FEBS Letters 553 (2003) 370–376 FEBS 27699

Ospdr9, which encodes a PDR-type ABC transporter, is induced by heavy metals, hypoxic stress and redox perturbations in rice roots[1]

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Received 28 July 2003; revised 10 September 2003; accepted 15 September 2003

First published online 26 September 2003

Edited by Ulf-Ingo Flügge

Abstract Little is known about the role of pleiotropic drug resistance (PDR)-type ATP-binding (ABC) proteins in plant responses to environmental stresses. We characterised *ospdr9*, which encodes a rice ABC protein with a reverse $(ABC\text{-}TMS_6)_2$ configuration. Polyethylene glycol and the heavy metals Cd (20 μM) and Zn (30 μM) rapidly and markedly induced *ospdr9* in roots of rice seedlings. Hypoxic stress also induced *ospdr9* in rice roots, salt stress induced *ospdr9* at low levels but cold and heat shock had no effect. The plant growth regulator jasmonic acid, the auxin α-naphthalene acetic acid and the cytokinin 6-benzylaminopurine triggered *ospdr9* expression. The antioxidants dithiothreitol and ascorbic acid rapidly and markedly induced *ospdr9* in rice roots; the strong oxidant hydrogen peroxide also induced *ospdr9* but at three times lower levels. The results suggested that redox changes may be involved in the abiotic stress response regulation of *ospdr9* in rice roots.

Key words: ATP-binding cassette protein; Pleiotropic drug resistance protein; Rice (*Oryza sativa* L.) root; Heavy metal stress; Jasmonic acid; Hypoxic stress; Redox perturbation

1. Introduction

The large ATP-binding cassette (ABC) protein family generally consists of membrane proteins, active in the ATP-powered transport of a broad range of structurally unrelated substrates across membranes in a wide variety of organisms [1–3]. The *abc* gene family has 51 members in the human genome, 30 in yeast [1,4,5], and 131 in *Arabidopsis thaliana* [2,3,6]. The higher necessity for ABC transporters in plants has been attributed to the sessile plant lifestyle, making it obligatory to cope with various environmental stresses [7]. However, the involvement of ABC transporters in plant responses to various adverse growth conditions remains poorly documented.

Gene inventory studies revealed that the *A. thaliana* [2,3,6] and the Japonica rice genomes [6] encode 54 and 45 full-size ABC transporters respectively, largely confined to the multidrug resistance proteins (MDRs), the multidrug resistance-associated proteins (MRPs) and the pleiotropic drug resistance (PDR) protein subfamilies. *Arabidopsis* MRPs and MDRs, the best characterised full-size plant ABC transporters to date, were found to participate in detoxification processes and in plant growth and development [2,3]. AtMRP1 [8], AtMRP2 [9] and AtMRP3 [10] are functional glutathione-conjugate Mg^{2+}-ATPases, that can transport herbicides, anthocyanins and other substrates [11,12]. AtMRP5 controls stomatal movement [13], AtMDR1 regulates hypocotyl elongation [14] and AtMDR11 is required for auxin transport [15]. CjMDR of *Coptis japonica* specifically transports the alkaloid berberine [16]. Expression of wheat *TaMDR1* is induced by aluminium and calcium channel inhibitors in roots [17].

The PDR protein subfamily, which is the subject of this study, is absent in mammalians, has 10 members in yeast [1,4,5] and 15 members in *Arabidopsis* [2,3,6,18] and rice [6]. Yeast PDRs proved to be the functional homologues of mammalian MDRs, in spite of their structural differences. The well-characterised PDR5 transporter and SNQ2 are plasma membrane efflux pumps for hundreds of structurally and functionally unrelated compounds, including various antifungal and anticancer drugs, and confer a MDR phenotype when overexpressed [1,4,5]. The normal, physiological substrates and roles of most PDRs remain elusive but may be linked to cellular stress responses, as suggested by various findings. PDR5 and SNQ2 also transport steroids [19]. SNQ2 also modulates resistance to the metal ions Na^+, Li^+ and Mn^+ [20]. Expression of the plasma membrane transporters PDR10 and PDR15 is markedly induced by various stresses [5]. PDR12 was found to function as a plasma membrane efflux pump for water-soluble carboxylate anions and mediates resistance to weak organic acids used as food preservatives [21], but also transports structurally unrelated substrates such as fluorescein [22]. Expression of most yeast *pdr* genes is controlled by the zinc finger transcriptional regulators PDR1 and PDR3 [4,5,23] but stress response regulators, including the bZip transcription factor Yap1, modulate the expression of individual genes of the PDR network [5].

