Osgstu3 and *osgtu4*, encoding tau class glutathione *S*-transferases, are heavy metal- and hypoxic stress-induced and differentially salt stress-responsive in rice roots[1]

Ann Moons*

Department of Biology, Faculty of Science, University of Ottawa, 30 Marie Curie, Ottawa, ON, Canada K1N 6N5

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.

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

*Present address: Biotechnology Research Institute (BRI), National Research Council Canada (NRC), 6100 Royalmount Ave, Montreal, QC, Canada H4P 2R2. Fax: (1)-514-4966213.
E-mail address: ann.moons@nrc.ca (A. Moons).

Abbreviations: ABA, abscisic acid; BA, 6-benzylaminopurine; BAC, bacterial artificial chromosome; DTT, dithiothreitol; EST, expressed sequence tag; GA₃, gibberellic acid; GSH, γ-glutamyl-cysteinyl-glycine; GST, glutathione *S*-transferase; GSTU, tau class GST; JA, jasmonic acid; NAA, α-naphthalene acetic acid; OSGSTU3 and OSGSTU4, *Oryza sativa* GSTU 3 and 4; PEG, polyethylene glycol; POX, peroxidase; RACE, rapid amplification of cDNA ends; SA, salicylic acid

detoxification. GSTs 'tag' herbicides for vacuolar sequestration by ATP-binding cassette transporters that function as glutathione-conjugate Mg^{2+}-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 vacuolar anthocyanin sequestration respectively. Other GSTs catalyze alternative isomerization or peroxidase reactions and are involved in tyrosine metabolism or hydroxyperoxide 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 (T 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 *Atgstu8*, which was attributed to secondary oxidative stress effects [19]. Redox perturbations were found to induce a number of λ class and DHAR *gst* 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 *Zmgstu4*, 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), $ZnSO_4 \cdot 7H_2O$, KCl, $MgSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, ethanol, NaCl (all from BDH, VWR Canlab, Mississauga, ON, Canada), heavy metal-free dithiothreitol (DTT, Sigma, St. Louis, MO, USA), a 30% (w/w) H_2O_2 solution (Sigma), ascorbic acid (Sigma) or the plant growth regulators α-naphthalene acetic acid (NAA), 6-benzylaminopurine (BA, Sigma), SA (Sigma), ABA (Invitrogen, Carlsbad, CA, USA), (±)-jasmonic acid (JA, Sigma) and gibberellic acid (GA_3, Invitrogen). All media were filter-sterilized. Other seedlings were completely submerged or exposed to 4°C or 42°C. For the Cd treatments, 15-day-old seedlings were incubated on aqueous dilutions of a 1 g/l cadmium solution in 2% HNO_3 (Fisher Scientific). The durations of the treatments are indicated. All plant treatments were performed and analyzed twice in independent experiments.

2.3. Protein microsequencing

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

5′ and 3′ RACE (rapid amplification of cDNA ends) were performed on 1 μg RNA from roots of PEG-treated Pokkali seedlings following Invitrogen protocols.

The 3′ RACE *osgstu4* primer 5′-CGTGTTCATCCAAT-CATTCTC-3′ (1GA) was based on D41737, a 386-bp expressed sequence tag (EST) of the Japonica rice variety Nipponbare that correctly encoded three tryptic 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′-CGTCAATGTACTGCAC-3′ and 5′-GATGATCGACGACTCGCAGA-3′ respectively. The overlapping 5′ *osgstu4* cDNA was 300 bp long, including a 5′ UTR of 81 bp.

