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# Ten Rice Peroxidases Redundantly Respond to Multiple Stresses Including Infection with Rice Blast Fungus

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**Class III plant peroxidases are believed to function in diverse physiological processes including disease resistance and wound response, but predicted low substrate specificities and the presence of 70 or more isoforms have made it difficult to define a specific physiological function(s) for each gene. To select pathogen-responsive *POX* genes, we analyzed the expression profiles of 22 rice *POX* genes after infection with rice blast fungus. The expression of 10 *POX* genes among the 22 genes was induced after fungal inoculation in both compatible and incompatible hosts. Seven of the 10 *POX* genes were expressed at higher levels in the incompatible host than in the compatible host 6–24 h after inoculation by which time no fungus-induced lesions have appeared. Organ-specific expression and stress-induced expression by wounding and treatment with probenazole, an agrichemical against blast fungus, jasmonic acid, salicylic acid and 1-aminocyclopropane-1-carboxylate, a precursor of ethylene, indicated that rice POXs have individual characteristics and can be classified into several types. A comparison of the amino acid sequences of POXs showed that multiple isoforms with a high sequence similarity respond to stress in different or similar ways. Such redundant responses of *POX* genes may guarantee POX activities that are necessary for self-defense in plant tissues against environmental stresses including pathogen infection.**

**Keywords**: Classification — Peroxidase — Rice — Rice blast fungus — Stress.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; EST, expressed sequence tag; HR; hypersensitive reaction or response, JA, jasmonic acid; PBZ, probenazole; POX, class III plant peroxidase; SA, salicylic acid; UTR, untranslated region.

## Introduction

POX (Class III plant peroxidase; EC 1.11.1.7) catalyzes the oxidoreduction of various substrates using hydrogen peroxide. Many reports have suggested that POXs play roles in resistance to pathogens such as lignification and suberization (Dean and Kolattukudy 1976, Quiroga et al. 2000), cross-linking of cell wall proteins (Bradley et al. 1992, Showalter 1993), xylem wall thickening (Hilaire et al. 2001), generation of reactive oxygen species (Bolwell et al. 1995, Wojtaszek 1997, Bestwick et al. 1998), hydrogen peroxide scavenging (Kawaoka et al. 2003), phytoalexin synthesis (Kristensen et al. 1999, Stoessl 1967), antifungal activity of POX itself (Caruso et al. 2001) and auxin metabolism (de Forchetti and Tigier 1990, Lagrimini et al. 1990, Lagrimini et al. 1997). However, we know no clear evidence indicating that POX contributes to self-defense at the molecular level.

Some *POX* genes are activated by infection with pathogens such as fungi (Thordal-Christensen et al. 1992, Harrison et al. 1995, Curtis et al. 1997), bacteria (Young et al. 1995, Bestwick et al. 1998), viruses (Lagrimini and Rothstein 1987, Hiraga et al. 2000b) and viroids (Vera et al. 1993). Therefore, it is classified to the pathogenesis-related (PR) protein-9 family (van Loon et al. 1994). In rice, POX activity increased after inoculation with rice blast fungus (Matsuyama and Kozaka 1981), and the expression of *POX8.1* and *POX22.3* genes was induced by infection with *Xanthomonas oryzae* pv. *oryzae*, a bacterial leaf blight disease, especially in a resistant rice cultivar (Chittoor et al. 1997). Wounding also induced *POX* gene expression in horseradish (Kawaoka et al. 1994), rice (Hiraga et al. 2000a, Ito et al. 2000), tobacco (Sasaki et al. 2002a) and tomato plants (Mohan et al. 1993).

*POX* genes constitute a multigene family; 73 different *POX* expressed sequence tags (ESTs) in *Arabidopsis* (Tognolli et al. 2002, Welinder et al. 2002) and at least 42 *POX* cDNAs in rice (Yamamoto and Sasaki 1997). Six or more independent *POX* genes were found in alfalfa, tobacco, tomato and wheat (Chittoor et al. 1997, Lagrimini and Rothstein 1987). The redundancy of *POX* genes in a single plant species has made it difficult to evaluate the function of each *POX* gene.

We have reported on the diverse expression profiles of 21 *POX* genes (Hiraga et al. 2000a), which were randomly selected from the 42 rice *POX* genes (Yamamoto and Sasaki

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**Table 1** Information on 22 *POX* genes examined in this study

| Clone [a] | Accession number [b] | Source of library [c] | Positive response to [d] |
|---|---|---|---|
| S14493 | D48344 | Green shoot | Wounding |
| R2576 | D16442, D24800 | Root | JA, Wounding |
| R2329 | D24657 | Root | Et, JA, Wounding |
| R2184 | AU031855, D24571, D24354 | Root | Et, JA |
| R2693 | AU031932, D24879, D24300 | Root | Et, JA |
| prxRPN | D14482 | Green shoot | N |
| C52903 | C23550, C97179 | Gibberellin-treated callus | Et, JA, Wounding |
| S11222 | AU032599, D46505 | Green shoot | Et, JA, Wounding |
| S10927 | AU076282, C20524–C20543, D46324 | Green shoot | Et, JA, Wounding |
| R1744 | AU083492, D24331 | Root | – |
| R2151 | AU031848, D24550 | Root | N |
| S4325 | AU033167, D41670 | Root | N |
| R1617 | AU031774, C20488–C20496, D24271 | Root | N |
| R2877 | AU031963, D24977 | Root | N |
| R1420 | AU031744, D24140 | Root | N |
| prxRPA | D14481 | Green shoot | JA, Wounding |
| R2391 | D39141, D24695 | Root | N |
| R3025 | AU031983, D39025 | Root | N |
| C62847 | AU076038, AU063563 | Heat-shocked callus | N |
| S13316 | AU032675, D47678 | Green shoot | N |
| R0317 | D14997, D23835 | Root | N |
| S14082 | D23611 | Green shoot | N |

[a] Clone names of rice *POX* genes used in previous papers (Yamamoto and Sasaki 1997, Hiraga et al. 2000a) and this study.
[b] DDBJ/EMBL/GenBank accession number of each *POX* gene.
[c] Source of cDNA library described by Ito et al. (1994) and Yamamoto and Sasaki (1997).
[d] Responses to various treatments using leaf blades of 16-day-old rice seedlings (Hiraga et al. 2000a) except for *R1744*, which is an additive gene used in this experiment. Et: ethephon. JA: methyl jasmonate. N: no response. –: not tested. The first 10 clones are *POX* genes that were responsive to infection with rice blast fungus in this study.

