Diverse expression profiles of 21 rice peroxidase genes

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Abstract Secretory class III plant peroxidases (POXs) catalyze the oxidation of various reductants, and are encoded by a large multigene family. In rice, 42 independent expressed sequence tags for POXs have been identified. By RNA gel blot analysis using specific probes, we show here that 21 rice *POX* genes are unique in their developmental, organ specific and external stimuli-responsive expression. This would suggest that encoded POX isoenzymes are involved in a broad range of physiological processes in rice plants, individually.

Key words: Peroxidase; Expression profile; Gene family; Expressed sequence tag; *Oryza sativa* L.

1. Introduction

Secretory class III plant peroxidases (POXs) (EC 1.11.1.7) are found in multiple molecular forms in individual plant species. Each POX catalyzes the oxidoreduction between H_2O_2 and several different reductants, suggesting its contribution to diverse physiological processes such as lignification [1], suberization [2], crosslinking of cell wall proteins [3], auxin degradation [4], defense against pathogen infection [5], salt tolerance [6] and senescence [7]. Multiple forms and low substrate specificity also make it difficult to define the specific biological functions of individual POXs.

Some POX cDNAs and genomic DNA fragments have been isolated from single plant species. For example, more than six *POX* genes have been identified in alfalfa, tomato and wheat [5]. Chittoor et al. [8] isolated three independent cDNAs and a genomic DNA fragment encoding highly homologous rice POXs, and demonstrated that they had different induction patterns after infection by *Xanthomonas oryzae* pv. *oryzae*. Ito et al. [9] isolated the cDNAs encoding two structurally related rice POXs (prxRPA and prxRPN) whose corresponding transcripts exhibited slight different distribution in organs. In tobacco plants, 12 POX isoenzymes were detected by isoelectric focusing followed by activity staining. They showed differences in organ specific expression and in response to wounding or TMV infection [10].

Recent advances in the genome sequencing of rice and *Arabidopsis* have provided large scale expressed sequence tags (ESTs). Because cDNA libraries are prepared from various tissues with or without treatments, many independent POX ESTs have been isolated. Consequently, 42 and more than 40 different POX ESTs have been found in rice and *Arabidopsis*, respectively [11,12]. Here, we show the expression profiles of 21 rice *POX* genes identified by the rice genome research program. Interestingly, the expression profiles of all rice *POX* genes were unique. This is the first report on the expression analysis of a large number of *POX* genes in single plant species all at once.

2. Materials and methods

2.1. ESTs encoding rice POXs

ESTs for putative rice POX isoenzymes were selected from the EST population isolated from several cDNA libraries derived from rice organs or cultured cells with or without treatments [11]. ESTs covering the putative open reading frame of POX were further selected and sequenced from both sides. Invariable two histidine and eight cysteine residues, and three well conserved domains [13] were found in predicted amino acid sequences indicating that these sequences are POX cDNAs. Among these, 19 randomly selected ESTs, in addition to prxRPA and prxRPN cDNAs which we have previously isolated [9], were subjected to further analyses.

2.2. Rice plants and treatments

Five-day-old rice plants (*Oryza sativa* L. cv. Nipponbare) grown in a greenhouse were used as the materials for roots and aerial parts of the younger seedlings. Sixteen-day-old plants were used as the materials for roots, leaf sheaths and leaf blades of the older seedlings. For cutting treatment, the tips of leaf blades were cut off with a pair of scissors. For rubbing, the whole leaf blade was rubbed with carborundum #600 by hand. For ethephon and methyl jasmonate (MeJA) treatments, a 1 mM ethephon solution and a 25 μM MeJA solution containing 0.05% ethanol and 0.125% Triton X-100, respectively, were sprayed onto whole plants. Untreated plants were harvested at the same time as a control for cutting, rubbing and ethephon treatments (Cont 1). Seedlings sprayed with a solution containing 0.05% ethanol and 0.125% Triton X-100 were used as a control for MeJA treatment (Cont 3). For UV treatment, leaf blades were cut into pieces, floated on sterile water and irradiated with UV light (0.74 kJ/m^2) from a germicidal lamp (GL-15, NEC Corporation, Tokyo, Japan). For paraquat treatment, leaf blade pieces were floated on a 1 μM paraquat solution. Equivalent leaf blade sections were incubated on sterile water without UV or paraquat treatments and sampled as a control for these treatments (Cont 2). All treated plants or leaf blade sections were incubated for 48 h at 25°C under continuous illumination (200 $\mu E/m^2/s$) until sampling.

