. The gene-specific PCR primers were 1GA and 5’-CGTCTTGGCTCTGAAGTAGTGC-3’ (2GA) for osgstu4, 1GB and 2GB for osgstu5, and 5’-ATCCATAGACATCATACAAC-3’ (1AC) and 5’-TAGAAAGATCTTCTGTTGCACA-3’ (2AC) for rice actin 1 (accession number X15865). The primer pairs 1GA/2GA, 1GB/2GB and 1AC/2AC spanned introns of 228 bp, 97 bp and 82 bp respectively in the osgstu4, osgstu5 and rac1 genes of the rice variety Pokkali (data not shown). Specificity of amplification was confirmed by an analogous PCR on genomic DNA with each of the above primer pairs, which yielded single, gene-specific amplification products, that were cloned and sequenced. Specificity of amplification was also confirmed through direct sequencing of RT-PCR products (data not shown).

3. Results

3.1. PEG induces a GST protein in roots of rice

In the course of a study on the molecular responses to salt and osmotic stress, we analyzed changes in two-dimensional protein patterns in roots of rice in response to ABA, NaCl and PEG [25,26]. In the present study, a protein of about 29 kDa and pI 5 was found to be induced in roots but not in the shoot of rice seedlings, incubated on 15% PEG solutions for 60 h (Fig. 1).

Internal peptides were generated by in situ trypsin digestion of the 29-kDa protein, immobilized on 12 two-dimensional protein blots. The amino acid sequences of six tryptic peptides were determined as WPSPFVT, GLSYEYVK, SELLASNPVK, APLLAAWAQR, FGELDVAEK, and VLPDV-DGVVEFk. These peptide sequences exhibited significant homology to GSTU proteins of the monocotyledonous plants rice, maize, Aegilops sp. and Aloepecurus sp.

3.2. Osgstu4 and osgstu3 encode GSTU proteins

Osgstu4 and osgstu3 (O. sativa genes for GSTUs 3 and 4) were cloned by RT-PCR, including 5’ and 3’ RACE. We followed the nomenclature for a rice gst EST inventory that was introduced in GenBank. The osgstu4 and osgstu5 cDNA sequences of 1200 bp and 943 bp have open reading frames of 717 bp and 699 bp and 5’ UTRs of 81 bp and 56 bp respectively. The positions of the initiation codons were based on homology with other plant GST proteins. Three types of 3’ end osgstu4 cDNAs that only varied in the length of the 3′ UTRs were isolated (data not shown). Three potential polyadenylation addition signal sequences AATAAT, which are single base variants of the consensus AATAAA sequence,
Ser-16, an Arg-21 and a Ser-70, the latter in the 'signature motif' for plant GSTs: the putative active site
233 amino acids contain the conserved amino acids that form
which indicates that they originated by gene duplication.
the same location and exhibit 80% coding sequence identity,
clone AP003450) contains a tandem repeat of two
 genes. Chromosome 1 (bacterial artifical chromosome (BAC)
which has been best resolved so far, encodes at least 22
ences do not exist amongst the OsGSTU3proteins of Pokkali
AF309378) in GenBank. The
coding sequence of Pok-
are located 46 bp, 25 bp and 47 bp upstream of the three
detected polyadenylation sites (data not shown). Differential
polyadenylation processing has also been reported for the An9
gst gene of petunia [27]. The 3’ UTR of osgstu3 contains a
single AATAAA sequence, 32 bp upstream of the only poly-
denylation site.
We compared osgstu4 and osgstu3 isolated from O. sativa L. sp.
Indica cv. Pokkali to the corresponding ESTs and
genes of the evolutionarily distinct Japonica rice variety Nip-
ponbare [28] and the genome draft from other Indica rice
varieties [29]. The osgstu4 cDNA from Pokkali is 10 bp and
205 bp longer in the 5’ and 3’ UTRs respectively than the
osgstu4 cDNA from Nipponbare (accession number
AF309378) in GenBank. The osgstu4 coding sequence of Pok-
kali differs in 5 bp from Nipponbare, which results in one
amino acid substitution, but is identical to the Indica sp.
gene after conceptual splicing. The osgstu3 coding sequence from Pokkali differs in 3 bp from the osgstu3 cDNA from
Nipponbare (accession number AF309379) in GenBank, which
results in two amino acid substitutions. These differ-
ences do not exist amongst the OsGSTU3 proteins of Pokkali
and Indica sp., although only 85% of the coding region was
present in that genome draft. The comparisons indicated that
all observed differences in sequence were of varietal origin.