The 3′ RACE *osgstu3* primer 5′-TCTGCGAGTCGCAGGTCATC-3′ was based on D40283, a 305-bp EST from the Japonica rice variety Nipponbare that was homologous to *osgstu4*. The 3′ *osgstu3* cDNAs were typically 688 bp long and all included a 188-bp 3′ UTR. RT-PCR using the primers 5′-AGCTAGCAAAAGACAACTCAAG-3′ (1GB) and 5′-CGCATCGAACAGCCTCATC-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 *T7*-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′-CGTCTTGCCTCTGAAAGTCG-3′ (2GA) for *osgstu4*, 1GB and 2GB for *osgstu3*, and 5′-ATCCATGAGACTA-CATACAACT-3′ (1AC) and 5′-TAGAAGCATTTCCTGTGCACA-3′ (2AC) for *rice actin 1* (accession number X15865). The primer pairs 1GA/2GA, 1GB/2GB and 1AC/2AC overspan introns of 228 bp, 97 bp and 82 bp respectively in the *osgstu4*, *osgstu3* 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 p*I* 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, SELLLAS-NPVHK, APLLAAWAQR, FGELDVAEK, and VLPDV-DGVVEFAK. These peptide sequences exhibited significant homology to GSTU proteins of the monocotyledonous plants rice, maize, *Aegilops* sp. and *Alopecurus* 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 *osgstu3* 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 poly-adenylation addition signal sequences AATAAT, which are single base variants of the consensus AATAAA sequence,
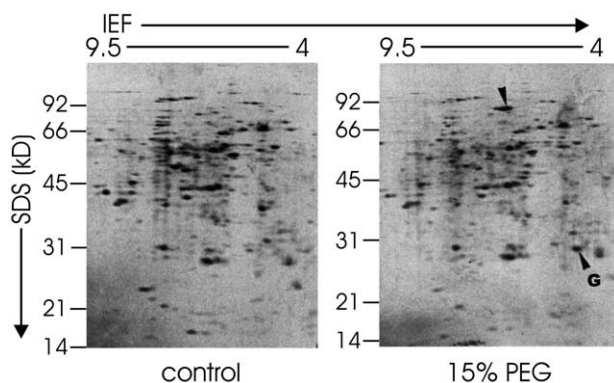
Fig. 1. Changes in two-dimensional protein patterns in response to PEG in roots of rice seedlings. Proteins were isolated from roots of rice seedlings, incubated on growth medium (control) or on growth medium supplemented with 15% PEG for 60 h. The two-dimensional isoelectric focusing gels were stained 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.

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 polyadenylation site.

We compared *osgstu4* and *osgstu3* isolated from *O. sativa* L. ssp. Indica cv. Pokkali to the corresponding ESTs and genes of the evolutionarily distinct Japonica rice variety Nipponbare [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 Pokkali 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 differences 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 related *gstu* genes, comprised on a 148-kb BAC clone (accession number AC091680). *Osgstu3* and *osgstu4* are tandemly arranged 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 τ class Glu-

Ser configuration [15,30]. OsGSTU4 and OsGSTU3 have predicted molecular masses and pI of 25.6 kDa, 5.0 and 25.3 kDa, 5.5 respectively and each contain one potential *N*-glycosylation 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-terminal 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, OsGSTU4 was most homologous to TtGSTU2 of *Aegilops tauschii*
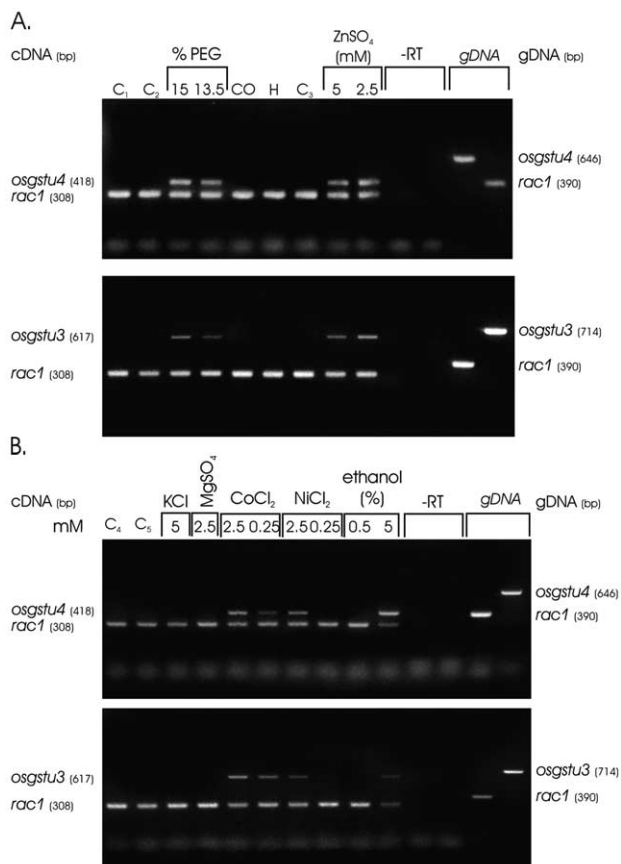