2.3. RNA gel blot analysis

Total RNAs isolated by the ATA (aurin tricarboxylic acid) method [14] were subjected to RNA gel blot analysis as described [15]. As

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specific probes, approximately 500 bp fragments containing 3′ untranslated regions were prepared by polymerase chain reaction or digestion with restriction enzymes. After hybridization, the membranes were washed once for 5 min and twice for 10 min each in 2×SSC and 0.1% SDS at room temperature and then three times in 1×SSC and 0.1% SDS for 15 min each at 65°C, before being subjected to autoradiography at −80°C.

3. Results and discussion

3.1. ESTs encoding rice POXs

From the EST population for putative full length POXs, we randomly selected 19 ESTs whose analyses had not been previously reported. *poxA* and *poxN* genes corresponding to prxRPA and prxRPN cDNAs that we previously isolated and characterized [9] were included as reference *POX* genes in the present study. All EST clones encoded proteins containing N-terminal signal peptide which confers the secretion to the vacuole or outside cells with or without the C-terminal extension, respectively (Table 1).

Two *Arabidopsis* POXs, ATP A2 and ATP 1a, have been reported to be 95% identical to horseradish HRP A2 and 87% to cotton GHP 1a, respectively [16,17]. In contrast, *Arabidopsis* ATP 2a shared less than 35% with POXs (ATP Ea and ATP Cb) from the same plant [16]. These observations suggest that respective plant species contain several common POX families and POX isoenzymes with low homology fall into distinct families within the single plant species. On POX proteins deduced from selected cDNAs, amino acid sequence identities ranged from 32.7 to 75.1% and most comparisons resulted in matching 35–50% (Table 2), suggesting that the

Table 1
Annotations of 21 rice POXs

Clone No.	Library[a] Accession No.[c]		Localization[b]
R2693	root	A	AU031932, D24879, D24300
R2329	root	A	D24657
S11222	green shoot	A	AU032599, D46505
R2151	root	A	AU031848, D24550
S4325	etiolated shoot	A	AU033167, D41670
S10927	green shoot	A	AU076282, C20524 to C20543, D46324
R1617	root	A	AU031774, C20488 to C20496, D24271
R2877	root	A	AU031963, D24977
R1420	root	A	AU031744, D24140
prxRP-A	green shoot	A	D14481
prxRP-N	green shoot	A	D14482
R2391	root	A	D39141, D24695
R2576	root	A	D16442, D24800
R3025	root	A	AU031983, D39025
S14493	green shoot	A	D48344
C62847	heat-shocked callus	A	AU076038, AU063563
R2184	root	V	AU031855, D24571, D24354
S13316	green shoot	A	AU032675, D47678
R0317	root	A	D14997, D23835
S14082	green shoot	A	C23611
C52903	gibberellin-treated callus	V	C23550, C97179

[a] Details for each cDNA library were described in [9,11].

[b] Predicted from the presence of putative signal peptides at N-termini of the coding regions (apoplastic: A) or both putative signal peptides and C-terminal extensions (vacuole: V).

[c] DDBJ/EMBL/GenBank accession No.

Table 2
Amino acid sequence identities between 21 rice POXs (%)[a]