The genome of O. sativa ssp. Japonica cv. Nipponbare [28],
which has been best resolved so far, encodes at least 22 gstu
genes. Chromosome 1 (bacterial artificial chromosome (BAC)
clone AP003450) contains a tandem repeat of two gstu genes,
whereas chromosome 10 contains a cluster of 20 closely re-
lated gstu genes, comprised on a 148-kb BAC clone (accession
number AC091680). Osgstu3 and osgstu4 are tandemly ar-
ranged within that gstu gene cluster on chromosome 10,
with 1.8 kb of DNA sequence between the two genes. The
osgstu3 and osgstu4 genes are interrupted by a single intron at
the same location and exhibit 80% coding sequence identity,
which indicates that they originated by gene duplication.

The encoded OsGSTU4 and OsGSTU3 proteins of 239 and
233 amino acids contain the conserved amino acids that form
the 'signature motif' for plant GSTs: the putative active site
Ser-16, an Arg-21 and a Ser-70, the latter in the \(\tau\) class Glu-
Ser configuration [15,30]. OsGSTU4 and OsGSTU3 have pre-
dicted molecular masses and pI of 25.6 kDa, 5.0 and 25.3
kDa, 5.5 respectively and each contain one potential N-glyco-
sylation site (data not shown). The six internal peptides of the
PEG-induced 29-kDa GST protein (Fig. 1) were in complete
agreement with OsGSTU4 whereas each of them differed from
OsGSTU3. The observed migration at 29 kDa after sodium
dodecyl sulfate–polyacrylamide gel electrophoresis (Fig. 1)
may be due to glycosylation.
OsGSTU3 and OsGSTU4 share 64% amino acid identity
and 75% similarity, not only in the typically conserved N-ter-
mal G site for GSH activation and binding but also in the
C-terminal H domain for co-substrate binding, which suggests
functional similarities. Compared to other plant species, OsG-
STU4 was most homologous to TiGSTU2 of Aegilops tauschii

![Fig. 1. Changes in two-dimensional protein patterns in response to PEG in roots of rice seedlings. Proteins were isolated from roots of rice seedlings, incubated on growth medium (control) or on growth medium supplemented with 15% PEG for 60 h. The two-dimension-
al isoelectric focusing gels were stained with Coomassie blue dye. Two proteins whose abundance markedly increased in response to PEG are indicated with arrowheads. A PEG-induced protein, which migrated at about 29 kDa and pI 5, was identified by microsequencing as a GST protein (G) in this study.](Image 47x643 to 277x783)

![Fig. 2. Early osgstu4 and osgstu3 expression in rice roots in re-
sponse to various stresses. RT-PCR products of osgstu4 and rac1 (upper panels) and osgstu3 and rac1 (lower panels) were separated on 1.1% agarose gels and stained with ethidium bromide. –RT lanes show typical –RT reactions, in which reverse transcriptase was omitted. gDNA lanes show PCR amplifications in the same conditions on genomic DNA with each primer pair. Sizes of the
cDNA (left) or gDNA products (right) are indicated. A: RNA was prepared from the roots of rice seedlings that were incubated on growth medium (controls C1, C2 and C3), incubated on growth medium supplemented with PEG (15 or 13.5%), kept in the cold (4‡C, C4), exposed to heat (42‡C, H), or incubated on growth medium supplemented with ZnSO4 (5 or 2.5 mM) for 2 h. B: Rice seedlings were incubated on growth medium (controls C1, C2 and C3,) or on growth medium supplemented with KCl (5 mM), MgSO4 (2.5 mM), CoCl2 (250 \(\mu\)M or 2.5 mM), NiCl2 (250 \(\mu\)M or 2.5 mM), or ethanol (0.5 or 5%) for 2 h. Equal amounts of rac1 were detected in all lanes, except during ethanol toxicity (5%) which caused a decline of the rac1 messenger.](Image 311x279 to 542x604)
Fig. 3. Osgstu4 and osgstu3 expression in response to zinc and cadmium. RT-PCR expression patterns of osgstu4 and rac1 (upper panels) and osgstu3 and rac1 (lower panels). A: RNA was extracted from the roots of rice seedlings incubated on growth medium (control C0), on water (control C1), on water pH 4.5 (control C2), on aqueous solutions of zinc sulfate (30, 150 and 750 μM ZnSO4, pH 4.5) or on aqueous solutions of cadmium (20, 100 and 500 μM Cd) for 3 h. B: RNA was extracted from the root of shoot seedlings incubated on growth medium (control C0) or on 750 μM ZnSO4 for 3 h. Equal amounts of rac1 were detected in all lanes.