1997), using specific 3′UTR probes. These genes were unique in their developmental, organ specific and external stress-inducible expression profiles in rice plants at the juvenile stage (Hiraga et al. 2000a). Extending this work, we report here on the responses of these *POX* genes to infection with rice blast fungus, one of the most devastating fungal diseases in the world (Valent and Chumley 1991). We found that 10 genes among the 22 genes were induced by infection with rice blast fungus in adult rice plants. These *POX* genes were classified into several types by the expression profiles after blast fungus infection in both compatible and incompatible hosts, treatment with defense signal compounds and wounding. One type contains multiple *POX* genes, indicating that their roles would be important in rice plants at least in response to stresses.

## Results

*Induced expression of rice POX genes by infection with rice blast fungus*

Information on 22 rice *POX* genes used in this study is shown in Table 1. The positive responses of 21 rice *POX* genes to various treatments in 16-day-old rice plants (cv. Nipponbare) described in our previous paper (Hiraga et al. 2000a) are shown in the right column. One extra rice *POX* gene, *R1744* (Yamamoto and Sasaki 1997) was used in addition to the 21 *POX* genes.

To confirm the development of disease symptom induced by rice blast fungus (Fig. 1), we first examined the responses of the 22 rice *POX* genes plants at the eight-leaf stage after inoculation with *Magnaporthe grisea* race 003 by RNA blot analysis. After spraying an aliquot of spore suspension ($3\times10^5$ conidia $ml^{-1}$) onto rice plants (time 0), the plants were kept at 25°C for 20 h in the dark, and then transferred to a greenhouse at 25°C. The compatible host (wild-type cv. Nipponbare, which lacks the *Pi-i* resistance gene against *M. grisea* race 003) exhibited sensitive response with whitish developed lesions in the fully developed upper leaves 3–4 d post-inoculation (dpi) (Fig. 1A, left), which grew to 31.0±11.0 mm in diameter with conidia formation at 8 dpi (Fig. 1B). In the incompatible host (IL-7, an isogenic line of Nipponbare which contains the *Pi-i* gene), hypersensitive reaction (HR; Goodman and Novacky 1994) occurred with small dark brown lesions (2.8±1.0 mm in diameter) at 2 dpi, which hardly increased in size thereafter, as the resistance response. As the typical lesion phenotypes in both

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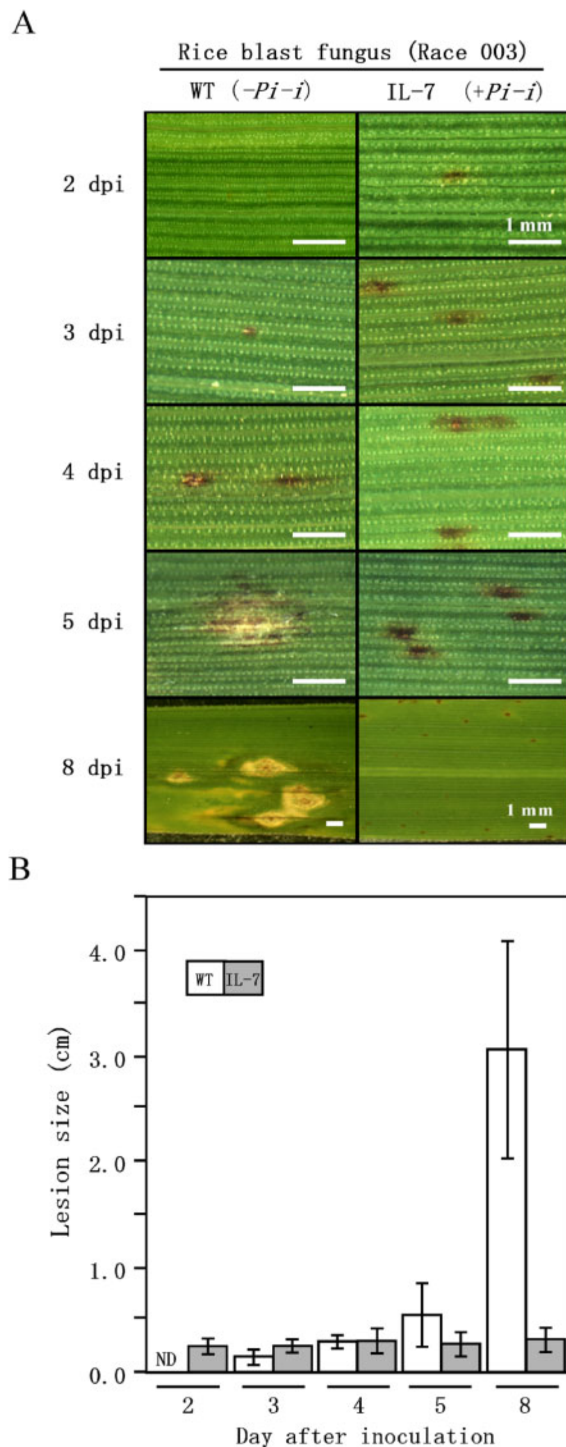


**Fig. 1** Local lesions that formed on rice leaves infected with rice blast fungus. *M. grisea* race 003 was inoculated on a compatible cultivar, wild-type Nipponbare (left panel) and an incompatible cultivar IL-7, which contains the resistance gene *Pi-i* against the rice blast fungus in the Nipponbare background (right panel). (A) Photographs of the inoculated leaves 2–8 dpi. (B) The size of the lesions developed after inoculation with *M. grisea* was measured. Open bar, wild-type Nipponbare (WT); gray bar, IL-7. The error bars indicate the standard deviation for the measurement of individual lesions.

compatible and incompatible hosts were commonly found on fully developed 8th leaf blades (the uppermost position) at the eight-leaf stage (Fig. 1B), we used these leaves for RNA gel blot analyses.

When we analyzed expression of the 22 *POX* genes, detectable levels of transcript were found for only the 10 *POX* genes at the top of Table 1, while those of the 12 genes below were not detectable before or after inoculation with rice blast fungus in both compatible and incompatible hosts.