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[1] The nucleotide sequence reported in this paper has been submitted to the EMBL, GenBank and DDBJ nucleotide sequence databases with the accession number AY271618.

Abbreviations: ABA, abscisic acid; ABC, ATP-binding cassette; BA, 6-benzylaminopurine; DTT, dithiothreitol; EST, expressed sequence tag; GA_3, gibberellic acid; JA, jasmonic acid; MDR, multidrug resistance; MRP, MDR-associated protein; NAA, α-naphthalene acetic acid; OsPDR9, *Oryza sativa* PDR-type ABC protein 3; PAC, P1-derived artificial chromosome; PDR, pleiotropic drug resistance; PEG, polyethylene glycol; RACE, rapid amplification of cDNA ends; RAP-PCR, RNA arbitrarily primed polymerase chain reaction; SA, salicylic acid; TMS, transmembrane-spanning α-helix

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)01060-3

Three *pdr* cDNAs from plants have been characterised so far. The plasma membrane transporter *NpABC1* from *Nicotiana plumbaginifolia* is induced by the elicitor analogue sclareolide and is most likely involved in the secretion of the antifungal diterpene sclareol [24]. The *Sptur2* gene from *Spirodela polyrrhiza* is induced by abscisic acid (ABA), cold, salinity and dormant bud formation [25]. Expression of the plasma membrane transporter Sptur2 in *Arabidopsis* also conferred resistance to sclareol but not to various other compounds [26]. *NtPDR1* from *Nicotiana tabacum* is induced by methyl jasmonate, microbial and yeast elicitors and may be involved in pathogen defence as well [27]. ABC1, a fungal PDR-type protein, is required for invasive growth of the phytopathogenic fungus *Magnaporthe grisea*, presumably to cope with phytoalexins [28].

Yeast ABC transporters have well-defined roles in heavy metal stress tolerance. The MRP-type yeast cadmium factor 1 (YCF1) protein confers resistance to Cd^{2+} through the vacuolar sequestration of oxidised glutathione–Cd complexes [29]. The heavy metal tolerance factor 1 (HMT1) of fission yeast, which is a half-molecule ABC transporter, confers Cd^{2+} tolerance through the vacuolar sequestration of phytochelatin–Cd^{2+} complexes [30]. Plant ABC transporters are being investigated for their involvement in metal stress responses as well. *Arabidopsis* AtMRP3 can partially restore the cadmium resistance of a *ycf1* yeast mutant [10] and was recently found to be Cd-induced in roots and, after Cd translocation, also in the shoot of *Arabidopsis* seedlings [31].

In this study, we present the characterisation of *ospdr9*, encoding a PDR-type ABC transporter of rice. *Ospdr9* was identified by RNA differential display. We studied *ospdr9* expression in response to various environmental stresses while focussing on roots, which have important roles in early plant responses to soil stresses. We also studied the effect of various plant growth regulators and chemicals that cause redox perturbations, in an attempt to identify signals that might regulate *ospdr9* gene expression during stress.

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-sterilised.

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 polyethylene glycol (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), salicylic acid (SA, Sigma), ABA (Invitrogen, Carlsbad, ON, Canada), (±) jasmonic acid (JA, Sigma) and gibberellic acid (GA_3, Invitrogen). All media were filter-sterilised.

Other seedlings were completely submerged or exposed to 4°C or 42°C. For the Cd treatments, 15-day-old seedlings were incubated on the appropriate 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 analysed twice in independent experiments.