	R2693	R2329	S11222	R2151	S4325	S10927	R1617	R2877	R1420	prxRPA	prxPRN	R2391	R2576	R3025	S14493	C62847	R2184	S13316	R0317	S14082
R2329	42.0																			
S11222	34.2	44.7																		
R2151	33.5	44.1	42.5																	
S4325	36.0	40.2	44.3	41.9																
S10927	33.9	39.4	44.8	42.3	58.0															
R1617	37.7	51.6	45.0	48.5	45.5	41.6														
R2877	39.7	50.2	43.0	41.8	42.9	41.3	48.7													
R1420	41.8	49.0	40.8	41.9	41.3	40.7	47.2	64.6												
prnRPA	32.7	43.3	44.8	44.0	47.2	41.8	46.4	40.0	41.2											
prxRPN	36.5	44.2	49.9	48.5	49.5	44.0	49.5	42.4	42.6	69.6										
R2391	34.2	41.2	45.3	41.3	55.6	49.7	45.6	39.9	45.3	48.9	50.3									
R2576	34.3	44.4	44.1	45.5	53.0	52.7	47.2	41.2	42.4	48.8	49.0	56.4								
R3025	30.6	42.0	45.4	44.2	56.4	54.2	46.5	42.3	43.4	48.5	47.9	55.1	57.1							
S14493	33.1	44.0	43.5	43.1	58.8	52.9	49.0	40.1	41.7	47.4	49.8	59.6	63.8	53.0						
C62847	32.0	43.1	36.1	39.4	38.7	41.1	40.8	38.1	35.4	37.7	37.4	37.4	39.1	37.3	39.0					
R2184	34.4	40.2	40.0	42.5	37.7	39.3	43.7	40.5	38.2	40.7	42.3	36.8	43.2	37.4	39.6	40.8				
S13316	37.0	48.2	45.2	43.7	44.9	46.5	49.7	43.6	41.4	42.7	43.7	45.4	40.4	42.0	42.4	40.4	41.2			
R0317	33.9	49.7	44.4	43.8	42.6	41.8	47.7	44.6	42.4	42.9	42.7	41.4	41.4	42.7	40.5	39.1	42.3	72.0		
S14082	35.4	47.8	44.7	43.8	42.5	42.3	48.0	41.4	38.6	41.1	41.5	42.3	40.5	42.6	41.4	38.1	43.7	75.9	69.0	
C52903	40.1	66.8	44.2	42.9	43.7	42.7	54.0	49.2	48.2	40.6	44.3	45.3	45.2	43.7	46.9	41.2	41.3	45.0	45.6	42.4

[a] Identity was calculated with predicted amino acid sequences deduced from each POX cDNA.

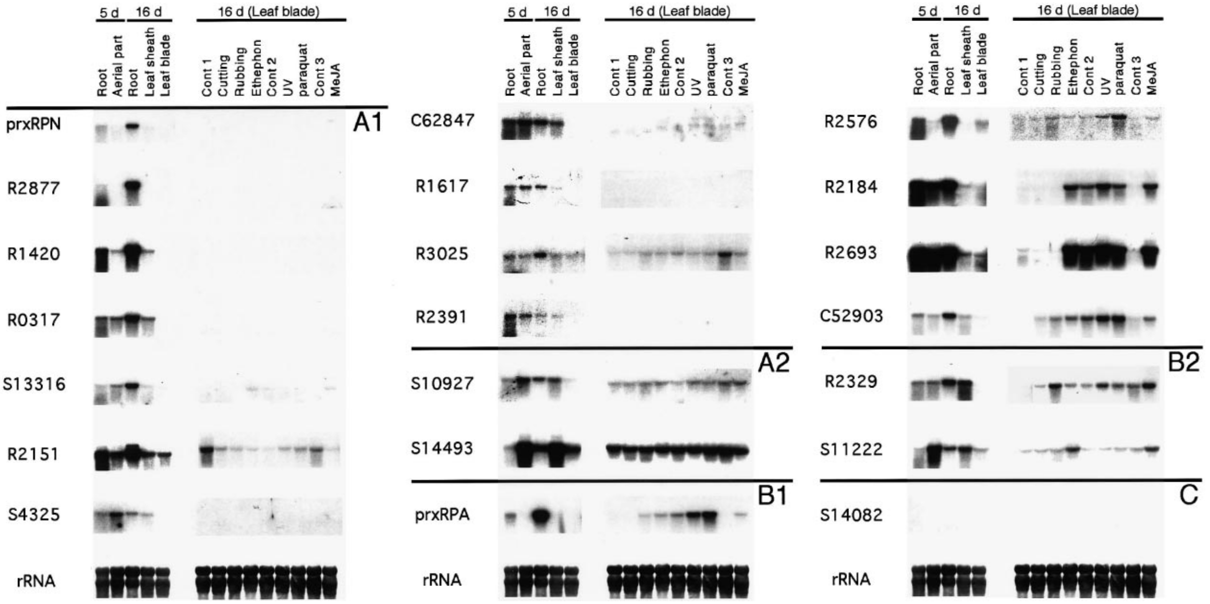

Fig. 1. RNA gel blot analysis of the 21 rice *POX* genes. Transcript levels were monitored with specific probes for each *POX* gene. 20 μg RNA samples were loaded on each lane and equal loading of RNAs was confirmed for all membranes by methylene blue staining of ribosomal RNAs [26].

analyzed POXs are fundamentally members of different families and that some POXs are structurally related to each other. Such diversity in amino acid sequences may reflect differences in enzymatic properties and biological functions.