Fig. 2. Early *osgstu4* and *osgstu3* expression in rice roots in response to various stresses. RT-PCR products of *osgstu4* and *rac1* (upper panels) and *osgstu3* and *rac1* (lower panels) were separated on 1.1% agarose gels and stained with ethidium bromide. −RT lanes show typical −RT reactions, in 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 C_1, C_2 and C_3), incubated on growth medium supplemented with PEG (15 or 13.5%), kept in the cold (4°C, CO), exposed to heat (42°C, H), or incubated on growth medium supplemented with ZnSO$_4$ (5 or 2.5 mM) for 2 h. B: Rice seedlings were incubated on growth medium (controls C_4 and C_5) or on growth medium supplemented with KCl (5 mM), MgSO$_4$ (2.5 mM), CoCl$_2$ (250 μM or 2.5 mM), NiCl$_2$ (250 μM 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.
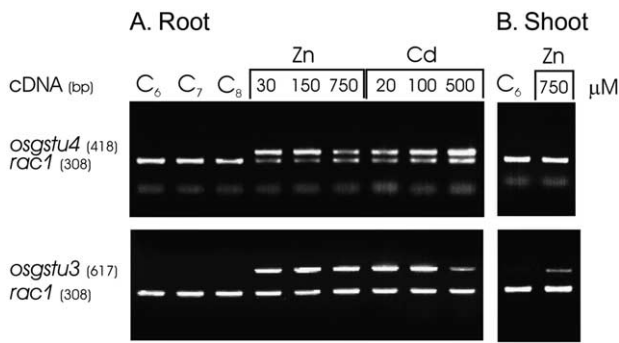
Fig. 3. *Osgstu4* and *osgstu3* expression in response to zinc and cadmium. RT-PCR expression patterns of *osgstu4* and *rac1* (upper panels) and *osgstu3* and *rac1* (lower panels). A: RNA was extracted from the roots of rice seedlings incubated on growth medium (control C_6), on water (control C_7), on water pH 4.5 (control C_8), on aqueous solutions of zinc sulfate (30, 150 and 750 μM ZnSO$_4$ pH 4.5) or on aqueous solutions of cadmium (20, 100 and 500 μM Cd) for 3 h. B: RNA was extracted from the shoot of rice seedlings incubated on growth medium (control C_6) or on 750 μM ZnSO$_4$ for 3 h. Equal amounts of *rac1* were detected in all lanes.

(accession 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.

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 C_1, C_2, C_3, C_4 and C_5) 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 ZnSO$_4$ (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 MgSO$_4$) 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 *ogstu4* and *osgtu3* 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 *osgtu4* and *osgtu3* in rice seedling roots at all concentrations tested, down to levels as low as 20 μM Cd and 30 μM Zn. ZnSO$_4$ solutions are acidic, but a pH of 4.5 did not induce *osgstu4* or *osgstu3* (Fig. 3A, control C_8). 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
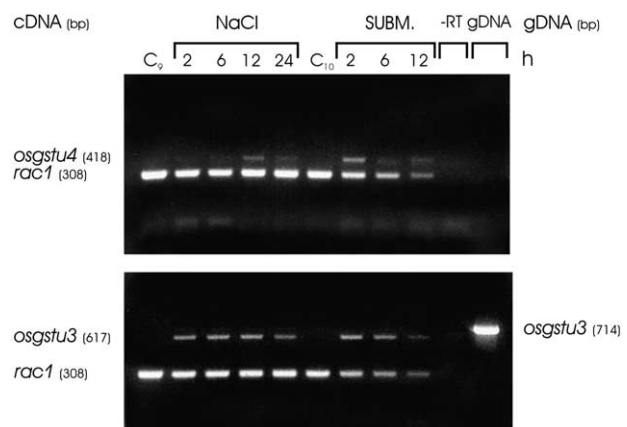

