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ORIGINAL PAPER

Characterization of two rice peroxidase promoters that respond to blast fungus-infection

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Abstract Peroxidase (*POX*) genes consist of a large gene family possibly contributing to self-defense, however constitutive and stress-induced expression patterns of individual gene were poorly understood in rice. We studied here the characteristic expression of two representative rice *POX* genes, *R2329* and *R2184*, which are blast fungus-inducible (Sasaki et al. in Plant Cell Physiol 45:1442–1452, 2004). Basal GUS activity in *R2329* promoter::*GUS* rice plants was 100-fold higher than that in *R2184* promoter::*GUS* plants, and these levels reflected the transcript levels monitored by quantitative real-time RT-PCR. *R2329* promoter was activated by blast fungus-infection and wounding, and *R2184* promoter was activated by the fungal-infection and methyl jasmonate (MeJA)-treatment. By histochemical GUS staining analysis, constitutive *R2329* and *R2184* expression was commonly found in vascular bundle and exodermis in leaves and roots, while the precise expression profile was characteristic. In blast fungus inoculated *R2329* promoter::*GUS* leaves, GUS staining was induced just around fungus-induced local lesions. Analysis of the 5′ deleted promoters suggests the presence of many kinds of stress-responsive elements in the regions between −1798 and −748 of *R2329* promoter and between −1975 and −548 of *R2184* promoter. These results revealed the stress-responsive characteristics of *R2329* and *R2184* promoters, and indicated the possible use for generation of useful transgenic plants.

Keywords Rice · Peroxidase · Promoter assay · Rice blast fungus · GUS · Histochemistry

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Introduction

Characteristics of many abiotic stress-responsive promoters have been analyzed in monocotyledonous plants. However, the reports on blast fungus infection-inducible promoters are limited to the preliminary studies on rice lipid transfer protein (LTP, Guiderdoni et al. 2002), wheat puroindoline-a gene (Evrard et al. 2007) and maize *PR* (pathogenesis-related) genes (Moreno et al. 2005). To understand the response of rice defensive genes to biotic and abiotic stresses, we used blast fungus-induced peroxidase genes as the representatives, and studied in more detail the precise characteristics of these promoters including the histochemical

and fluorometric analysis of the *GUS* reporter gene in transgenic rice plants carrying the introduced 2-kb and the deleted promoters.

Class III plant peroxidase (POX; EC 1.11.1.7) genes consist of a large family in plants; 138 members in rice (Passardi et al. 2004a) and 73 members in the *Arabidopsis* genome (Welinder et al. 2002). POXs, which are induced by infection with pathogens, belong to pathogenesis-related (PR) protein 9 family, indicating defense-related proteins (van Loon et al. 1994). Indeed, POX activity or *POX* gene expression in higher plants were reportedly induced by fungi (Sasaki et al. 2004; Thordal-Christensen et al. 1992), bacteria (Young et al. 1995), viruses (Hiraga et al. 2000) and viroids (Vera et al. 1993). POX catalyzes the oxidoreduction of various substrates with hydrogen peroxide (for review see Hiraga et al. 2001; Passardi et al. 2004b), possibly conferring resistance to both biotic and abiotic stresses through lignification and suberization (Dean and Kolattukudy 1976; Quiroga et al. 2000), cross-linking of cell wall proteins (Showalter 1993), xylem wall thickening (Hilaire et al. 2001), generation of reactive oxygen species (Bolwell et al. 1995; Wojtaszek 1997), 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 (Lagrimini et al. 1997).

POXs are known to have similar substrate specificity each other (for review, Hiraga et al. 2001; Passardi et al. 2004b) and contain the amino acid sequences, which carry sugar-conjugate. These characters make it difficult to distinguish each other and to get specific antibodies for each POX, causing a slow progress in the study on the characteristics of individual POX at protein levels. On the other hand, studies on the expression profiles of individual *POX* gene would give us the information on the characteristics of *POX* gene whose nature has been poorly reported especially in rice plants.

In previous studies, we analyzed the expression profiles of 22 rice *POX* genes, and found that ten genes among them responded to blast fungus-infection in both compatible and incompatible interactions (Sasaki et al. 2004). From the gene expression profiles after blast fungus-inoculation and some treatments, the ten *POX* genes were classified into six types. Among the ten *POX*s, *R2329* and *R2184* were induced rapidly in both compatible and incompatible rice-blast fungus interaction (Sasaki et al. 2004). Then, we selected *R2329* and *R2184* as the representative rice *POX* genes, and studied here on their characteristics such as tissue specific expression profiles and the effect of 5′-deletion on the promoter activities. As materials, we produced transgenic rice plants carrying *POX* promoter::*GUS*-fusion genes, and monitored the GUS reporter activity. Histochemical and quantitative GUS analysis of these plants showed the characteristics of the *R2329* promoter are defense-related, and only partly similar to those of *R2184* promoter. Further, we indicate that the biotic or abiotic stress-induced expression is conferred by the nature of the 5′ flanking regions (−1798 to −748 of *R2329* promoter and −1975 to −548 of *R2184* promoter), that contain many kinds of potential stress-responsive *cis*-elements.

Materials and methods

Plant materials

Rice plants (*Oryza sativa* L. cv. Nipponbare and Chiyohonami), and IL7, which is a near isogenic line of Nipponbare containing the *R* gene *Pi-i* against blast fungus (*Magnaporthe oryzae*) race 003 (isolate, Kyu89-241; Yamada et al. 1976), or transgenic rice plants carrying *POX* promoter::*GUS*-fusion genes were grown for 3–6 weeks in a greenhouse (25°C). Rice genomic DNA was prepared from young leaf blades of Nipponbare according to the method of Murray and Thompson (1980) for isolation of the *POX* promoters by genomic PCR.