2.3. Differential display polymerase chain reaction (PCR)

RNA fingerprinting was performed by RNA arbitrarily primed PCR (RAP-PCR). The first cDNA strand was synthesised on 1 μg RNA from various root samples using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen) and the artificial 18-mer A3 primer 5′-AATCTAGAGCTCTCCTGG-3′ (Stratagene, La Jolla, CA, USA). Reactions without reverse transcriptase (−RT) were performed in parallel. The second cDNA strand was synthesised on one twentieth of the first strand cDNA products, using the same A3 primer and *Taq* DNA polymerase (Invitrogen). A single round of low stringency PCR: 94°C (5 min), 94°C (1 min), 36°C (5 min), 72°C (5 min), which allowed annealing of the A3 primer at a second site within the cDNA, was followed by 40 amplification cycles of high stringency PCR: 94°C (1 min), 55°C (2 min), 72°C (2 min) and 1 cycle of 72°C (10 min). 1.2 μCi [α-^{32}P]dCTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was included in each 50 μl PCR reaction. The RT-PCR samples were separated on a 4% acrylamide gel and exposed to autoradiography film overnight. Differentially displayed cDNA bands were excised, eluted, reamplified by PCR and cloned in pGEM-T (Promega, Madison, WI, USA).

2.4. 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) and analysed 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.

2.5. RT-PCR cloning of ospdr9

3′ RACE (rapid amplification of cDNA ends, Invitrogen protocols) was performed on 1 μg RNA from PEG-treated rice roots, using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen). The 3′ RACE primer 5′-GCTTGATGCACGAGCAGCTG-3′ was based on the *dd1* and *dd2* cDNA sequence. A 1559 bp 3′ end cDNA was isolated. An overlapping 2246 bp cDNA was cloned by RT-PCR using the direct primer 5′-CTTCATGGACGAGATCTCAACTG-3′, based on the short expressed sequence tag (EST) sequence D22472 (*O. sativa* ssp. Japonica var. Nipponbare) and the inverse primer 5′-TGATCAGCTCCGAAGAATGATG-3′. An overlapping 1204 bp 5′ end cDNA was cloned by RT-PCR on poly(A)$^+$ RNA, using the RT-primer 5′-ACACAATCTGACCGTC-3′ and the nested inverse primer 5′-CTGCAGCAGGGAGATAACAGC-3′. The direct primer 5′-GTTTTGGTGGTGGTGGGAGA-3′ was based on the P1-derived artificial chromosome (PAC) clone AP002844.2 from *O. sativa* ssp. Japonica cv. Nipponbare. The PCR products were cloned in pGEM-T (Promega).

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^- RT (Invitrogen). Reactions without RT were included 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: 5′-CAAGACTTGTTCAATGCCATG-3′ (1AB) and 5′-CAGGTAGCGTTTGCAACTCA-3′ (2AB) for *ospdr9*, 5′-ATCCATGAGACTACATACAACT-3′ (1AC) and 5′-TAGAAGCATTTCCTGTGCACA-3′ (2AC) for *rice actin 1* (accession number X15865) and 5′-GTACTTGCTGAGATGACCAATG-3′ (1AD) and 5′-CTAGTTCTCCATGCGGATGATG-3′ (2AD) for *rice alcohol dehydrogenase 1* (*adh1*) (accession number X16296). The primer pair 1AB/2AB overspans two introns of 81 bp and 92 bp in the *ospdr9* gene, 1AC/2AC overspans an intron of 82 bp in the *rac1* gene, and 1AD/2AD overspans two introns of 82 bp and 159 bp in the *adh1* gene. 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 (data not shown). Specificity

of amplification was also confirmed through direct sequencing of RT-PCR products (data not shown).

3. Results

3.1. Identification of an ABC cDNA in rice roots by RNA differential display

We previously used two-dimensional protein screens for the detection and characterisation of proteins and genes involved in ABA, salt and osmotic stress responses in rice [32–34]. Complementary to that approach, we recently utilised RNA differential display to characterise early stress-induced low abundant transcripts in rice roots. The RNA fingerprinting was performed by RAP-PCR, in which a single 18-mer arbitrary primer was used for reverse transcription and for PCR amplification [35]. Fourteen cDNAs induced by salt, osmotic, heavy metal or low oxygen stress were identified, two of which are described here. Fig. 1 shows two cDNAs, designated *dd1* and *dd2* (differential display cDNA products 1 and 2), that were induced in roots of rice seedlings in response to PEG within 2 h of exposure. Cloning and sequence analysis indicated that *dd1* and *dd2* had an identical cDNA insert of 310 bp, which encoded a peptide from an ABC transporter protein homologue.