3.2. Expression profiles of 21 rice POX genes

We obtained diverse expression profiles of the 21 rice *POX* genes using specific probes for each gene in this study (Fig. 1). Expression profiles of the genes for prxRPA and prxRPN cDNAs were essentially identical to the results we reported previously [9], indicating that the observed expression profiles of the *POX* genes are reproducible. Except for *S14082*, all *POX* genes analyzed here were expressed constitutively in roots of both 5- and 16-day-old rice plants, although the levels were variable. Transcripts for 17 *POX* genes were detected in the aerial parts of 16-day-old plants. Among 21 genes, seven genes responded to external stimuli such as wounding, UV-irradiation and treatments with ethephon, paraquat and MeJA, while the remaining 14 genes did not respond at all. According to the expression profiles, we categorized the 21 genes into five groups as shown in Table 3. Genes in group A were not induced by the stimuli whereas group B genes were induced. Expression of *S14082* in group C was not detected in either healthy or treated plants under our experimental conditions. Genes in groups A1 and B1 were preferentially or almost equally expressed in roots compared to aerial parts of 16-day-old healthy plants. Genes in groups A2 and B2 were preferentially expressed in aerial parts. The number of genes in groups A1 and B1 is 16, while that in groups A2 and B2 is only four (Table 3). This suggests that many *POX* genes are expressed in roots, although the levels were highly variable. This agrees with the fact that most POX ESTs originated the root cDNA library [11].

3.3. Possible functions of rice POXs

Expression profiles of individual POX isoenzymes could at least partly reflect their biological functions. Namely, accumulated transcripts in organs or after certain treatments are important determinants of the function of each POX. For convenience, the expression profiles of each *POX* gene are illustrated in Fig. 2.

Transcripts for the nine genes in group A1 were not detectable in the healthy leaf blades of 16-day-old plants, and various treatments did not change this. For *R2151* and *R3025*, other genes in group A1, considerable amounts of transcript were found in healthy leaf blades, but these levels were rather

Table 3
Classification of the 21 rice *POX* genes by the expression profiles

Group	Induction by stimuli[a]	Spatial distribution[c]	Number[d]
A1	no	root≥aerial part	11
A2	no	root < aerial part	2
B1	yes	root≥aerial part	5
B2	yes	root < aerial part	2
C	nd[b]	nd	1
		Total	21

[a] Leaf blades of 16-day-old plants.
[b] Not detectable.
[c] 16-day-old plants.
[d] Number of *POX* genes in the group.