Fig. 4. Time course analysis of *osgstu4* and *osgstu3* expression in rice roots during salt shock and submergence. RT-PCR expression patterns of *osgstu4* and *rac1* (upper panel) and *osgstu3* and *rac1* (lower panel). A: Rice seedlings were incubated on growth medium (controls C_9 and C_{10}), incubated on growth medium supplemented with 300 mM NaCl for 2, 6, 12 and 24 h or were submerged (SUBM.) for 2, 6 and 12 h. Equal amounts of *rac1* were detected in all lanes except during submergence, which caused a gradual decline of the *rac1* messenger.
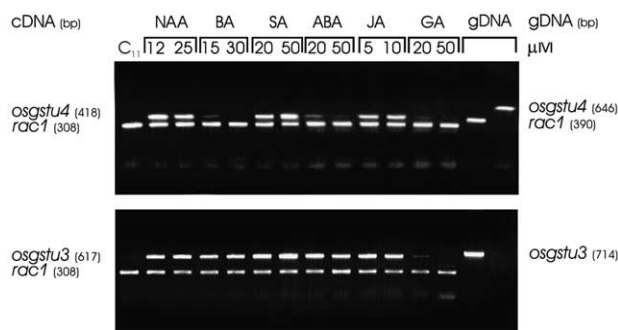
Fig. 5. *Osgstu4* and *osgstu3* expression in rice roots in response to plant growth regulators. RT-PCR expression patterns of *osgstu4* and *rac1* (upper panel) and *osgstu3* and *rac1* (lower panel). Rice seedlings were incubated on growth medium (control C_{11}) or on growth medium supplemented with 12 and 25 µM NAA, 15 and 30 µM BA, 20 and 50 µM SA, 20 and 50 µM ABA, 5 and 10 µM JA or 20 and 50 µM GA_3 for 5 h. Equal amounts of *rac1* were detected in all lanes.

various plant growth regulators. The auxin NAA (12 and 25 µM) markedly induced *osgstu4* and *osgstu3*. The cytokinin BA (15 and 30 µM) induced *osgtu3* but not *osgstu4*. The plant hormone ABA (20 and 50 µM), which regulates many plant responses to water-limiting stresses, induced *osgstu3* 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*. GA_3 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. 6 shows *ogstu4* and *osgtu3* expression in roots of rice seedlings in response to the antioxidants DTT and ascorbic acid and the oxidant hydrogen peroxide (H_2O_2) 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 H_2O_2 (5 mM) rapidly and markedly induced *osgstu4* and *osgtu3* 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
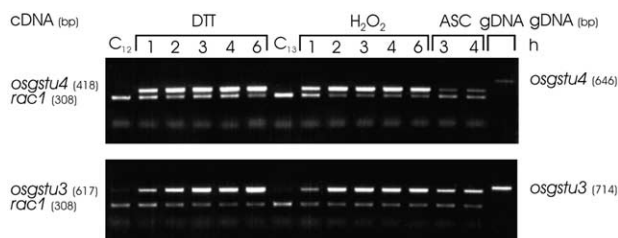

Fig. 6. *Osgstu4* and *osgstu3* expression in rice roots in response to DTT, hydrogen peroxide and ascorbic acid. RT-PCR expression patterns of *osgstu4* and *rac1* (upper panel) and *osgstu3* and *rac1* (lower panel). Rice seedlings were incubated on growth medium (controls C_{12} and C_{13}) or on growth medium supplemented with 5 mM DTT or 5 mM H_2O_2 for 1 h, 2 h, 3 h, 4 h and 6 h, or 5 mM ascorbic acid (ASC) for 3 h and 4 h. Equal amounts of *rac1* were detected in all lanes.

acid (5 mM) also induced *osgstu4* and *osgtu3* 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 *osgtu4* (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). Hypoxic stress, 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* (*erd13*) 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 *osgstu3* and *osgstu4* 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 λ class *Atgstl1* 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 *osgstu4* and *osgstu3* 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]. *Osgstu4* and *osgstu3*, 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 amongst 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 *osgstu4* and *osgstu3* 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 *osgstu4* and *osgstu3* expression.

References

[1] Marrs, K.A. (1996) Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 127–158.

[2] Droog, F. (1997) J. Plant Growth Regul. 16, 95–107.

[3] Dixon, D.P., Cummins, I., Cole, D.J. and Edwards, R. (1998) Cur. Opin. Plant Biol. 1, 258–266.

[4] Edwards, R., Dixon, D.P. and Walbot, V. (2000) Trends Plant Sci. 5, 193–198.

[5] Dixon, D.P., Lapthorn, A. and Edwards, R. (2002) Genome Biol. 3, 30004.1–30004.10.

[6] Kreuz, K., Tommasini, R. and Martinoia, E. (1996) Plant Physiol. 111, 349–353.