3.3. PEG rapidly induces osgstu4 and osgstu3 in rice roots

Because of the high degree of cDNA sequence homology amongst osgstu3 and osgstu4, we used semi-quantitative RT-PCR expression analysis with gene-specific primers [31] to compare relative amounts of the osgstu3 and osgstu4 messengers in various root samples. The rice actin 1 gene, which is usually constitutively expressed, was monitored in parallel as an internal standard for the RT and PCR reactions.

Fig. 2 (controls C1, C2, C3, C4 and C5) shows that osgstu4 and osgstu3 were not expressed in roots of rice seedlings in the absence of stress. The RT-PCR expression analysis results confirmed that PEG (13.5 and 15%) caused a marked induction of the osgstu3 and osgstu4 transcripts in rice roots within 2 h (Fig. 2A), in agreement with the PEG-induced accumulation of the OsGSTU4 protein observed before (Fig. 1). PEG, which is generally used to impose osmotic stress, can also provoke other adverse effects, e.g. effects on root respiration [32] and/or toxic effects [33]. We therefore proceeded to test the effect of various other stresses on osgstu3 and osgstu4 expression. A 2-h exposure to low or high temperatures (4°C and 42°C) did not induce osgstu4 nor osgstu3 in rice roots (Fig. 2A).

3.4. Heavy metals induce osgstu4 and osgstu3 in rice roots

The toxic chemical ZnSO4 (5 mM or 2.5 mM) markedly induced osgstu4 and osgstu3 in rice roots within 2 h of exposure (Fig. 2A). Fig. 2B shows that cobalt and nickel chloride (250 μM and 2.5 mM) also induced osgstu4 and osgstu3 in rice roots within 2 h. Equimolar chloride concentrations (5 mM KCl) had no effect, indicating that Ni and Co were the inducers. Analogously, Zn was an inducer (Fig. 2A) since equimolar sulfate concentrations (2.5 mM MgSO4) had no effect (Fig. 2B). Responses to Co were generally stronger than responses to Ni. Exposure to toxic ethanol concentrations (5%) caused an induction of osgstu4 and osgstu3 and a decline in transcript levels of the rice actin 1 housekeeping gene.

Fig. 3A shows the osgstu4 and osgstu3 expression in roots of rice seedlings, incubated on a concentration range of zinc (30, 150 and 750 μM) and cadmium (20, 100 and 500 μM) solutions for 3 h. The heavy metals markedly induced osgstu4 and osgstu3 in rice seedling roots at all concentrations tested, down to levels as low as 20 μM Cd and 30 μM Zn. ZnSO4 solutions are acidic, but a pH of 4.5 did not induce osgstu4 or osgstu3 (Fig. 3A, control C0). Fig. 3B shows that osgstu3 but not osgstu4 was expressed in the shoot of rice seedlings in response to Zn (750 μM) and Cd (data not shown). Osgstu3 and osgstu4 were not expressed in the shoot of non-stressed seedlings (Fig. 3B).

3.5. Osgstu4 and osgstu3 are hypoxic stress-induced and differentially salt shock-responsive in rice roots

Fig. 4 shows the expression of osgstu4 and osgstu3 in roots of rice seedlings that were exposed to salt shock (300 mM) or a completely submergence for different times. Salt shock-induced osgstu3 within 2 h and at all longer durations. Salt stress only caused a maximal induction of osgstu4 after 12 h, which generally indicates a secondary response. Submergence, which imposed hypoxic stress, induced osgstu4 and osgstu3 expression in rice roots within 2, 6 and 12 h. Submergence caused a gradual decline of rice actin 1 transcript levels, a well-known effect of hypoxic stress on housekeeping genes. No osgstu4 or osgstu3 expression was detected in the shoot of control seedlings nor in the shoot of seedlings exposed to salt stress or submergence (data not shown).