3.2. Structural analysis of ospdr9

The 4700 bp *ospdr9* cDNA was cloned by RT-PCR, including 3′ RACE. *Ospdr9* is the first characterised cDNA for a rice ABC transporter. We followed the nomenclature that was recently introduced in an inventory of rice and *Arabidopsis* genes for ABC transporters [6]. The *ospdr9* cDNA sequence fully corresponded to the 310 bp *dd1* and *dd2* cDNA sequence, isolated by RNA differential display. *Ospdr9* has an open reading frame of 4371 bp and a 3′ untranslated region of 313 bp, including a poly-A tail of 18 bp. The position of the initiation codon was based on homology to other plant ABC transporters. The first encountered stop codon (TGA) was followed by two more TGA stop codons in tandem.

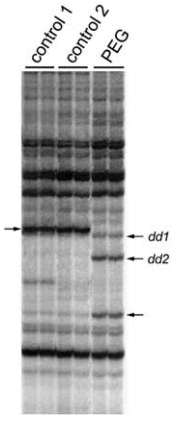

Fig. 1. Identification of *ospdr9* cDNAs in rice roots by RNA fingerprinting. RNA was isolated from roots of rice seedlings, incubated on growth medium (controls 1 and 2) or on growth medium supplemented with 15% PEG for 2 h. A single 18 base arbitrary primer was used for first and second strand cDNA synthesis, during which [α-^{32}P]dCTP was incorporated. The cDNA products were separated on 4% polyacrylamide gels and exposed to X-ray film. cDNAs whose abundance markedly changed in response to PEG are indicated with arrows. Two PEG-induced cDNAs, *dd1* and *dd2*, were found to encode an ABC protein homologue.

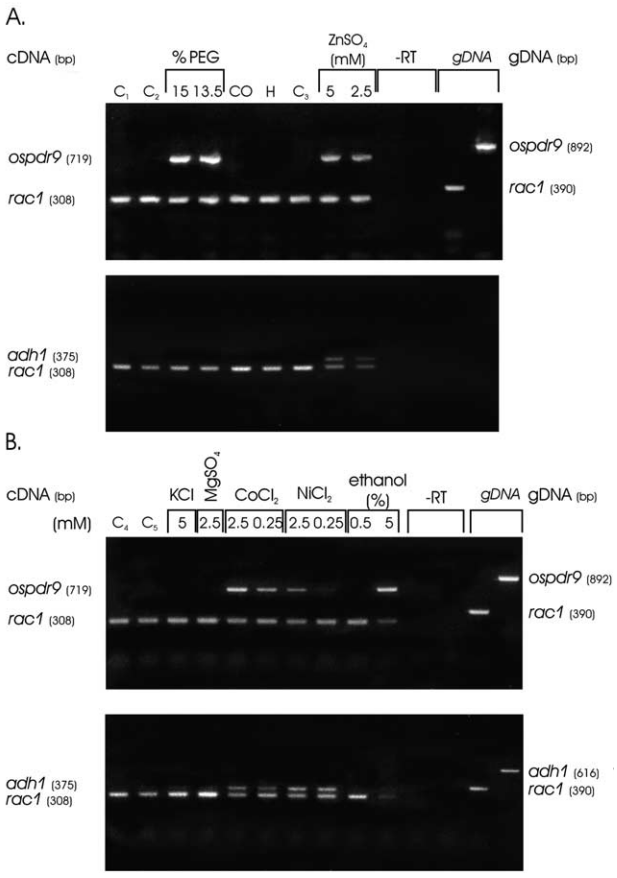

Fig. 2. Early *ospdr9* expression in rice roots in response to various stresses. RT-PCR products of *ospdr9* and *rac1* (upper panels) and *adh1* and *rac1* (lower panels) were separated on 1.1% agarose gels and stained with ethidium bromide. −RT lanes show typical −RT reactions in which reverse transcriptase was omitted. gDNA lanes show PCR amplifications on genomic DNA with each primer pair in the same conditions. Sizes of 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 and 2.5 mM), $NiCl_2$ (250 μM and 2.5 mM), or ethanol (0.5% and 5%) for 2 h. Equal amounts of *rac1* were detected, except during ethanol toxicity (5%) which caused a decline of the *rac1* messenger.