[7] Rea, P.A., Li, Z.-S., Lu, Y.-P., Drozdowicz, Y.M. and Martinoia, E. (1998) Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 727–760.

[8] Zettl, R., Schell, J. and Palme, K. (1994) Proc. Natl. Acad. Sci. USA 91, 689–693.

[9] Marrs, K.A., Alfenito, M.R., Lloyd, A.M. and Walbot, V. (1995) Nature 375, 397–400.

[10] Mueller, L.A., Goodman, C.D., Silady, R.A. and Walbot, V. (2000) Plant Physiol. 123, 1561–1570.

[11] Roxas, V.P., Smith Jr., R.K., Allen, E.R. and Allen, R.D. (1997) Nat. Biotechnol. 15, 988–991.

[12] Dixon, D.P., Davis, B.G. and Edwards, E. (2002) J. Biol. Chem. 277, 30859–30869.

[13] Loyall, L., Uchida, K., Braun, S., Furuya, M. and Frohmeyer, H. (2000) Plant Cell 12, 1939–1950.

[14] Kampranis, S.C., Damianova, R., Atallah, M., Toby, G., Kondi, G., Tsichlis, P.N. and Makris, A.M. (2000) J. Biol. Chem. 275, 29207–29216.

[15] McGonigle, B., Keeler, S.J., Lau, S.-M.C., Koeppe, M.K. and O'Keefe, D.P. (2000) Plant Physiol. 124, 1105–1120.

[16] Xu, F., Lagudah, E.S., Moose, S.P. and Riechers, D.E. (2002) Plant Physiol. 130, 362–373.

[17] Wagner, U., Edwards, R., Dixon, D.P. and Mauch, F. (2002) Plant Mol. Biol. 49, 515–532.

[18] Kiyosue, T., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1993) FEBS Lett. 335, 189–192.

[19] Bianchi, M.W., Roux, C. and Vartanian, N. (2002) Physiol. Plant. 116, 96–105.

[20] Ulmasov, T., Ohmiya, A., Hagen, G. and Guilfoyle, T. (1995) Plant Physiol. 108, 919–927.

[21] Mauch, F. and Dudler, R. (1993) Plant Physiol. 102, 1193–1201.

[22] Marrs, K.A. and Walbot, V. (1997) Plant Physiol. 113, 93–102.

[23] Klinedinst, S., Pascuzzi, P., Redman, J., Desai, M. and Arias, J. (2000) Plant Mol. Biol. 42, 679–688.

[24] Bauw, G., Van Den Bulcke, M., Van Damme, J., Puype, M., Van Montagu, M. and Vandekerckhove, J. (1989) in: Methods in Protein Sequence Analysis (Wittmann-Liebold, B., Ed.), pp. 220–233, Springer-Verlag, Heidelberg.

[25] Moons, A., Bauw, G., Prinsen, E., Van Montagu, M. and Van Der Straeten, D. (1995) Plant Physiol. 107, 177–186.

[26] Moons, A., Valcke, R. and Van Montagu, M. (1998) Plant J. 15, 89–98.

[27] Alfenito, M.R., Souer, E., Goodman, C.D., Buell, R., Mol, J., Koes, R. and Walbot, V. (1998) Plant Cell 10, 1135–1149.

[28] Goff, S.A., Ricke, D. and Lan, T.H. et al. (2002) Science 296, 92–100.

[29] Yu, J., Hu, S. and Wang, J. et al. (2002) Science 296, 79–92.

[30] Board, P.G., Coggan, M., Wilce, M.C.J. and Parker, M.W. (1995) Biochem. J. 311, 247–250.

[31] Gause, W.C. and Adamovicz, J. (1995) The use of PCR to quantitate gene expression, in: PCR Methods and Applications, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. S123–S135.

[32] Verslues, P.E., Ober, E.S. and Sharp, R.E. (1998) Plant Physiol. 116, 1403–1412.

[33] Plaut, Z. and Federman, E. (1985) Plant Physiol. 79, 559–561.

[34] Moons, A. (2003) FEBS Lett. 553, 370–376.

[35] Misra, H.P. (1974) J. Biol. Chem. 249, 2151–2155.

[36] Levine, A., Tenhaken, R., Dixon, R. and Lamb, C. (1994) Cell 79, 583–593.

[37] Mou, Z., Fan, W. and Dong, X. (2003) Cell 113, 935–944.