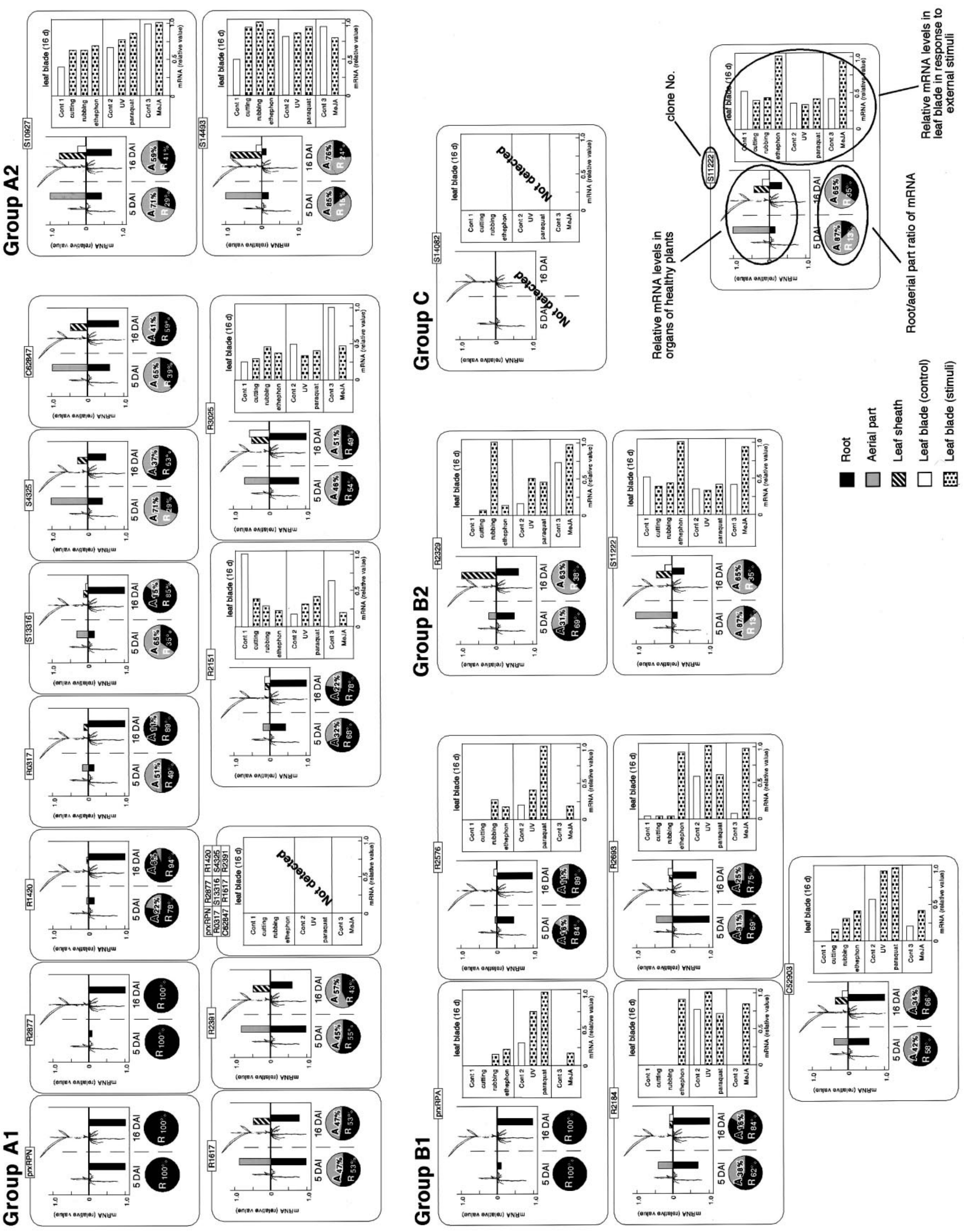

Fig. 2. Illustration of the expression profiles of 21 rice POX genes. Signals for each POX transcripts shown in Fig. 2 were quantified, calculated and illustrated in a circle according to the ratio of total intensity of the signal in root and aerial parts. The aerial parts contain both leaf sheath and leaf blade in 16-day-old plants. Signals in treated leaf blades of 16-day-old plant were quantified, calculated and shown as relative values compared to the maximal value. An explanation of the illustration is indicated on the right bottom of this figure. DAI: days after inhibition. A: aerial part. R: root.

decreased by the treatments. These results suggest possible involvement of group A1 *POX* genes in the basal metabolism in plant growth such as crosslinking of cell wall proteins and feruloylated polysaccharides, lignification, suberization and auxin degradation. Among the 11 genes belonging to A1, *R2151*, *S4325*, *R2877*, *R1420*, *prxRPN*, *S13316* and *R0317* were predominantly expressed in roots, while *R1617*, *R2391*, *R3025* and *C62847* were similarly expressed in both roots and aerial parts, suggesting specific roles in these organs.

The two genes in group A2, *S10927* and *S14493*, were constitutively expressed like the genes in group A1, although their expression levels were higher in aerial parts than roots and were not influenced by the stress treatments. Thus, in aerial parts, these POXs would have basal functions that are not inhibited by environmental stresses.