Chemical treatment and mechanical wounding

For GUS assays of the MeJA treatment, 1 cm cut leaf blades of transgenic rice plants (adult stage) were floated on an aliquot of 50 μM MeJA (pH 7.0) solution containing 0.02% ethanol, and the cut leaf blades were subsequently incubated in a greenhouse (25°C) for 48 h. Solution of 0.02% ethanol was used as a control for MeJA treatment. For wound treatment, the uppermost leaf blades were cut by a pair of scissors (into 1 cm length sections), and the wounded leaf blades were incubated on water for the indicated time periods.

For quantitative real-time RT-PCR (qRT-PCR) analysis, a pot with 12 seedlings was put in an air-tight clear plastic box, and a cotton pad with MeJA was put at the corner of the box at 100 μM concentration. After incubation for 24 h, the 4th leaves were used for RNA extraction.

Plasmid construction

pBI221 (Clontech) was digested with *Xba*I/*Sac*I to obtain a fragment with the *GUS* coding region, and the *GUS* fragment was introduced into the corresponding sites of pIG121-Hm (Ohta et al. 1990) to generate the pIG*35S-GUS* plasmid (Fig. 1A). The pIG*35S-GUS* plasmid was used for the generation of the binary vectors used in this paper.

Promoter sequences of the *POX* genes were obtained from KOME (Knowledge-based Oryza Molecular biological Encyclopedia; http://cdna01.dna.affrc.go.jp/cDNA/) and RGP (Rice Genome research Program; http://rgp.dna.affrc.

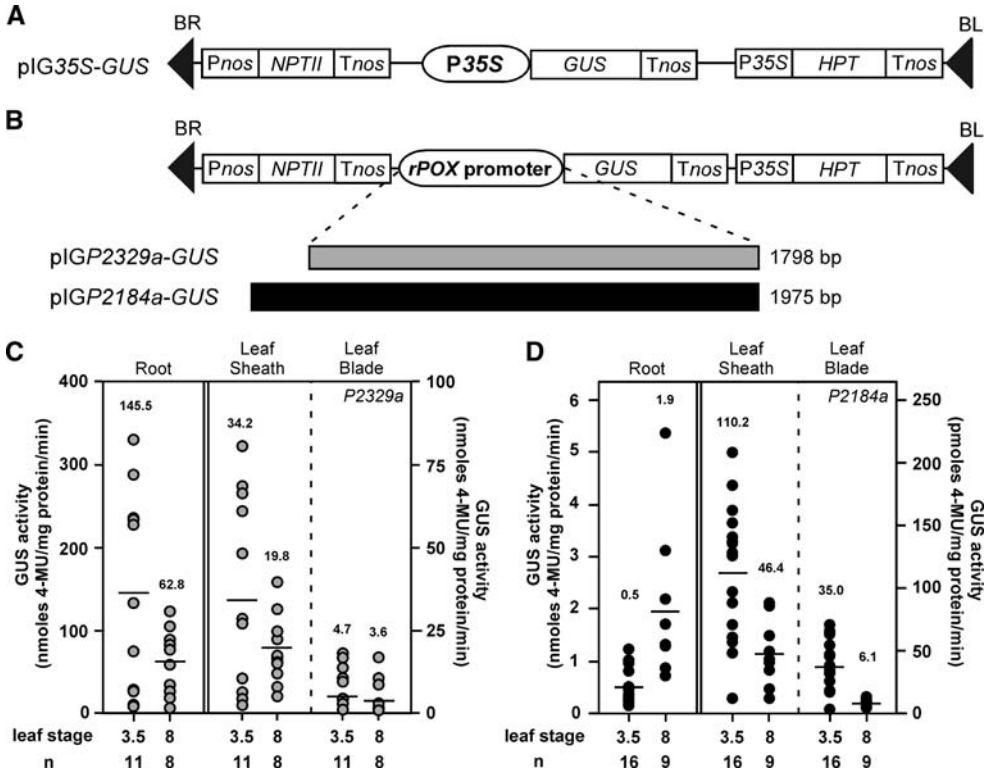

Fig. 1 The generation of transgenic plants carrying *R2329* or *R2184* promoter::*GUS*-fusion genes. **A** A binary vector, pIG*35S-GUS*, was used as the base for the construction of *POX* promoter::*GUS* plasmids. **B** Promoters of *R2329* (1,798 bp) and *R2184* (1,975 bp) were inserted into pIG*35S-GUS* instead of the *35S* promoter to generate plasmids pIG*P2329a-GUS* and pIG*P2184a-GUS*. **C**, **D** Organ specific GUS activity in two *POX* promoter::*GUS* transgenic rice plants. GUS activity in roots, leaf sheaths and leaf blades were measured in 3.5- and 8-leaf stages using 8–16 independent transgenic rice plants carrying **A** pIG*P2329a-GUS* (*gray circles*) or **B** pIG*P2184a-GUS* (*black circles*). The *circles* indicate the value of GUS activity from independent lines. *n* Means the number of transgenic plants used. The average values of the GUS activities in transgenic plants are shown by *horizontal bars* and the *numbers above the columns*

go.jp/J/toppage.html). The promoter region of *R2329* was amplified with primer sets R2329FP1H3 (5′-CCCAAGC TTGGCAGGTGCTACGTACTGTACTAGG-3′) and R2329RP1Xba (5′-GCTCTAGAGAAGAGGCACTTCAT GGCC-3′) using the rice genomic DNA (cv. Nipponbare) as a template for PCR. The amplified fragment was introduced into the *Eco*RV site of pBluescript SKII (Stratagene) to generate plasmid pSK*P2329*. After checking the sequence, the pSK*P2329* plasmid was digested with *Hin*dIII/*Xba*I and the promoter fragment exchanged into the corresponding sites of pIG*35S-GUS* to generate pIG*P2329a-GUS*. pIG*P2329c-GUS* and pIG*P2329e-GUS* plasmids were prepared in a similar way to pIG*P2329a-GUS* using products amplified by primer sets R2329H3-300 (5′-CCCAAGCTTTCCTTACCCGTCATGTAGTAATG GG-3′) and R2329H3-150 (5′-CCCAAGCTTATTTC TAGTGTAAATCCACTCCTTTTTC-3′) as forward primers and R2329RP1Xba as the reverse primer, respectively.