Then, we analyzed the expression profiles of the 10 *POX* genes. As shown in the left panel of Fig. 2A, the transcripts of four *POX* genes (*R2576*, *R2329*, *R2184* and *R2693*) were transiently detected 1 and 2 d after mock-inoculation in wild-type leaves (Fig. 2A, left side panel). The transcripts of *R1744* and *PBZ1*, which is a positive control gene whose expression was induced by rice blast fungus infection (Midoh and Iwata 1996), were found constitutively at low levels. No transcripts were found for the other five *POX* genes (*S14493*, *prxRPN*, *C52903*, *S11222* and *S10927*).

After inoculation, the accumulation of transcripts for eight *POX* genes, especially *S14493* and *R2576*, was detected 6–12 h post-inoculation when no lesion formation was observed, and the transcript levels decreased thereafter in wild-type compatible host plants (Fig. 2A, middle panel). The maximum levels of transcripts for seven *POX* genes (*S14493*, *R2576*, *R2329*, *R2184*, *R2693*, *prxRPN* and *C52903*) were found 5 dpi, when spreading-type whitish lesions had developed. In IL-7 incompatible plants, the expression profiles of almost all *POX* genes were similar to those of the wild-type plants except at 5 dpi, but the levels were considerably higher (Fig. 2A, right panel). When the data in Fig. 2A were quantified, the accumulation profiles of these transcripts were clarified, allowing for the classification of the 10 genes into six types (Fig. 2B). Responses of *S14493* and *R2576* in type 1 were found as early as 6 h post-inoculation and returned to basal levels 2 dpi in the incompatible IL-7 host and the compatible wild-type hosts. *R2329* in type 2 similarly exhibited a quick response, but its transcript level did not return to the basal level even 5 dpi. Compared with the three *POX* genes *S14493*, *R2576* and *R2329* in types 1 and 2, the response of the four genes in type 3, *R2184*, *R2693*, *prxRPN* and *C52903*, was slightly delayed, peaking at 1 dpi in both rice cultivars. The expression of *S11222* in type 4 and *S10927* in type 5 took longer than that for the seven *POX* genes mentioned above, and they also exhibited different expression profiles. The response of *R1744* was similar in compatible and incompatible hosts, and its transcript level increased 1 dpi, and was maintained at this level thereafter. The expression of *PBZ1*, a positive marker gene expressed upon PBZ treatment, was observed 2 dpi, just after HR lesion formation in the IL-7 plants and increased thereafter. In compatible hosts, *PBZ1* transcript levels increased along with the expression of types 1, 2 and 3 *POX* genes 5 dpi after spreading-type lesions had developed. The transcripts of three *POX* genes in types 1 and 2 were found 6 h post-inoculation, and returned

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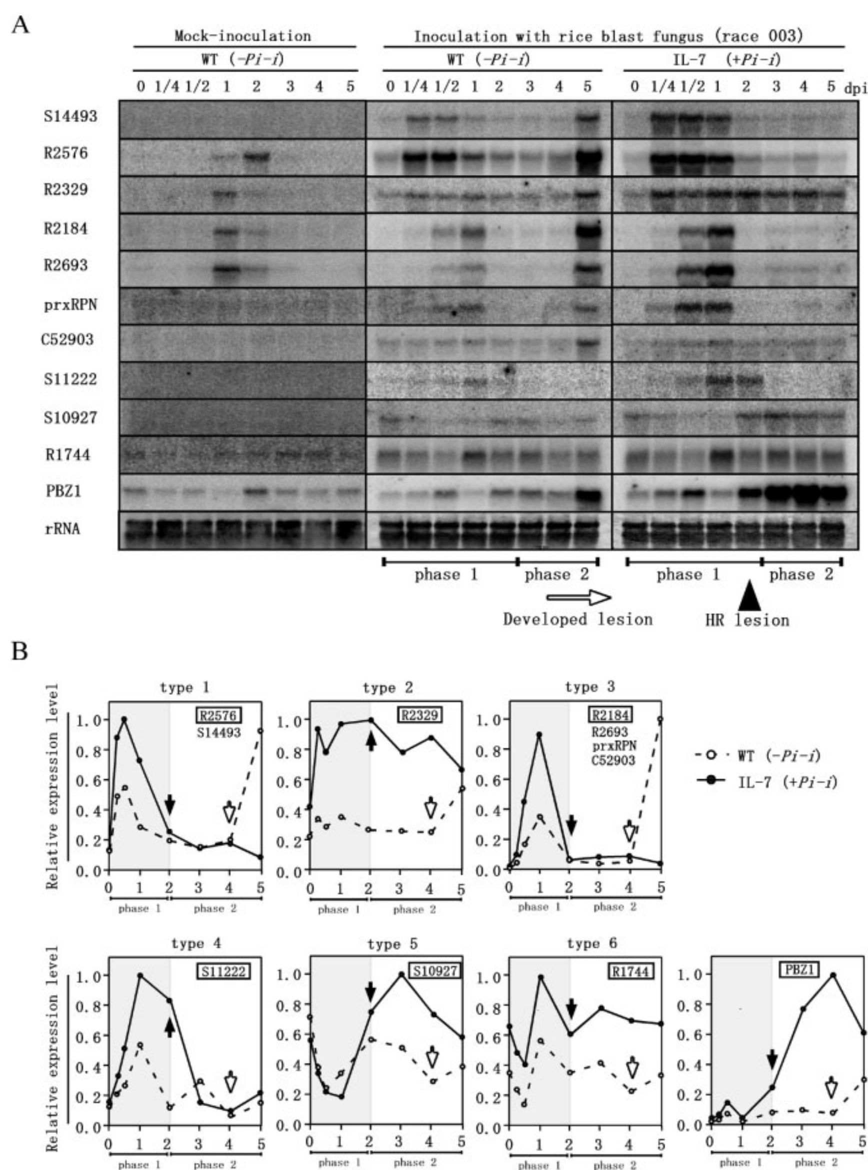


**Fig. 2** Responses of rice *POX* genes to infection with rice blast fungus. (A) Left panel: time course analysis after mock-inoculation by spraying a 0.05% Tween-20 solution onto compatible wild-type (WT) Nipponbare plants. Middle and right panels: the response after inoculation with a conidial suspension of rice blast fungus race 003 in compatible host Nipponbare and in incompatible host IL-7, respectively. Phase 1 indicates 0–2 dpi, at which time HR lesions had not formed in the incompatible host IL-7. Phase 2 follows phase 1. Total RNA (20 µg lane$^{-1}$) from the uppermost leaf blades at the 8th leaf stage was subjected to RNA gel blot analysis using 3′ UTR-specific probes for each gene. RNA gel blot analyses were repeated three times using independent RNA samples, and similar expression patterns were observed in all cases. (B) Relative quantification of *POX* transcripts. The signal strength was quantified using a PhosphorImager and normalized against the maximum level (relative expression level = 1.0). Closed arrow, the time of HR lesion visualization in IL-7; open arrow, the time of compatible lesion visualization in the wild type. Data for the *POX* gene, whose name is enclosed like that for R2576 in type 1, were used as a representative example. Open circle, wild-type; closed circle, IL-7.

to basal levels 2 dpi, by which time an HR lesion had developed in the incompatible host. Thus, the expression profiles of each *POX* gene after fungal infection were quite different and unique. Such rapid and transient *POX* gene expression has not been reported until now.