The genes in group B were induced differently by external stimuli in the leaf blades of 16-day-old plants. *R2693*, *prxRPA*, *R2576*, *R2184* and *C52903* genes in group B1 were expressed constitutively at higher levels in roots than in aerial parts, while *R2329* and *S11222* in group B2 had higher levels in aerial parts. These genes, which responded differently to the stimuli, would be involved in self-defense against physical, chemical and biological stresses.

Both cutting and rubbing treatments are known to cause wound stress which inhibits normal growth and reproduction of plants, and enables pathogens to penetrate easily into plant tissues. Expression of *C52903* gene in group B1 and *R2329* gene in B2 was induced by both cutting off the tips of the leaf blades and rubbing the whole leaf blades, while that of *prxRPA* and *R2576* in group B1 was induced only by rubbing. Expression levels of *R2693* and *R2184* in group B1 were increased by cutting leaf blades into small pieces (compare Cont 1 and 2 in Fig. 1) but not by cutting off the tips of leaf blades or by rubbing. Thus, the wound response of these *POX* genes is complex, indicating specific roles in the restoration of damaged tissues for example in suberization, lignification and crosslinking of cell wall proteins. Some wound-inducible *POX* genes were also induced by pathogen infection [8,18], suggesting that the wound-inducible rice *POX* genes studied here are involved in the defense against pathogen attack.

UV-irradiation was reported to cause accumulation of H_2O_2 [19]. Paraquat, a non-selective contact herbicide, disturbs proton translocation through the thylakoid membrane leading to production of active oxygen species and energy depletion [20]. Among several types of POXs, only ascorbate POX, one of the class I plant POXs, has been considered a H_2O_2-scavenging enzyme in plants [21]. Recent biochemical studies suggested that some class III plant POXs, referred to as POXs in this paper, are also involved in H_2O_2-scavenging systems [22,23]. Thus, UV- or paraquat-inducible *POX* genes such as *R2693*, *R2329*, *prxRPA*, *R2576*, *R2184* and *C52903* would be involved in the detoxification of H_2O_2 which accumulates as a result of UV-irradiation, exposure to air pollutants or high intensity light.

Treatment with MeJA or ethephon, which is an ethylene-releasing agent, resulted in the induction of the *POX* genes *R2693*, *R2329*, *S11222*, *prxRPA*, *R2576*, *R2184* and *C52903* which were also induced by wounding, UV-irradiation or paraquat treatment, suggesting that JAs and ethylene are the signal compounds for the stress-induced expression of the rice *POX* genes. Actually, JA has been shown to accumulate both locally and systemically in wounded rice plants [24]. Many pathogen-inducible rice genes were also induced by exogenous application of JA [25]. Thus, seven MeJA-inducible *POX* genes are possibly pathogen-inducible.

All *POX* genes in groups B1 and B2 were also expressed constitutively in roots at various levels in both 5- and 16-day-old plants. Except for *prxRPA*, transcripts of these genes were also constitutively detected in aerial parts at various levels. From these results, we speculate that genes in groups B1 and B2 participate in the developmentally regulated basic metabolism and stress-responsive reactions in rice plants.

The expression profiles of the 21 *POX* genes suggest that they have diverse biological functions in growth and response to environmental stresses in rice plants. Interestingly, many different *POX* genes were induced by a single stimulus: wounding induced expression of *R2693*, *R2329*, *prxRPA*, *R2576*, *R2184* and *C52903*, UV-irradiation that of *R2693*, *R2329*, *prxRPA*, *R2576*, *R2184* and *C52903*, and paraquat treatment that of *R2329*, *prxRPA*, *R2576* and *C52903*. Amino acid sequences of R2184 and C52903 in group B1 contained putative N-terminal signal peptides and C-terminal extension, suggesting that these POXs are targeted to vacuoles. In contrast, those of R2693, prxRPA, R2576 and R3025 in group B1, and R2329 and S11222 in group B2 contained only putative N-terminal signal peptides, suggesting that they are secreted outside the cells. Wounding and/or paraquat treatment induced the expression of both putative apoplastic (R2329, prxRPA and R2576) and vacuolar (R2184 and C52903) POXs. These observations suggest that multiple POXs function differently or cooperatively in the same physiological reactions. Thus, this may be one of the reasons why such a huge number of POX isoenzymes work in a single plant species. Analyses of pathogen-induced and tissue/organ specific expression of these *POX* genes would be one of the most interesting studies to do in the future, as well as experiments using transformation techniques. More precise expression analyses, promoter analysis, reverse genetics and biochemical studies would further enhance our understanding of the biological functions of each POX.