The promoter region of *R2184* was amplified with primer sets R2184FP2NAE (5′-TTTCGCCGGCCTTTG CCGATTTGATTGGAGGTCATAG-3′) and R2184RP1BH (5′-CGGGATCCCAGTAGCACAACCATACCCAGCTT GG-3′) using the rice genome (Nipponbare) as a template for PCR. The amplified fragment was introduced into the *Eco*RV site of pT7Blue (Novagen) to generate plasmid pT7B*P2184*. The pBI221 vector was digested with *Bam*HI/*Sac*I to obtain the *GUS* gene fragment, and the fragment was introduced into the corresponding sites of pT7B*P2184* to generate plasmid pT7B*P2184-GUS*. After checking the sequence, pT7B*P2184-GUS* was digested with *Xba*I/*Sac*I and exchanged with the corresponding sites of pIG*35S-GUS* to generate plasmid pIG*P2184a-GUS*. pIG*P2184c-GUS* and pIG*P2184e-GUS* plasmids were prepared in a similar way to the pIG*P2184a-GUS* using products amplified by primer sets R2184H3-300 (5′-CCCAAGCTTGCC TTTGATGTAGGCATATGCC-3′) and R2184H3-150 (5′-CCCAAGCTTGTAGTAGGAGTATACATTATATTC CAC-3′) as forward primers, and R2184RP1BH as the reverse primer, respectively.

Generation of transgenic plants

Agrobacterium tumefaciens EHA101 (a gift from Dr. Elizabeth Hood, VP Technology Prodigene) was transformed

with the constructed vectors as described by Holsters et al. (1978). The transformation of rice (*O. sativa* L. cv. Chiyohonami) was performed as described by Toki et al. (2006) or Hiei et al. (1994). Selection of each transgenic rice plant was conducted using 50 μg/ml hygromycin B.

For calli formation, T_0 seeds of *P2329a* and *P2184a* plants were incubated at 30°C on callus formation medium (N6 medium containing N6 vitamin; Chu 1978 and 2 mg/L 2, 4-D) for 1 week.

Inoculation with pathogen

Magnaporthe grisea race 003 (Kyu89-241) (Yamada et al. 1976) was grown on an oatmeal medium (DIFCO) for 2 weeks at 26°C in the dark, and then, spore formation was induced under a 20 W BLB light (FL20S.BLB: TOSHIBA, Japan) for 2–3 days at 24°C. Spore suspension (3×10^5 conidia/ml) containing 0.05% Tween-20 was sprayed on transgenic rice plants. Transgenic plants were derived from Chiyohonami, which is a compatible host against *M. grisea* race 003. 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 the measurement of GUS activity at 5 days post inoculation (dpi), and performed GUS staining at 3 or 5 dpi.

For infection with *Xanthomonas oryzae pv. oryzae*, the 4th leaves of rice seedlings cv. Nipponbare as a compatible host were infected by cutting the leaf top with scissors that had been dipped in a suspension containing 1×10^8 cfu/ml of *X. oryzae pv. oryzae*, strain T7174 (race I, MAFF 311018).

Assays of GUS activity

GUS activity was analyzed according to Kosugi et al. (1990) with some modifications. For quantitative analysis, plant tissues were homogenized in a GUS assay buffer (50 mM potassium phosphate, 10 mM EDTA, 0.1% Triton X-100, 0.1% Sarcosyl, 2 mM DTT, and 10 μg/ml cycloheximide), and an aliquot of the supernatant was incubated after 4-methylumbelliferyl-β-D-glucuronide (4-MUG) was added as the substrate at 37°C for 2 h. The amount of 4-methylumbelliferone (4-MU) formed by the GUS reaction was determined using an F-2500 fluorescence spectrophotometer (HITACHI). Protein concentrations were determined by a method described by Bradford (1976), using a Coomassie protein assay kit (Bio-Rad) with BSA as the standard.

Histochemical GUS analysis

GUS activity was analyzed histochemically according to Kosugi et al. (1990) with some modifications. Plant tissues were vacuum-infiltrated for 1 h in the GUS reaction mixture containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc), 50 mM potassium phosphate buffer (pH 7.0), 20% (v/v) methanol, 10 μg/ml cycloheximide, an inhibitor of protein synthesis, and 6 mM dithiothreitol and incubated at 37°C for 3–12 h (*P2329a* plants) or 1 day (*P2184a* plants). After the reaction was stopped by the addition of 70% ethanol, the pigments and chlorophylls were removed by repeated ethanol treatments. From the GUS staining of four independent lines each of *P2329a* and *P2184a* plants, representative samples are shown in Figs. 2, 3, and 4. Each tissue of *P2329a* and *P2184a* plants was cross-cut 80–100 μm in thickness by Plant Microtome MTH-1 (NK system, Japan).

Quantitative real-time PCR analysis

Total RNA of roots, flowers and leaf blades was prepared by TRIzol (Invitrogen), and cDNAs synthesized from the RNA using ReverTra Ace-α (TOYOBO) were used for the quantification of transcripts. The real-time PCR reaction was performed using iQ™ SYBR Green Supermix (BIO RAD), and the signals were detected on an iCYCLER (BIO RAD), according to the manufacturer's instructions. The sequence of each specific primer is described in Table S1. The real-time PCR reaction was normalized using the rice *actin* gene (AK060893) as an internal control. The results shown are mean values ± SD of at least three independent RNA samples.