### *Localization of 22 POX genes in the rice genome*

In *Arabidopsis*, the genome sequencing project has recently been completed (Mayer et al. 1999, Lin et al. 1999, Salanoubat et al. 2000, Tabata et al. 2000, Theologis et al. 2000), and 73 *POX* genes were identified (Tognolli et al. 2002, Welinder et al. 2002). Rice genome sequencing has also proceeded (Feng et al. 2002, Sasaki et al. 2002b), and the information is now available by KOME (http://cdna01.dna.affrc.go.jp/cDNA/). Using the genome sequence data, we localized the 22 rice *POX* genes by referring to data on rice gene markers (Yamamoto and Sasaki 1997) or by using their original Bac or Yac clones (Fig. 3). Ten of the *POX* genes, which are shown as the first 10 clones in Table 1, are responsive to rice blast fungus, and located on seven chromosomes. Tandem repeat localizations of *POX* genes were found in five loci; a similar situation was previously reported in *Arabidopsis* genome (Tognolli et al. 2002).

### *Comparison of the amino acid sequences of the POX proteins*

The amino acid sequence homology between 21 POXs and R1744 ranged between 32.7% and 75.1% (Hiraga et al. 2000a and our unpublished data). The diversity in amino acid sequences suggests that these proteins have different biological functions. The sequences of the 22 genes were then aligned using the coding region and compared with POXs from other plant species (Fig. 4). They were found to contain eight con-

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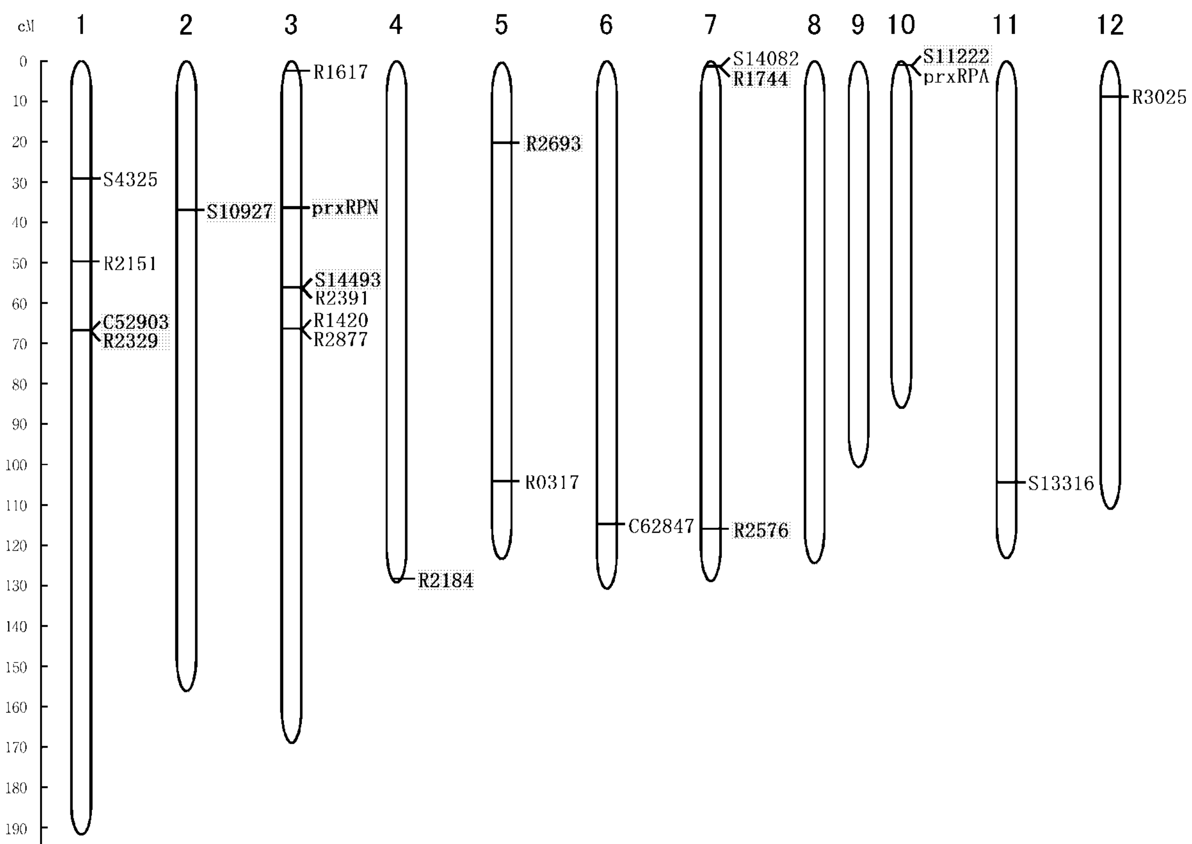


**Fig. 3** Localization of 22 *POX* genes on rice chromosomes. The positions were registered as rice markers, or deduced from the position of their original Bac or Yac clones registered at KOME (http://cdna01.dna.affrc.go.jp/cDNA/). The clone number in the gray background indicates the *POX* genes that responded to inoculation with rice blast fungus.

served cysteine residues and two histidine residues as described previously by Welinder et al. (2002) (data not shown), and were subsequently classified into four groups (G1–G4). S10927 was classified into G1, into which an anionic tobacco POX likely related to auxin metabolism (Lagrimini et al. 1990, Lagrimini et al. 1997) was also classified. R2329, R2184, R2693, C52903 and R1744 as well as TPX1, TPX2 and RCI3 were classified into G2, and are thought to participate in cell wall-reinforcement activities including lignin biosynthesis (Amaya et al. 1999, Llorente et al. 2002, Quiroga et al. 2000). R2576, S14493 and prxRPN as well as POC1, which was detected at the protein level earlier in incompatible interaction than in compatible interaction after infection with *Xanthomonas oryzae*, a bacterial blight disease (Young et al. 1995), were classified into G3. These genes are expected to participate in cell wall reinforcement (Young et al. 1995). S11222 was classified into G4 with horseradish prxC1 (HRPC), which reportedly has ascorbate peroxidase activity (Kawaoka et al. 2003).