The encoded OsPDR9 protein of 1457 amino acids has a predicted molecular mass of 163 kDa and a calculated p*I* of 6.7. OsPDR9 is a full-size ABC transporter protein, containing two hydrophilic ABC domains of about 150 amino acids long, each with a well-conserved Walker A motif and less conserved Walker B and ABC signature sequences [36,37]. OsPDR9 also has two hydrophobic integral membrane domains, each with six potential transmembrane-spanning α-helices (TMS), as predicted using the TMHMM2.0 program [38]. The reverse $(ABC\text{-}TMS_6)_2$ configuration is typical for PDR-type ABC transporter proteins from yeast and plants.

The *ospdr9* cDNA that we isolated from *O. sativa* L. spp. Indica cv. Pokkali is 307 bp longer in the 3′ untranslated regions than the *ospdr9* cDNA that was obtained by conceptual splicing of a gene from the evolutionarily distinct Japon-

ica rice variety Nipponbare (accession number AJ35046) [6,39]. The coding region of *ospdr9* from Pokkali has five base pair substitutions compared to *ospdr9* of Nipponbare, resulting in four amino acid differences amongst the OsPDR9 proteins from Pokkali and Nipponbare (CAD59568, revised from BAB21276). A comparison of *ospdr9* cDNA from Pokkali to a recent draft of the genome from other Indica rice varieties [40] showed seven base pair substitutions in the coding region after conceptual splicing, resulting in three amino acid differences.

The *ospdr9* gene has 21 exons and 20 introns [39]. *Ospdr9* is part of a cluster of four closely related rice *pdr* genes, i.e. *ospdr10*, *ospdr11*, *ospdr9* and *ospdr8*, located on a 165 kb PAC clone (AP002844) of chromosome I, in which *ospdr11* and *ospdr9* form a tandem. OsPDR9 exhibits identities of 82%, 80%, 74% and 68% and similarities of 90%, 88%, 85% and 82% to the hypothetical rice PDR proteins OsPDR11 (CAD59566, revised from BAB21275), OsPDR10 (CAD59567, revised from BAB21273), OsPDR8 (CAD59569 revised from BAB21279) and OsPDR7 (CAD59570), the latter of which is encoded on chromosome II [6,39]. OsPDR9 furthermore exhibits identities of 71%, 69% and 68% and similarities of 82%, 81% and 81% to NtPDR1 of *N. tabacum* (accession number BAB92011), NpABC1 of *N. plumbaginifolia* (accession number CAC40990) and SpTUR2 of *S. polyrrhiza* (accession number CAA94437). For the structural relationships amongst OsPDR9 and the 15 putative PDR-type ABC transporters of *Arabidopsis*, we refer to a phylogenetic tree presented by Jasinski et al. [6]. The nearest yeast equivalents of OsPDR9 are PDR5 and PDR15, both with 27% identity and 44% similarity.

3.3. *PEG rapidly induces* ospdr9 *in rice roots*

Because of the high degree of coding sequence homology amongst rice *abc* genes, we used semi-quantitative RT-PCR expression analysis with gene-specific primers [41] to compare relative amounts of the *ospdr9* messenger 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. *Rice adh1* gene expression was monitored as a marker for low oxygen stress.

Fig. 2 (controls C_1, C_2, C_3, C_4 and C_5) shows that *ospdr9* was 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 *ospdr9* transcript in rice roots within 2 h (Fig. 2A), in agreement with the PEG induction of *ospdr9* found by differential display (Fig. 1). Adverse effects of PEG can be complex. PEG can provoke not only osmotic but also hypoxic [42] and/or toxic effects [43]. We therefore proceeded to test the effect of various other stresses on *osdpr9* expression. *Ospdr9* was not induced by a 2 h exposure to low or high temperatures (4°C and 42°C) (Fig. 2A). This indicated that the rapid *ospdr9* induction was not a general response to a variety of abiotic stresses.

3.4. *Toxic chemicals markedly induce* ospdr9 *in rice roots*

The toxic chemical $ZnSO_4$ (2.5 or 5 mM) caused a marked induction of *ospdr9* in rice roots (Fig. 2A). Fig. 2B shows that cobalt and nickel chloride (250 μM and 2.5 mM) induce *ospdr9* within 2 h. Equimolar chloride concentrations (5 mM KCl) had no effect, indicating that Ni and Co were the inducers. Analogously was Zn the inducer (Fig. 2A) since equimolar sulphate concentrations (2.5 mM $MgSO_4$) had no effect (Fig. 2B). Co generally caused a stronger *ospdr9* expression than Ni. Zn, Ni and Co also induced *rice adh1* in the seedling roots within 2 h (Fig. 2A,B). Toxic ethanol concentrations (5%) induced *ospdr9* and caused a decline in transcripts of the *rice actin 1* housekeeping gene.