Results

Isolation of *POX* promoters and generation of transgenic rice plants containing *POX* promoter::*GUS* genes

To study the characteristics of rice *POX* promoters, which respond to infection with rice blast fungus, we selected two representative genes with different expression profiles (Sasaki et al. 2004). One was *R2329* (AK099241), which was induced 6 h post inoculation (hpi) and the high transcript level was maintained for a longer time period in both compatible and incompatible interactions. *R2329* was responsive to wounding and treatments with probenazole, which is an agrochemical to protect against blast fungus-attack by activating plant self-defense, but not to treatment with JA. The other gene was *R2184* (AK102307), which was expressed transiently at 12–24 hpi in both compatible and incompatible interactions, and was responsive to probenazole and JA but not to wounding.

Promoter sequences of the *POX* genes were obtained from KOME and RGP. The 1,798 bp *R2329* and 1,975 bp *R2184* promoters were amplified by genomic PCR, and designated as *P2329a* and *P2184a*, respectively (Fig. 1B).

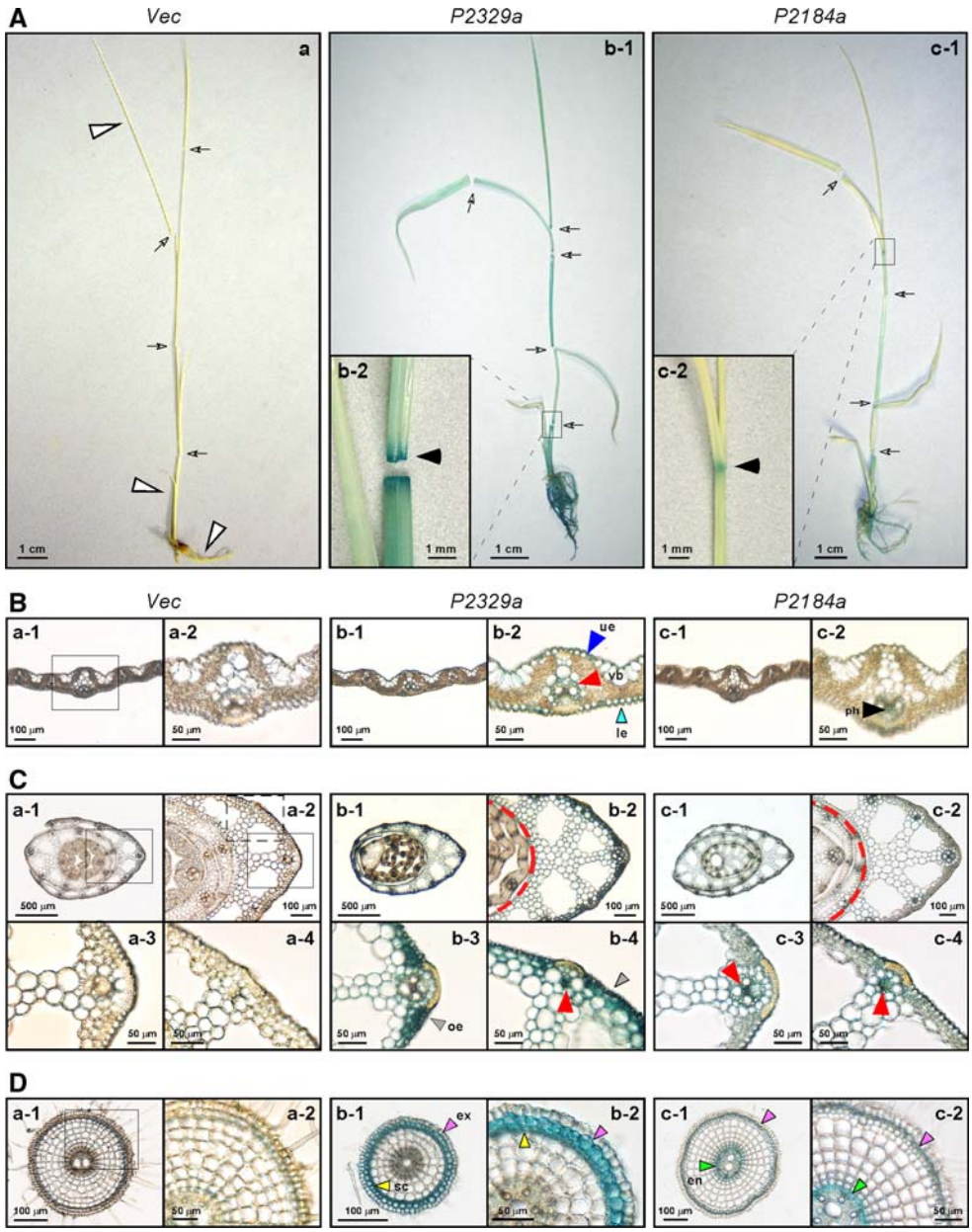

Fig. 2 GUS staining assay of whole Vec, *P2329a* and *P2184a* plants. Three-week-old Vec (*A-a*), which is a negative control, *P2329a* (*A-b-1*, *A-b-2*) or *P2184a* (*A-c-1*, *A-c-2*) plants were cut into four to five pieces (*small open arrow*) and subjected to GUS staining for 24, 8 and 24 h, respectively. *A-b-2* Wounded surfaces of *P2329a* plants were stained (*black arrowhead*). *A-c-2* Lamina joints of *P2184a* plants were stained (*black arrowhead*). Four independent 3-week-old transgenic plants are subjected to GUS staining. Representative staining images of the *P2329a* and *P2184a* plants are shown. Histochemical GUS analysis of Vec, *P2329a* and *P2184a* plants. *B-a-1*, *B-a-2* Cross-section of leaf blades of Vec plants. *B-b-1*, *B-b-2* Cross-section of leaf blades of *P2329a* plants. *B-c-1*, *B-c-2* Cross-section of leaf blades of *P2184a* plants. Inner regions (a *square* as shown in *B-a-1*) of *B-a-1*, *B-b-1* and *B-c-1* were magnified and shown in *B-a-2*, *B-b-2* and *B-c-2*, respectively. *C-a-1* to *C-a-4* Cross-section of leaf sheathes of Vec plants. *C-b-1* to *C-b-4* Cross-section of leaf sheaths of *P2329a* plants. *C-c-1* to *C-c-4* Cross-section of leaf sheathes of *P2184a* plants. Inner regions (*squares* as in *C-a-1* and *C-a-2*) of *C-a-1*, *C-b-1*, *C-c-1*, *C-a-2*, *C-b-2* and *C-c-2* were magnified and shown in *C-a-2*, *C-b-2*, *C-c-2*, *C-a-3*, *C-b-3* and *C-c-3*, respectively. Inner regions (a *dotted square* as in *C-a-2*) of *C-a-2*, *C-b-2* and *C-c-2* were magnified and shown in *C-a-4*, *C-b-4* and *C-c-4*, respectively. *D-a-1*, *D-a-2* Cross-section of roots of Vec plants. *D-b-1*, *D-b-2* Cross-section of roots of *P2329a* plants. *D-c-1*, *D-c-2* Cross-section of roots of *P2184a* plants. Inner regions (a *square* as in *D-a-1*) of *D-a-1*, *D-b-1* and *D-c-1* were magnified and shown in *D-a-2*, *D-b-2* and *D-c-2*, respectively. After the GUS reaction was stopped, each tissue of Vec, *P2329a* and *P2184a* plants was cross-cut into 80–100 µm thick sections. Representative staining images of Vec, *P2329a* and *P2184a* plants are shown. *ue* Upper epidermis (*blue arrowhead*), *vb* vascular bundle (*red arrowhead*), *le* lower epidermis (*light-blue arrowhead*), *ph* phloem (*black arrowhead*), *oe* outer epidermis (*gray arrowhead*), *ex* exodermis (*purple arrowhead*), *sc* sclerenchyma (*yellow arrowhead*), *en* endodermis (*green arrowhead*)

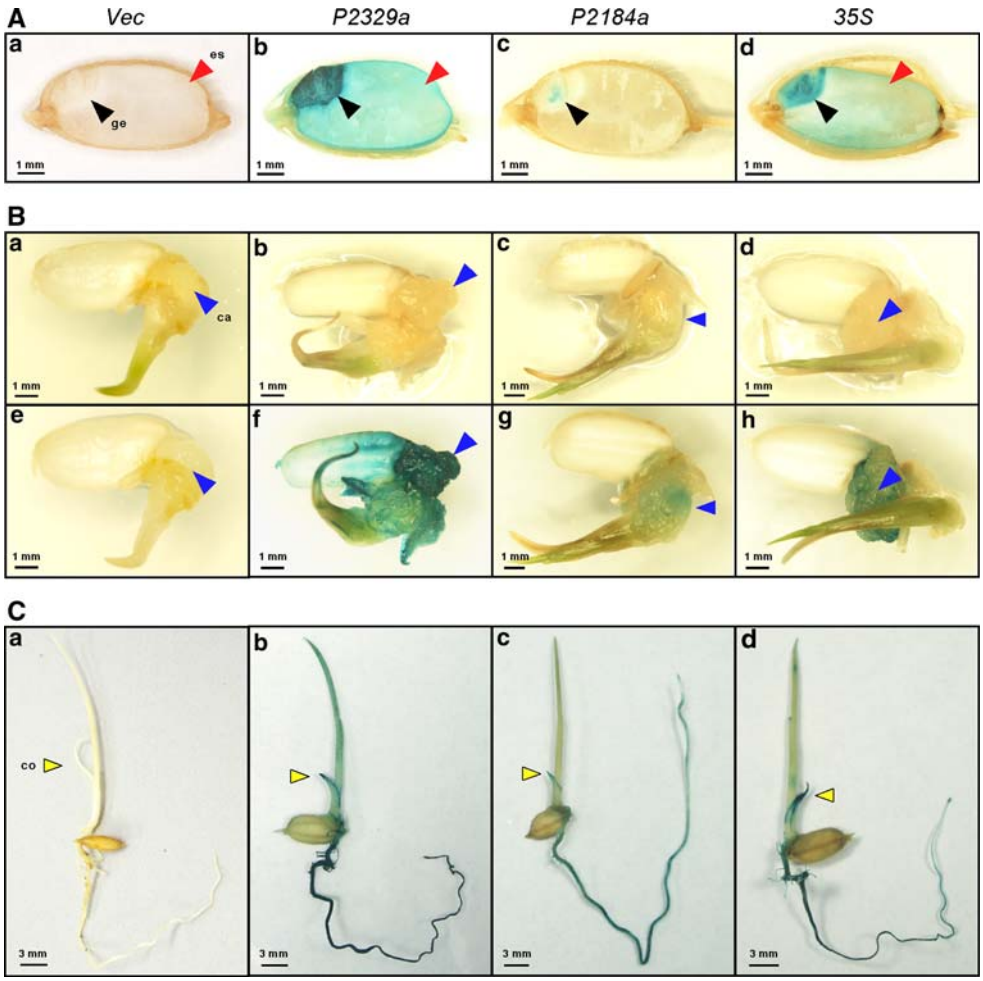

Fig. 3 GUS staining of seeds, calli and coleoptiles of two *POX* promoter::*GUS* transgenic plants. **A** The seeds of Vec, *P2329a*, *P2184a* and *35S* plants were incubated at 20°C for 3 days for water absorption, and subjected to GUS staining for 12, 3, 8 and 4 h, respectively. **B** The callus (*blue arrowhead*) was prepared from seeds of T_0 transgenic plants after generation on callus formation medium for 1 week at 30°C (*B-a* to *B-d*). The calli of Vec, *P2329a*, *P2184a* and *35S* plants were subjected to GUS staining for 12, 3, 12 and 4 h, respectively (*B-e* to *B-h*). **C** One-week-old seedlings of Vec, *P2329a*, *P2184a* and *35S* plants were subjected to GUS staining for 12, 3, 12 and 6 h, respectively. Representative GUS staining among three independent transgenic lines is shown in seeds (**A**) and seedlings (**C**). *ge* Germ (*black arrowhead*), *es* endosperm (*red arrowhead*), *ca* callus (*blue arrowhead*), *co* coleoptile (*yellow arrowhead*)