*Organ-specific expression of rice POX genes*

The organ-specific expression patterns of 10 *POX* genes were analyzed in healthy wild-type rice plants (Fig. 5). Transcripts of all *POX* genes except for *S14493* were detected at considerable levels in the roots. In coleoptiles, the transcript levels of *S14493*, *R2184*, *R2693* and *S1122* were high, but those of *R2576*, *R2329*, *prxRPN*, *C52903* and *R1744* were low. When leaf blades were prepared from the uppermost leaf blades of rice plants at the 3rd, 5th, 6th and 9th leaf stages, respectively, *R2576*, *R2184* and *R2693* genes were found to be constitutively expressed at high levels in the 3rd leaf, which had already developed during the seed stage, but not in the 5th, 6th and 9th leaves, which are true leaves developing after germination. No significant expression of the other rice *POX* genes was observed in the uppermost leaves except for the 3rd leaf. In the panicles, all *POX* genes except for *S14493* and *S11222* were expressed at low levels. The *PBZ1* gene was expressed at considerable levels in the panicle, and at low levels in the root, coleoptile and the top leaf during the 3rd and 9th leaf stages. A different expression profile of *PBZ1* was also observed after infection with rice blast fungus (Fig. 2). Thus, the 10 *POX* genes exhibited characteristic constitutive expression profiles in various organs of healthy rice plants.

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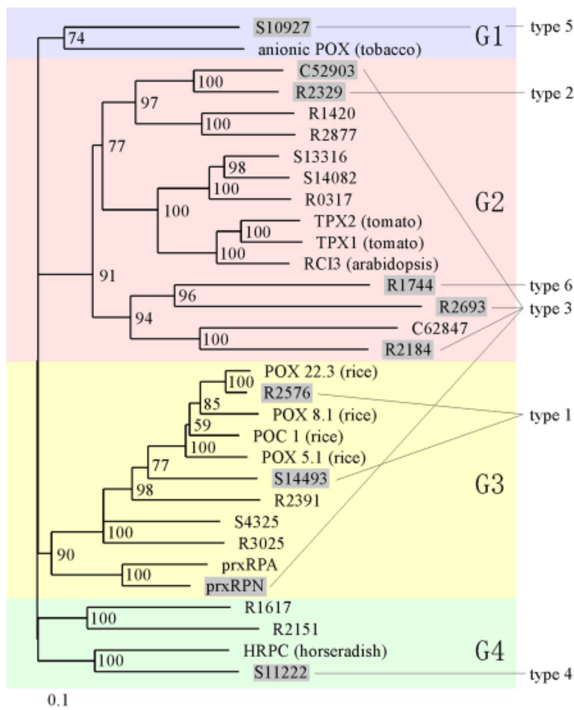


**Fig. 4** Phylogenic relationship of 22 rice POXs and other plant POXs. The amino acid sequences of 22 POXs were compared with those of other plant POXs which have been examined using transgenic plant, such as *TPX1* and *TPX2* (Amaya et al. 1999, Quiroga et al. 2000), horseradish *prxC1* (HRPC; Kawaoka et al. 2003), *Arabidopsis RCI3* (Llorente et al. 2002) and tobacco anionic *POX* (Lagrimini et al. 1990, Lagrimini et al. 1997). In rice, *POC1* (Young et al. 1995), *POX5.1*, *POX8.1* and *POX22.3* (Chittor et al., 1997) expression was induced by inoculation with *X. oryzae*. The bootstrapped tree method was used with Genetyx version 6. The numbers on the branches refer to bootstrap probabilities and values <50% are not shown. The scale bar indicates the number of amino acid substitutions per site. Clone names in gray backgrounds indicate the *POX* genes that responded to inoculation with rice blast fungus. G1-4 indicates the classified group; 'type 1–6' indicates the classified type seen in Fig. 2.

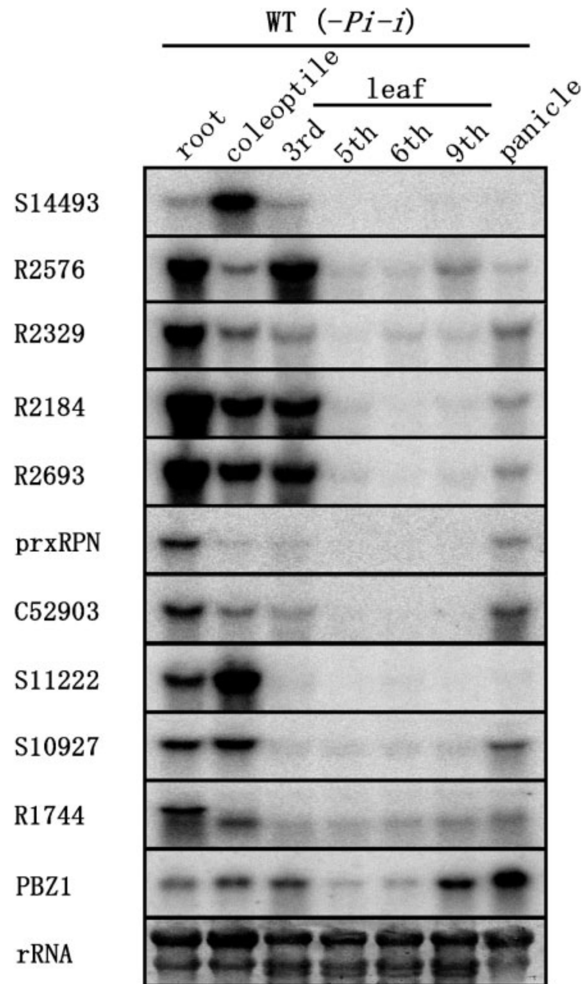


**Fig. 5** Organ specific expression of 10 *POX* genes. The organ-specific expression of 10 *POX* genes that responded to infection with rice blast fungus was investigated using healthy wild-type Nipponbare. As organs, roots, sheaths, uppermost leaf blades of the plants at the 3rd and 4th, 5th, 6th and 9th leaf stages, respectively, and panicles were used. Total RNA was subjected to RNA gel blot analysis as described in the legend of Fig. 2A.