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References

[1] Whetten, R. and Sederoff, R. (1995) Plant Cell 7, 1001–1013.
[2] Espelie, K.E., Franceschi, V.R. and Kolattukudy, P.E. (1986) Plant Physiol. 81, 487–492.
[3] Fry, S.C. (1986) Ann. Rev. Plant Physiol. 37, 165–186.
[4] Hinman, R.L. and Lang, J. (1965) Biochemistry 4, 144–158.
[5] Chittoor, J.M., Leach, J.E. and White, F.F. (1999) in: Pathogenesis-Related Proteins in Plants (Datta, S.K. and Muthukrishnan, S., Eds.), pp. 171–193, CRC Press, Boca Raton, FL.
[6] Amaya, I., Botella, M.A., de la Calle, M., Medina, M.I., Heredia, A., Bressan, R.A., Hasegawa, P.M., Quesada, M.A. and Valpuesta, V. (1999) FEBS Lett. 457, 80–84.
[7] Abeles, F.B., Dunn, L.J., Morgens, P., Callahan, A., Dinterman, R.E. and Schmidt, J. (1988) Plant Physiol. 87, 609–615.
[8] Chittoor, J.M., Leach, J.E. and White, F.F. (1997) Mol. Plant-Microbe Interact. 7, 861–871.
[9] Ito, H., Kimizuka, F., Ohbayashi, A., Matsui, H., Honma, M., Shinmyo, A., Ohashi, Y., Caplan, A.B. and Rodriguez, R.L. (1994) Plant Cell Rep. 13, 361–366.

[10] Lagrimini, L.M. and Rothstein, S. (1987) Plant Physiol. 84, 438–442.
[11] Yamamoto, K. and Sasaki, T. (1997) Plant Mol. Biol. 35, 135–144.
[12] Østergaard, L., Pedersen, A.G., Jespersen, H.M., Brunak, S. and Welinder, K.G. (1998) FEBS Lett. 433, 98–102.
[13] Buffard, D., Breda, C., van Huystee, R.B., Asemota, O., Pierre, M., Ha, D.B.D. and Esnault, R. (1990) Proc. Natl. Acad. Sci. USA 87, 8874–8878.
[14] Nagy, F., Kay, S.A. and Chua, N.H. (1988) in: Plant Molecular Biology Manual (Glevin, R.A., Schilperoot, S.B. and Verma, D.P.S., Eds.), pp. B4:1–29, Kluwer Academic Publishers, Dordrecht.
[15] Ausubel, F.A., Brent, R., Kingston, R.E., Moor, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1988) Current Protocols in Molecular Biology, Wiley, New York.
[16] Kjærsgård, I.V.H., Jespersen, H.M., Rasmussen, S.K. and Welinder, K.G. (1997) Plant Mol. Biol. 33, 699–708.
[17] Østergaard, L., Abelskov, A.K., Mattsson, O. and Welinder, K.G. (1996) FEBS Lett. 398, 243–247.
[18] Mohan, R. and Kolattukudy, P.E. (1990) Plant Physiol. 92, 276–280.
[19] Murphy, T.M. and Huerta, A.J. (1990) Physiol. Plant 78, 247–253.
[20] Babbs, C.F., Pham, J.A. and Coolbaugh, R.C. (1989) Plant Physiol. 90, 1267–1270.
[21] Asada, K. (1992) Physiol. Plant 85, 235–241.
[22] Mehlhorn, H., Lelandais, M., Korth, H.G. and Foyer, C.H. (1996) FEBS Lett. 378, 203–206.
[23] Kvaratskhelia, M., Winkel, C. and Thorneley, R.N.F. (1997) Plant Physiol. 114, 1237–1245.
[24] Schweizer, P., Buchala, A., Dudler, R. and Métraux, J.P. (1998) Plant J. 14, 475–481.
[25] Schweizer, P., Buchala, A., Silverman, P., Seskar, M., Raskin, I. and Métraux, J.P. (1997) Plant Physiol. 114, 79–88.
[26] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.