The sequence length of each promoter was calculated from the translation start point. The two *POX* promoters were introduced upstream of a *GUS* gene to prepare the binary vectors, pIG*P2329a-GUS* and pIG*P2184a-GUS*. The binary vectors containing the *GUS*-fusion genes were introduced into rice by an *Agrobacterium*-infection method. Regenerated hygromycin-resistant seedlings were grown in an isolated green house. The first generation of transformants (T_0) grew with normal phenotype and fertility. After confirmation of transgene insertion by genomic PCR in T_0, T_1 transgenic plants were prepared by self-pollination, and used for GUS activity assays. In this paper, we mainly used the self-pollinated T_1 transgenic plants after checking the hygromycin B-resistance. T_1 transgenic plants carrying *P2329a*:: or *P2184a*::*GUS*-fusion genes were designated as *P2329a* or *P2184a* plants, respectively.

Analysis of GUS activity in various organs in healthy transgenic rice plants

In healthy *P2329a* plants, GUS activity in roots was considerably high (Fig. 1C), confirming the previous result by RNA gel blot analysis (Sasaki et al. 2004). The GUS activity in roots and leaf sheaths was stronger in young plants at the 3.5-leaf stage than in adult plants at the 8-leaf stage. In *P2184a* plants, the highest GUS activity was also found in roots compared with other organs (Fig. 1D), and it was higher in adult plants than young plants. Conversely, the GUS activity in leaf sheaths and leaf blades of *P2184a* plants was stronger in young plants than adult plants. The levels of GUS activity in both *P2329a* and *P2184a* plants were high in roots, intermediate in leaf sheaths and low in leaf blades.

Organ and tissue specific GUS expression in healthy *P2329a* and *P2184a* plants

Four individual lines of negative control plants carrying a vector without *GUS* gene (Vec plant), *P2329a* and *P2184a* plants at the 3.5-leaf stage were subjected to GUS staining analysis using whole plants, and representative results are shown in Fig. 2. After cutting into 4–5 pieces at the indicated points (small open arrows in Fig. 2A-a, A-b-1, A-c-1), they were immediately incubated in GUS staining solution. Vec plant did not stained at all (Fig. 2A-a), while the blue