### *Response to PBZ treatment*

Probenazole (PBZ; 3-allyloxy-1,2-benzisothiazole-1,1-dioxide) is known as an effective agricultural chemical against *M. grisea*, inducing systemic acquired resistance in whole rice plants. It exhibits only weak antimicrobial activity by itself against the fungus in vitro (Watanabe et al. 1977). In PBZ-treated rice plants, the activities of defense-related enzymes such as POX, polyphenoloxidase, phenylalanine ammonia-lyase, tyrosine ammonia-lyase and catechol-*O*-methyltransferase markedly increased upon infection (Iwata et al. 1980). In our experimental system, pre-spraying a PBZ solution (100 mg liter$^{-1}$) onto wild-type plants 3 d before fungal inoculation altered the lesions from compatible-type whitish developed lesions to incompatible-type small HR lesions (data not shown). When the responses of the 10 *POX* genes to the treatment with PBZ were examined by RNA blot analysis, interestingly five *POX* genes (*S14493*, *R2576*, *R2184*, *R2693* and *prxRPN*) in type 1 and 3, responded rapidly to the treatment, and high levels of their transcripts were transiently detected at 6 h (Fig. 6A). *R2329* in type 2 also slightly responded 6 h after treatment, and *S10927* responded to PBZ more slowly than the other five genes 1–5 dpi. The transcript of *PBZ1* was detected at maximum level 1–2 dpi, and decreased thereafter. No detectable levels of *C52907* and *S11222* transcripts were found, and *R1744* did not respond to PBZ although constitutively low levels of the transcript were observed. It is notable that five PBZ-responsive *POX* genes belong to types 1 and 3, whose expression was rapid and superior in incompatible hosts compared with compatible hosts (see Fig. 2).

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*Response to treatment with defense signals*

In higher plants, jasmonic acid (JA), ethylene and salicylic acid (SA) are well known as defense-related signal compounds. Wounding induces an increment of JA (Creelman and Mullet 1997, Harms et al. 1995, Seo et al. 1995, Wasternack and Parthier 1997) and ethylene (Niki et al. 1998, Saltveit and Dilly 1978, Boller and Kende 1980). JA and ethylene have been reported to be involved in resistance to infection with necrotrophic pathogens (Vijayan et al. 1998, Knoester et al. 1999, Berrocal-Lobo et al. 2002). SA was also reported to be a defense-related signal compound for systemic acquired resistance to pathogen infection (Ryals et al. 1995, Shulaev et al. 1995). Then, the responses of the 10 *POX* genes to JA, SA and 1-aminocyclopropane-1-carboxylate (ACC), a precursor of ethylene, were examined to determine which defense compounds may affect the expression of the 10 genes (Fig. 6B). Spraying JA solution onto whole plants at the 8th leaf stage clearly induced the expression of six *POX* genes (*S14493*, *R2576*, *R2184*, *R2693*, *prxRPN* and *S11222*) at 1 d but not at 6 h. Treatment with SA and ACC slightly induced the expression of *S14493*, *R2184*, *R2693* and *prxRPN* genes, but the expression levels were not prominent compared with the treatment of JA. *S10927*, *R1744* and *PBZ1* were slightly expressed in healthy leaf blades, and no treatments tested here seemed to affect their expression levels after 1 d.

*Response to wounding*

Wounding induces the accumulation of JA, and the exogenous application of JA induces the expression of wound-inducible genes in higher plants (Farmer and Ryan 1992, Seo et al. 1995), including rice plants (Rakwal et al. 2002). Since expression of six *POX* genes was induced by JA treatment (Fig. 6B), we studied the response of the 10 *POX* genes to wounding (Fig. 6C). For wound treatment, we made nine holes per $cm^2$ of leaf using needles, and this induced the transient expression of three *POX* genes (*S14493*, *R2576* and *R2329*) in 6–12 h. Similar expression profiles of *R2184*, *R2693* and *S14493* were found while the levels of *S14493* were lower. *prxRPN* transcript accumulated at similar levels to that of *R2329* but was retained longer, to 3 d. *S10927* and *R1744* transcripts increased by 6 h and were kept for a longer time period, although the levels were lower. The *C52903*, *S11222* and *PBZ1* genes did not respond to wounding.

## Discussion

When we searched for homologous genes using the R2576 amino acid sequence as a probe, 100 genes among 28,469 rice cDNA clones (Kikuchi et al. 2003) were found as putative *POX* genes. Similarly, at least 73 *POX* genes were found in *Arabidopsis* (Tognolli et al. 2002, Welinder et al. 2002). What does the presence of such a large *POX* gene family in plants mean? Our data on 22 rice *POX* genes showed that organ-specific and stress-induced expression profiles of each *POX* gene were very characteristic (Fig. 2, 5, 6 in this paper, and Hiraga et al. 2000a). However, when we observed restricted responses such as to blast fungus infection and treatment with several chemicals, some *POX* genes exhibited a similar response. Considering these characteristics, we classified the 10 fungus-responsive *POX* genes as illustrated in Fig. 7. Among the 10 genes, seven are PBZ inducible, six are JA inducible and six are wound inducible, indicating all 10 genes except *C52903* were responsive to multiple stresses. These results suggest that the 10 *POX* genes were induced via complex signaling pathways. Interestingly, three *POX* genes, *S14493*, *R2576* (type 1, see Fig. 2B) and *prxRPN* (type 3), responded to all four treatments, belonging to G3 non-exceptionally (Fig. 4). However, the stress-induced expression profiles of the three genes were not similar, and furthermore, their organ-specific expression profiles were considerably different (Fig. 5). From these results, we could say that the three *POX* genes in G3 respond to the same stress at the same time in different organs in different manners. On the other hand, *R2184*, which is located on chromosome 4, and *R2693* on chromosome 5 belong to type 3 and G2, and were similarly expressed not only by the treatment with both PBZ and JA, but also in various organs. These diverse and/or redundant expression patterns indicate an indispensable function of each rice POX for resistance to environmental stresses. Among the 22 *POX* genes that we examined here, 12 genes were not responsive to the pathogen while they were expressed constitutively in juvenile plants (Hiraga et al. 2000a), suggesting that they may contribute to cellular homeostasis and development.