3.6. SA, JA and the auxin NAA induce osgstu4 and osgstu3 whereas ABA and the cytokinin BA induce osgstu3 but not osgstu4

Fig. 5 shows the expression of osgstu4 and osgstu3 in response to exogenously applied physiological concentrations of NaCl (data not shown).

Fig. 4. Time course analysis of osgstu4 and osgstu3 expression in rice roots during salt shock and submergence. RT-PCR expression patterns of osgstu4 and rac1 (upper panel) and osgstu3 and rac1 (lower panel). A: Rice seedlings were incubated on growth medium (control C0 and C10), incubated on water pH 4.5 (control C2), on aqueous solutions of zinc sulfate (30, 150 and 750 μM ZnSO4, pH 4.5) or on aqueous solutions of cadmium (20, 100 and 500 μM Cd) for 3 h. B: RNA was extracted from the root of shoot seedlings incubated on growth medium (control C0) or on 750 μM ZnSO4 for 3 h. Equal amounts of rac1 were detected in all lanes.

Fig. 3A shows the expression of osgstu3 and osgstu4 in roots of rice seedlings incubated on growth medium (control C0), on water (control C1), on water pH 4.5 (control C2), on aqueous solutions of zinc sulfate (30, 150 and 750 μM ZnSO4, pH 4.5) or on aqueous solutions of cadmium (20, 100 and 500 μM Cd) for 3 h. B: RNA was extracted from the root of shoot seedlings incubated on growth medium (control C0) or on 750 μM ZnSO4 for 3 h. Equal amounts of rac1 were detected in all lanes.

(acession number AY013753), GST1a and b of Alopecurus myosuroides (accession numbers AJ010448 and AJ010449) and GST42 of Zea mays (accession number AF244707), with amino acid identities of 72%, 67% and 65% respectively. OsGSTU3 was most homologous to GSTU42 and GSTU40 (accession number AF244705) of Z. mays and TtGSTU2 of A. tauschii, with identities of 71%, 65%, and 64% respectively.
Various plant growth regulators. The auxin NAA (12 and 25 μM) markedly induced osgstu4 and osgstu3. The cytokinin BA (15 and 30 μM) induced osgstu2 but not osgstu4. The plant hormone ABA (20 and 50 μM), which regulates many plant responses to water-limiting stresses, induced osgstu2 but not osgstu4. SA (20 and 50 μM), which controls the systemic acquired plant pathogen resistance response, markedly induced osgstu4 and osgstu3. JA, which controls plant responses to wounding and pathogen attack, also induced osgstu4 and osgstu3. GA3 was no inducer of osgstu4 nor osgstu3.

3.7. The antioxidants DTT and ascorbic acid and the oxidant hydrogen peroxide markedly induce osgstu4 and osgstu3 in rice roots

Fig. 5 shows osgstu4 and osgstu3 expression in roots of rice seedlings in response to the antioxidants DTT and ascorbic acid and the oxidant hydrogen peroxide (H₂O₂) for different times. The DTT used was heavy metal-free and the pH of all media was within the physiological range of 4.8–5. The thiol antioxidant DTT (5 mM) and the strong oxidant H₂O₂ (5 mM) rapidly and markedly induced osgstu4 and osgstu3 at all times. Maximal expression levels were reached within 1 h for osgstu3 and 2 h for osgstu4 and transcript levels remained high during the 6 h tested. The non-thiol antioxidant ascorbic acid (5 mM) also induced osgstu4 and osgstu3 in rice roots, reaching two times higher levels for osgstu3 than for osgstu4.

4. Discussion

The osgstu4 and osgstu3 genes and deduced proteins exhibited all hallmarks of GSTU proteins and genes: a single intron, an auxin response and the three conserved amino acids that form the plant GST signature motif [30]. Osgstu4 and osgstu3 were not constitutively expressed in the root or in the shoot of rice seedlings (Figs. 2–6), but were induced by a specific set of environmental stresses, which strongly suggests stress-related functions.