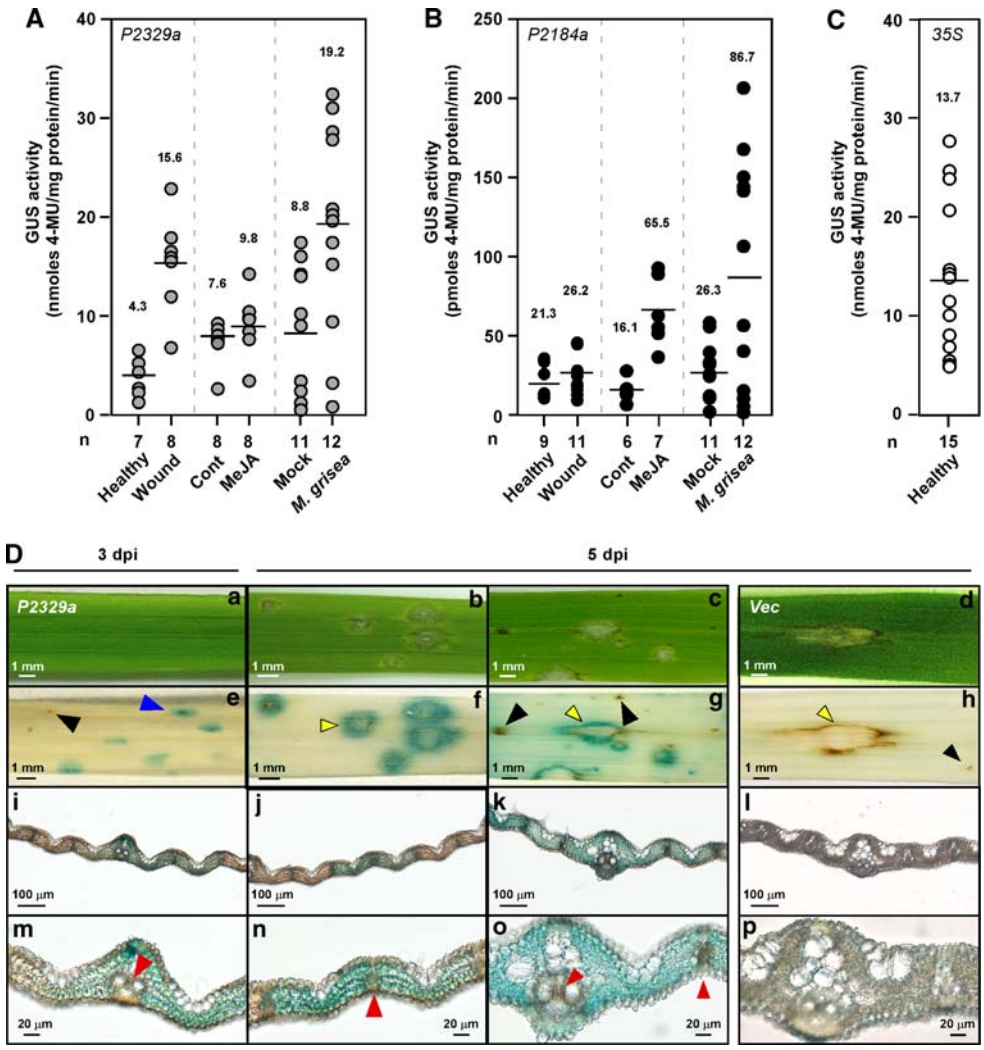

Fig. 4 Induced GUS activity in leaf blades of *P2329a* and *P2184a* plants. GUS activity in the leaf blades of *R2329* (**A**), *R2184* (**B**) or *35S* (**C**) plants was measured 2 days after wounding, floating on buffer solution as a control treatment for MeJA (cont) or on 50 μM MeJA, and mock inoculation with buffer (mock) and infection with rice blast fungus at 5 dpi. Details of each treatment for the GUS assays are described in "Materials and Methods". For the analysis, 6–15 independent transgenic plants were used. *n* Means the number of transgenic plants. The average values of the GUS activities in transgenic plants are shown by *horizontal bars* and the *numbers of above the columns*. **D** GUS staining after infection with rice blast fungus. Photographs of leaf blades at 3 (*D-a*) and 5 dpi (*D-b*, *D-c*) in 3-week-old *P2329a* plants, and 5 dpi (*D-d*) in 3-week-old Vec plants after the infection with rice blast fungus. The leaf blades (*D-a* to *D-d*) were subjected to GUS staining and are shown in *D-e* to *D-h*, respectively. *D-i* to *D-p* Cross section of leaf blades derived from *D-e* to *D-h*. *D-i*, *D-j*, *D-m*, *D-n* were derived from *D-e* and stained for 6 h at 37°C. *D-k*, *D-o* were derived from *D-g* and stained for 3 h at 37°C. *D-l*, *D-p* were derived from *D-h* and stained for 12 h at 37°C

GUS signal was found throughout *P2329a* plants at high levels, and it was notable in roots and cross-cut surfaces (Fig. 2A-b-1, A-b-2). In *P2184a* plants, roots and lamina joints were stained strongly (Fig. 2A-c-1, A-c-2).

To study the tissue specific expression, we next performed the histochemical analysis using pre-stained T_1 transgenic plants shown in Fig. 2A. In cross-section of the leaf blades, where the position was shown in Fig. 2A-a (upper open arrowhead), GUS signal was observed in the upper epidermis (ue; blue arrowhead), vascular bundle (vb; red arrowhead) and lower epidermis (le; light blue arrowhead) in *P2329a* plants (Fig. 2B-b-1, B-b-2). In *P2184a* plants (Fig. 2B-c-1, B-c-2), GUS signal was found in phloem (ph; black arrowhead). In cross-sections of leaf sheathes, where the position was shown in Fig. 2A-a (middle open arrowhead), strong GUS staining was observed in whole parts, but predominantly in the outer epidermis (oe; gray arrowhead) and vascular bundle (red arrowhead) in *P2329a* plants (Fig. 2C-b-3, C-b-4) except for the inner most part of leaf sheathes (left side of red dotted line in Fig. 2C-b-2). In *P2184a* plants, GUS signal was observed weakly in whole parts and predominantly in vascular bundles (red arrowhead in Fig. 2C-c-3, C-c-4) except for the inner most part of leaf sheathes (left side of red dotted line

in Fig. 2C-c-2). In cross-sections of roots, where the position was shown in Fig. 2A-a (lower open arrowhead), GUS signal was observed in exodermis (ex; purple arrowhead) and sclerenchyma (sc; yellow arrowhead) in *P2329a* plants (Fig. 2D-b-1, D-b-2), and exodermis and endodermis (en; green arrowhead) in *P2184a* plants (Fig. 2D-c-1, D-c-2). The results of the GUS staining were correlated to those of the fluorometric measurements on different tissues under the control of the two promoters (Fig. 1C, D).

GUS expression in seeds, seedlings and calli of transgenic plants

In seeds of Vec plants, no GUS signal was found (Fig. 3A-a), but in that of *P2329a* plants, strong GUS signal was found in germ (ge; black arrowhead) and endosperm (es; red arrowhead) (Fig. 3A-b), but it was detected weakly in the endosperm of *P2184a* seeds (Fig. 3A-c). In *35S* promoter::*GUS* plants, strong GUS signal was also found in germ and endosperm (Fig. 3A-d), but it was weaker than the GUS signal in *P2329a* plants. In calli (ca; blue arrowhead), which were generated from the seeds of T_0 on callus formation medium (Fig. 3B-a to B-d), GUS signal was detected in *P2329a*, *P2184a* and *P35S* transgenic plants (blue arrowhead in Fig. 3B-f to B-h). In calli of Vec plants, no GUS staining was found (Fig. 3B-e). The GUS staining was strong in *P2329a* plants, intermediate in *P35S* plants and weak in *P2184a* plants. When whole 7-day-old seedling was subjected to GUS staining, the GUS signal was found strongly in roots and coleoptiles (co; yellow arrowhead in Fig. 3C-b to C-d), while, Vec plants have no GUS signal (Fig. 3C-a). The GUS staining pattern in *P2329a* (Fig. 3C-b) and *P2184a* plants (Fig. 3C-c) was very similar to that in 3-week-old transgenic plants (Fig. 2A-b-1, A-c-1). In *35S* promoter::*GUS* plants (positive control plants for the GUS staining), strong GUS signal was also found in roots and coleoptiles (Fig. 3C-d). For GUS staining of seeds and calli, ten independent materials of each transgenic plant were performed GUS staining, and representative results are shown in Fig. 3A, B.