JA is a wound signal compound in rice plants (Rakwal et al. 2002) as well as in a broad range of higher plants. The level of JA is increased in incompatible interactions of pathogen and host plant such as in *Alternaria brassicicola*-infected *Arabidopsis* (Penninckx et al. 1996) and in tobacco mosaic virus-infected tobacco (Seo et al. 2001). However, infection of rice plant with *M. grisea* did not induce enhanced accumulation of JA in a compatible host (Schweizer et al. 1997). Because the evidence on JA accumulation has not been reported in an incompatible rice cultivar to our knowledge, the contribution of JA to the resistance to pathogen infection in rice plants is unclear now. As shown in Fig. 2, 5B, JA-inducible *POX* genes responded to infection with rice blast fungus. Thus, we could not rule out the role of JA in resistance to fungal attack. At least, JA contributes to the induction of basic type *PR* genes (Niki et al. 1998). Among the 10 pathogen-responsive rice *POX* genes, six genes in types 1, 3 and 4 clearly responded to JA, but not SA or ACC (Fig. 6B). Among the six wound-inducible *POX* genes, *R2329*, *S10927* and *R1744* hardly responded to JA treatment (Fig. 6B, C). Wound-inducible genes were not necessarily JA-inducible genes in *Arabidopsis* (Titarenko et al. 1997) and tobacco (Sasaki et al. 2002a). Our results also showed that the expression of wound-inducible genes such as *R2329*, *S10927* and *R1744* in rice was not induced by JA, and inversely JA-inducible genes such as *R2184*, *R2693* and *S11222* did not

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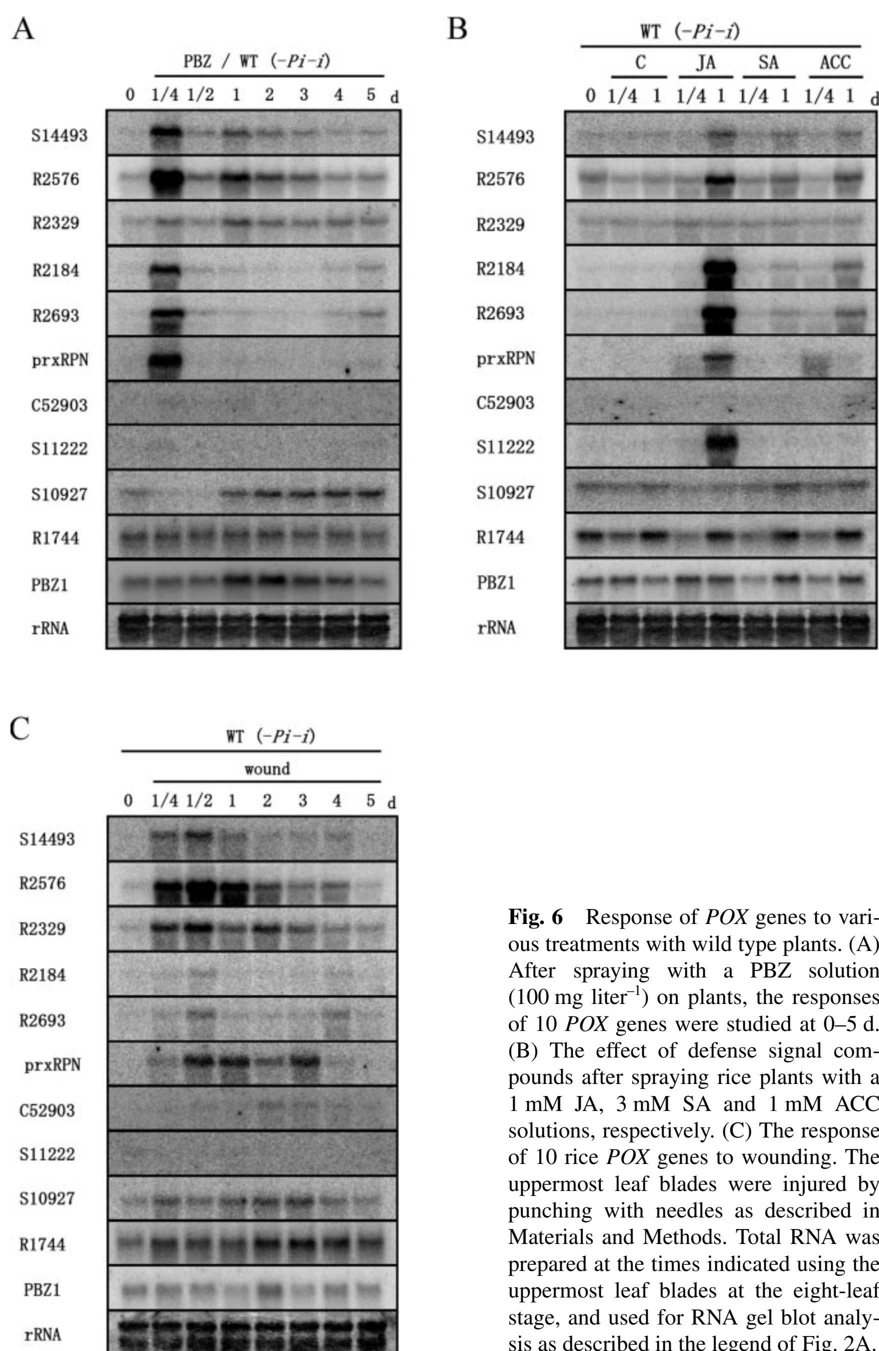


**Fig. 6** Response of *POX* genes to various treatments with wild type plants. (A) After spraying with a PBZ solution (100 mg liter$^{-1}$) on plants, the responses of 10 *POX* genes were studied at 0–5 d. (B) The effect of defense signal compounds after spraying rice plants with a 1 mM JA, 3 mM SA and 1 mM ACC solutions, respectively. (C) The response of 10 rice *POX* genes to wounding. The uppermost leaf blades were injured by punching with needles as described in Materials and Methods. Total RNA was prepared at the times indicated using the uppermost leaf blades at the eight-leaf stage, and used for RNA gel blot analysis as described in the legend of Fig. 2A.

respond to wounding. These results show the presence of JA-independent wound-signaling pathways.