Salt stress, which imposes osmotic and ion-toxic effects (Fig. 4), and dehydration by partial removal of the growth solution (data not shown) rapidly induced osgstu3 in rice roots, which indicated a water deficit response. Salt stress caused a late induction of osgstu4 (Fig. 4), rather due to secondary stress effects, and partial dehydration did not induce osgstu4 (data not shown). Consistently, the plant hormone ABA, which regulates many primary water deficit responses, induced osgstu3 but not osgstu4 (Fig. 5). The auxin response, imposed by seedling submergence, induced osgstu4 and osgstu3 in the seedling roots (Fig. 4), concomitant with a marked induction of rice alcohol dehydrogenase 1 [34]. A hypoxic stress response has so far not been reported for plant GSTs, which often are associated with oxidative stress. However, the observation that the early dehydration-induced Atgstf4 (erdi3) gene was expressed when whole plants were transferred to liquid medium [18] may indicate a hypoxic stress response as well.

Heavy metals were found to rapidly and markedly induce osgstu4 and osgstu3 at concentrations as low as 20 μM Cd and 30 μM Zn (Fig. 5A), which is generally considered a typical heavy metal response. Plants respond to heavy metals with a variety of detoxification pathways as well as responses to secondary stress effects. The observed stress responses of osgstu4 and osgstu3 suggest involvement in the detoxification of heavy metals or stress metabolites in rice roots. Analyzing the function of OsGSTU4 and OsGSTU3 is part of our continued research.

In an attempt to identify signals involved in the regulation of osgstu4 and osgstu3 expression, various plant growth regulators were tested. The auxin NAA markedly induced osgstu4 and osgstu3 (Fig. 5). Auxin responses were originally considered typical for gstu genes, but proved to vary considerably amongst the well-characterized Arabidopsis gstu genes [12,17]. The observation that osgstu4 and osgstu3 were markedly induced by SA and JA (Fig. 5) strongly suggests that these genes are also expressed as part of systemic and local plant pathogen defense responses. The versatility of GSTs allows an involvement in both biotic and abiotic stresses.

In a further search for cellular events that might regulate osgstu4 and osgstu3 expression, we studied compounds that differentially affect the cellular redox state. The antioxidant DTT caused a marked induction of osgstu4 and osgstu3 (Fig. 6), which suggested a response to redox perturbations that favor the reduced state. At high pH (> 8), autoxidation of thiols can generate superoxide radicals [35], but the experiments were performed at the physiological pH of 4.8–5. Moreover, the non-thiol antioxidant ascorbic acid also induced osgstu4 and osgstu3 (Fig. 6). A response to reducing condi-
tions may be consistent with the observed response to hypoxic stress in roots (Fig. 4). Low oxygen conditions in roots can partially prevent the reoxidation of NADH through the mitochondrial electron transport, which may cause perturbations of the cellular redox balance. Responses to antioxidants have been reported for the k class Atgstf1 gene, AtDHAR1 and AtDHAR2 with potential functions in redox homeostasis [12] and the early dehydration-induced Atgstf3 gene [12,18]. The strong oxidant hydrogen peroxide also rapidly and markedly induced osgst4 and osgst5 in rice roots (Fig. 6), which indicated an oxidative stress response as well. An apparently paradoxical response to contrasting redox perturbations has also been observed during plant pathogen responses. Plant pathogen infections cause biphasic cellular redox alterations, e.g., an initial oxidative burst that is part of the local hypersensitive response [36], followed by an accumulation of antioxidants [37], which triggers the SA-controlled systemic acquired resistance response through the redox activation of NPR1 [37]. Osgst4 and osgst5, which are SA-responsive, may be regulated in this way during biotic stresses. During abiotic stresses, the importance of these two triggers for gene activation, i.e., reactive oxygen species versus redox changes, may vary among photosynthetic tissues and roots and depend on the type (hypoxic, heavy metal, salt, etc.), the severity and the duration of the stress.

The findings indicated that osgst4 and osgst5 were induced by heavy metals and hypoxic stress and were differentially responsive to salt stress in rice roots, and suggested that reactive oxygen species and redox changes are involved in the complex stress response regulation of osgst4 and osgst5 expression.

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