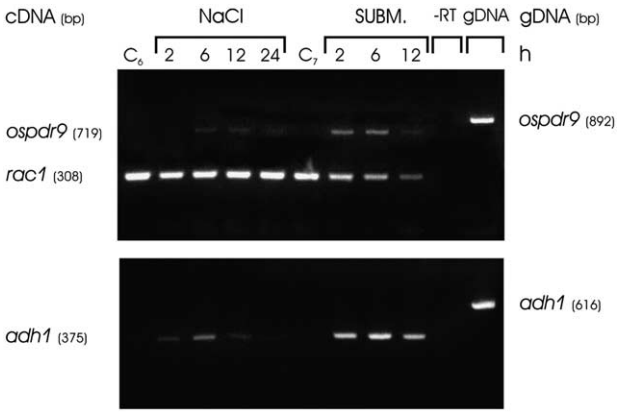

Fig. 3. Time course analysis of *ospdr9* expression in rice roots during salt shock and submergence. RT-PCR expression patterns of *ospdr9* and *rac1* (upper panel) and *adh1* (lower panel). A: Rice seedlings were incubated on growth medium (controls C_6 and C_7), incubated on growth medium supplemented with 300 mM NaCl for 2, 6, 12 and 24 h or 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.

3.5. *Low oxygen stress is a stronger inducer of* ospdr9 *than salt shock in rice roots*

Fig. 3 shows the expression of *ospdr9* in roots of rice seedlings exposed to salt shock (300 mM NaCl) or submergence for different times. Salt stress caused a delayed induction of *ospdr9* in rice roots at low levels after 6 h, 12 h and 24 h and a rapid, transient induction of *rice adh1*. Submergence of rice seedlings induced *ospdr9* expression in the roots within 2 h and at all other times tested. Submergence markedly induced *adh1*, indicating hypoxic stress in these roots. No *ospdr9* expression was detected in the shoot of control seedlings nor in the shoot of seedlings exposed to salt stress or submergence (data not shown). Submergence caused a gradual decline of *rice actin 1* transcripts, a well-known effect of hypoxic stress on transcripts of housekeeping genes.

3.6. *JA, the auxin NAA and the cytokinin BA induce* ospdr9

Fig. 4 shows the effect of exogenous application of low,

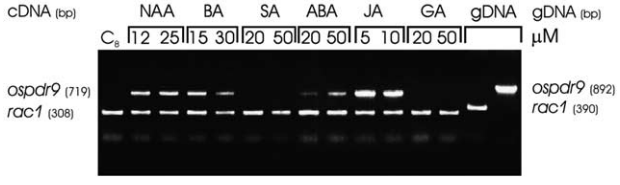

Fig. 4. *Ospdr9* expression in rice roots in response to plant growth regulators. RT-PCR expression patterns of *ospdr9* and *rac1*. Rice seedlings were incubated on growth medium (control C_8) or on growth medium supplemented with 12 and 25 μM NAA, 15 and 30 μM BA, 20 and 50 μM SA, 20 and 50 μM ABA, 5 and 10 μM JA or 20 and 50 μM GA_3 for 5 h. Equal amounts of *rac1* were detected in all lanes.

physiological concentrations of various plant growth regulators on the expression of *ospdr9*. The auxin NAA (12 and 25 μM) and the cytokinin BA (15 and 30 μM) induced *ospdr9*. SA did not induce *ospdr9* whereas JA (5 and 10 μM) caused a marked induction of *ospdr9*. ABA (20 and 50 μM) caused the *ospdr9* transcript to accumulate at relatively low levels. GA_3 did not induce *ospdr9*.

3.7. *Micromolar cadmium and zinc concentrations markedly induce* ospdr9 *in rice roots*

Fig. 5A shows *ospdr9* expression in roots of rice seedlings that were 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 *ospdr9* 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 *ospdr9* (Fig. 5A, control 11). Fig. 5B shows that *ospdr9* expression was not detected in the shoot of non-stressed 15-day-old rice seedlings, nor in the shoot of seedlings that were exposed to Zn or Cd (data not shown) for 3 h.