It is a well-known phenomenon that high levels of SA such as over 10 μg (g fresh weight)$^{-1}$ accumulates in the 6th leaf of 35-day-old healthy rice plants, which is 200 times higher than that in healthy tobacco and *Arabidopsis* leaves (Silverman et al. 1995). Furthermore, the level of SA in rice plants remains almost the same after inoculation with rice blast fungus (Silverman et al. 1995). In contrast, treatment with PBZ reportedly stimulated *NPR1*-mediated defense signaling in *Arabidopsis*, which works downstream of SA (Yoshioka et al. 2001). Rice plants overexpressing *Arabidopsis NPR1* were resistant to infection by *X. oryzae* pv. *oryzae*, a bacterial leaf blight (Chern et al. 2001). *S14493*, *R2576*, *R2184*, *R2693* and *prxRPN*, whose expression was induced by both fungal infection and PBZ treatment (Fig. 6A), did not respond to the treatment with SA (Fig. 6B), suggesting the presence of SA-independent signaling pathways for the expression of these *POX* genes.

The rice genome contains a variety of *POX* genes, and 100 or more are temporally, spatially and redundantly expressed.

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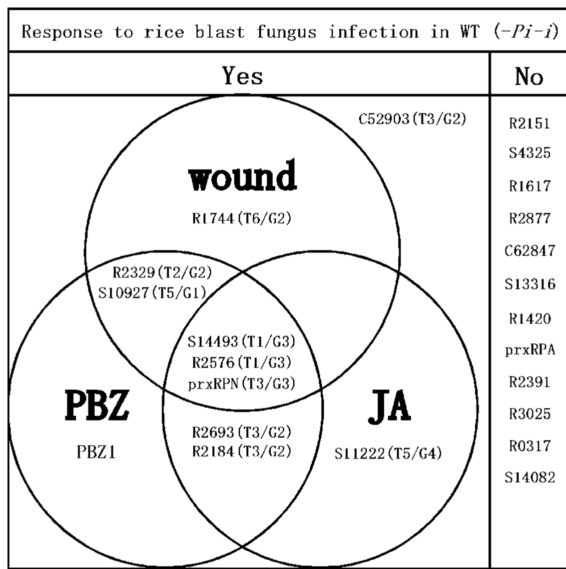


**Fig. 7** Classification of *POX* genes according to their response to various treatments. Twenty-two *POX* genes were classified first by inducibility after inoculation with rice blast fungus in wild-type plants as seen in Fig. 2. Next, 10 *POX* genes that responded to fungal inoculation in the leaves were classified by their responses to treatments with PBZ, JA and/or wounding seen in Fig. 6. T1–T6 and G1–G4 indicate the classified type (T) and group (G) in Fig. 2, 4, respectively.

The function of individual POXs may differ from each other, as suggested by their organ-specific or characteristic stress-responsive expression profiles seen in this study. The redundant expression after stress treatments indicated the necessity of POX activities for plant self-defense against pathogen attack and exposure to environmental stresses. We showed here the various expression profiles of rice *POX* genes. However, information is limited on the characteristics and roles of rice *POX* enzymes. For a better understanding of the roles of individual POXs, experiments using transgenic plants with suppression or enhancement of expression of each *POX* gene would be necessary. At the same time, studies on the localization of each *POX* gene product in organs, tissues and cells would be important, as well as the substrate specificity of each POX enzyme.

## Materials and Methods

*Plant materials*

Rice plants (*Olyza sativa* L. cv. Nipponbare) and the isogenic line (IL-7) carrying the resistance gene *Pi-i* against *M. grisea* race 003 (Kyu89-241) (Yamada et al. 1976) were grown for 6 weeks in a greenhouse (25°C). Six-week-old adult plants (eight-leaf stage) were mainly used as material except for Fig. 5.

*Inoculation with rice blast fungus*

*M. grisea* race 003 (Yamada et al. 1976) was grown on an oatmeal medium (Difco) for two weeks at 26°C in the dark, and then, spore formation was induced under a 20 W BLB light (FL20S.BLB; Toshiba, Tokyo, Japan) for 2–3 d at 24°C. Spore suspension ($3\times10^5$ conidia $ml^{-1}$) containing 0.05% Tween-20 was inoculated onto rice plants by spraying. The sprayed plants were incubated at 25°C with high humidity in the dark for 20 h, and then moved to a greenhouse (25°C). The uppermost leaf blades of the plants were harvested and used for RNA extraction and the measurement of the lesion size at the indicated time points.

*Chemical treatment and mechanical wounding*

For the treatments with chemicals, aliquots of PBZ solution (100 mg $liter^{-1}$), 1 mM JA, 3 mM SA and 1 mM ACC (pH 7.0) solutions containing 0.05% Tween-20 were sprayed onto whole rice plants at the eight-leaf (adult) stage, and the plants were subsequently maintained in a greenhouse (25°C). For wound treatment, the uppermost leaf blades of eight-leaf stage plants were injured by producing nine dots per $cm^2$ with needles (0.5 mm in diameter), after which the plants were incubated in a greenhouse (25°C).

*RNA gel blot analysis*

Total RNA isolated by the ATA method (Nagy et al. 1988) was applied to a 1.2% agarose gel containing formaldehyde at 20 µg per lane. After electrophoresis, the RNA was blotted onto Hybond-N nylon membranes (Amersham, Little Chalfont, U.K.). The 3′ UTR of each rice *POX* gene (Hiraga et al. 2000a) was $^{32}P$-labelled to produce specific probes using the Rediprime II DNA Labelling System (Amersham Biosciences). The membranes were hybridized with probes for each rice *POX* gene at 42°C for 16 h in 50 mM Tris–HCl (pH 7.5) buffer, containing 1 mM EDTA, 1× Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% BSA), 0.5% SDS, 3× SSC (1× SSC: 15 mM sodium citrate and 150 mM NaCl), 50% deionized formamide and 0.1 mg $ml^{-1}$ denatured salmon sperm DNA. The membranes were washed three times for 10 min at room temperature with 2× SSC containing 0.1% SDS, and then three times for 20 min at 65°C with 0.1× SSC containing 0.1% SDS. The images were visualized and gene expression levels were expressed numerically using a PhosphorImager SI (Molecular Dynamics, Tokyo, Japan). Equal loading of RNA was confirmed by monitoring the levels of ribosomal RNA stained with methylene blue (Sambrook et al. 1989).

*Phylogenic tree*

The amino acid sequence homology between each POX and R1744 was determined by referring to Welinder et al. (2002). These sequences were aligned by Genetyx version 6 using the coding region between the eight conserved cysteine residues from C1 to C8, which contribute to the POX structure. A phylogenic tree was produced in accordance with the alignment of the POX sequences using neighbor-joining method (Saitou and Nei 1987), and the phylogenic relationship was estimated for a bootstrap trial value of 10,000.

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