3.8. *DTT and ascorbic acid are stronger inducers of* ospdr9 *than hydrogen peroxide in rice roots*

We questioned whether compounds that perturb the cellular redox balance would have an effect on *ospdr9* expression. Fig. 6 shows *ospdr9* expression in roots of rice seedlings in response to the antioxidants DTT and ascorbic acid and the oxidant hydrogen peroxide for different times. The DTT used was heavy metal-free. The pH of all media was within the physiological range of 4.8–5. The thiol antioxidant DTT (5 mM) caused a rapid, marked induction of *ospdr9* at all times. Maximal expression levels were reached within 2 h of exposure to DTT, and transcript levels remained high for at least 6 h. The antioxidant ascorbic acid (5 mM) likewise markedly induced *ospdr9* in rice roots. The strong oxidant H_2O_2 (5 mM) also induced *ospdr9* in rice roots, but expression levels were three times lower than in response to DTT and ascorbic acid.

4. Discussion

Ospdr9 encodes a full-molecule rice ABC protein with a typical PDR-type reverse $(ABC\text{-}TMS_6)_2$ configuration. Its subcellular localisation remains unknown, although all yeast and plant PDR proteins that have been localised so far reside in the plasma membrane. *Ospdr9* was not constitutively expressed in the roots (Figs. 2–6) nor in the shoot (Fig. 5B) of 15-day-old rice seedlings. PEG induced a rapid, marked and root-specific *ospdr9* transcript accumulation (Figs. 1 and 2A), which indicated a stress-related function.

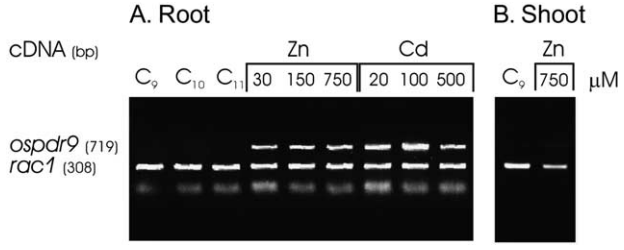

Fig. 5. *Ospdr9* expression in response to zinc and cadmium. RT-PCR expression patterns of *ospdr9* and *rac1*. A: RNA was extracted from roots of rice seedlings that were incubated on growth medium (control C_9), on water (control C_{10}), on water pH 4.5 (control C_{11}), on aqueous solutions of zinc sulphate (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_9) or on 750 μM $ZnSO_4$ for 3 h. Equal amounts of *rac1* were detected in all lanes.

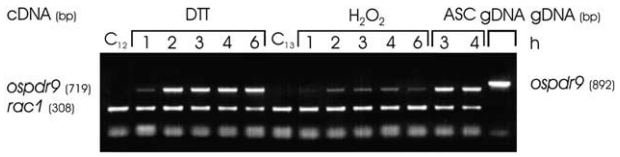

Fig. 6. *Ospdr9* expression in rice roots in response to DTT, hydrogen peroxide and ascorbic acid. RT-PCR expression patterns of *ospdr9* and *rac1*. Rice seedlings were incubated on growth medium (controls C_{12} and C_{13}) or on growth medium supplemented with 5 mM DTT or 5 mM H_2O_2 for 1, 2, 3, 4 and 6 h or on 5 mM ascorbic acid (ASC) for 3 and 4 h. Equal amounts of *rac1* were detected in all lanes.

PEG is generally used to impose osmotic stress, but can also cause adverse effects on root respiration [42] and/or toxic effects due to various contaminants [43], each of which was assessed further. Salt stress, which imposes osmotic and ion-toxic effects (Fig. 3), and dehydration, by partially removing the growth solution (data not shown), induced a delayed *ospdr9* expression at low levels in rice roots. Hypoxic stress, imposed by submergence as evident from a marked *rice adh1* response, induced *ospdr9* in rice seedling roots (Fig. 3). Toxic products, i.e. ethanol and the metal ions of zinc sulphate, cobalt and nickel chloride, also markedly induced *ospdr9* in rice roots (Fig. 2A,B). The results suggested that toxic and hypoxic PEG effects were more important than osmotic PEG effects in generating the marked PEG response of *ospdr9*.

The heavy metals cadmium and zinc rapidly and markedly induced *ospdr9* expression at concentrations as low as 20 μM Cd and 30 μM Zn (Fig. 5A), which is generally considered a typical heavy metal stress response. The Zn and Cd responses of *ospdr9* were root-specific (Fig. 5B), which could be due to root-to-shoot metal transport barriers, as observed in *Arabidopsis* [31] but could indicate root-specific regulations in addition. Primary defence responses that lead to Cd detoxification in plants include complexing the metal with phytochelatins, compartmentation in vacuoles or immobilisation at the cell wall by exclusion through the plasma membrane. Cd can also interact with photosynthesis, which typically generates secondary oxidative stress responses [44], or can interact with respiration or nitrogen metabolism. The tonoplast-localised MRP-type ABC transporter AtMRP3 of *Arabidopsis*, which can partially complement the yeast YCF protein involved in heavy metal detoxification [10], has recently been found to be Cd-induced in the roots of young *Arabidopsis* seedlings [31]. A role for a potentially plasma membrane-localised PDR-type ABC transporter in heavy metal stress responses in rice roots is intriguing but unknown, and is subject of our continued research.

In an attempt to identify signals that might be involved in the regulation of *ospdr9* expression during stress, we analysed the effect of various plant growth regulators. *Ospdr9* proved to be markedly induced by JA (Fig. 4), which suggested a pathogen or wounding response [45]. EST data in GenBank indicated that *ospdr9* was induced in rice leaves upon infection with the rice blast fungal pathogen *M. grisea*. JA may be

involved in regulating the expression of *ospdr9* in rice leaves during pathogen attack. *Ospdr9* was not SA-induced, and therefore most likely not part of a systemic acquired plant pathogen resistance response. *NtPDR1* was analogously methyl-JA-induced but not SA-responsive [27]. The plant hormone ABA, which controls many plant responses to water deficit, caused a moderate *ospdr9* transcript accumulation (Fig. 4). We previously observed that the ABA-induced transcripts of other rice genes, which were likewise both JA- and ABA-inducible and both salt- and biotic stress-responsive, were unstable and were not translated in the presence of ABA alone [32]. A putative involvement of OsPDR9 in biotic stress responses in leaves and in abiotic stress responses in roots is not unlikely for ABC transporters, which often have versatile functions.

In a further search for cellular events that might be involved in regulating *ospdr9* expression during stress, effects of redox perturbations were studied. The antioxidant DTT was found to markedly induce *ospdr9* in rice roots (Fig. 6), which suggested a response to redox perturbations that favour the reduced state. It is known that autoxidation of thiols, which occurs at a high pH (>8), can generate superoxide radicals [46], but the experiments were performed at a physiological pH of 4.8–5. Moreover, the non-thiol antioxidant ascorbic acid also induced *ospdr9* in rice roots (Fig. 6). The *ospdr9* response to reducing conditions might be consistent with the hypoxic stress response (Fig. 3). Low oxygen conditions can partially inhibit mitochondrial NADH reoxidations and might thus alter the cellular redox balance in roots. The induction of *rice adh1* by hypoxic stress (Fig. 3), heavy metals (Fig. 2A,B) and to some extent also by salt stress (Fig. 3) suggested effects on root respiration in all of these conditions. The strong oxidant hydrogen peroxide also induced *ospdr9* expression, though at three times lower levels than DTT (Fig. 6), which indicated a response to oxidative conditions as well. Cellular redox changes can affect the nuclear localisation or the activity of transcription factors or activators and thus regulate gene expression. Recently, cellular redox changes have been elegantly demonstrated to affect gene expression in plants through regulating the activity of the NPR1 protein, responsible for gene activation during the systemic acquired pathogen resistance response [47].

The expression of *ospdr9* was found to be markedly induced by the heavy metals Zn and Cd and hypoxic stress in rice roots, and the data suggested that redox changes may be involved in the complex stress response regulation of *ospdr9* in rice roots.

Acknowledgements: Thanks are due to the Faculty of Science and the Department of Biology of the University of Ottawa for logistic support. This work was supported in part by the Rockefeller Foundation Grant 2000 FS 184.

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