Induced GUS activity by blast fungus-infection and wound- and MeJA-treatments in *P2329a* and *P2184a* plants

To evaluate whether *P2329a* and *P2194a* respond to some stresses, GUS activity in *P2329a* and *P2184a* plants was fluorometrically measured after infection with rice blast at 5 dpi, and 2 days after wounding and MeJA treatment, when the two genes certainly respond to the treatments (Sasaki et al. 2004). In *P2329a* plants, GUS activity was increased after wounding, but no clear increment was shown after the treatment with MeJA (Fig. 4A). Inoculation of a spore suspension of blast fungus clearly increased the GUS activity, while mock-inoculation did slightly. In *P2184a* plants, both fungal-infection and the treatment with MeJA increased the GUS activity, while no clear induction was found after wounding (Fig. 4B). These results were consistent with the results of previous RNA gel blot analysis (Sasaki et al. 2004), indicating the two *POX* promoters isolated are really activated under these stresses in transgenic rice plants. The levels of induced GUS activity in *P2329a* plants after wounding and fungal-infection were higher than that in *P35S* plants (Fig. 4A, C).

To analyze the GUS expression profiles precisely, the localization of induced GUS activity was studied using blast fungus-infected leaf blades. In the compatible pathogen-host interaction, disease symptoms of the rice blast fungus-infected 4th leaf blades of *P2329a* plants was not clear by the naked eye at 3 dpi (Fig. 4D-a), but whitish developed lesions were found at 5 dpi with mycelium development and spore formation (Fig. 4D-b, D-c). When the leaf pieces were subjected to GUS staining assay, blue GUS signal was clearly shown around the whitish developed lesions at 5 dpi (yellow arrowhead in Fig. 4D-f, D-g). No clear GUS staining was shown at or around the necrotic lesions in dark brown (black arrowhead in Fig. 4D-e, D-g), which resembles the resistant type lesions that were found in an incompatible interaction upon HR (hypersensitive reaction; Goodman and Novacky 1994). At 3 dpi, no detectable whitish developed lesions had been observed yet (Fig. 4D-a), but GUS staining was already visible at the indicated positions (blue arrowhead in Fig. 4D-e), which were supposed to develop whitish lesions 2 days later (yellow arrowhead). In the cross-section of the leaf blades at 3 and 5 dpi (Fig. 4D-e to D-g), broad GUS staining was observed in the infected areas except for vascular bundles (red arrowhead in Fig. 4D-m to D-o). The tendency of GUS staining in *P2329a* plants was almost the same in four independent transgenic plants. No detectable GUS staining was found in mock or infected leaf blades of *P2184a* plants (data not shown). The result of *P2184a* plants was understandable, because the level of GUS activity in infected *P2184a* plants was only less than 1/100 of that in *P2329a* plants at 5 dpi (Fig. 4B).

Quantitative real-time RT-PCR analysis of *R2329* and *R2184* expression in wild-type rice plants

Promoter activity of *R2329* was clearly higher than that of *R2184*, but it was not clear whether the difference was derived from the intact promoter activity or artificial promoter activity, which was caused by plasmid construction. Then, we quantified the transcript levels of *R2329* and *R2184* accumulated in wild-type rice plants by qRT-PCR (Fig. 5) using specific primers (Table S1), because the

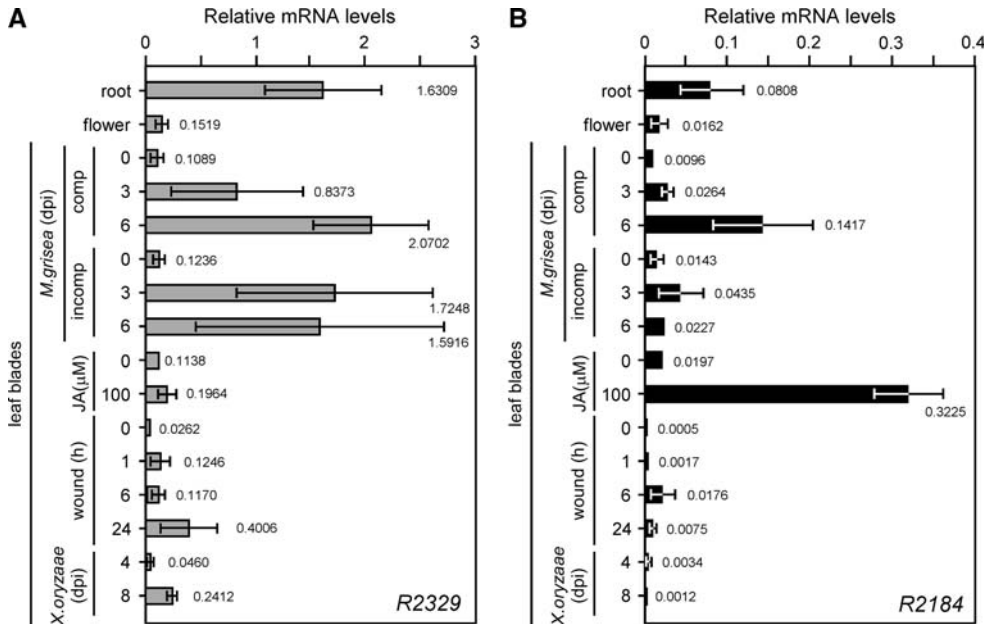

Fig. 5 Quantitative real-time RT-PCR of *R2329* and *R2184* genes. Transcript accumulation of *R2329* and *R2184* was analyzed by qRT-PCR in roots, flowers and leaf blades of wild-type Nipponbare. In leaf blades, qRT-PCR analysis was performed after infection with *M. grisea* race 003 in compatible (Nipponbare) and incompatible (IL7) hosts at 0, 3 and 6 dpi, treatment with or without 100 μM MeJA for 24 h, wounding at 0, 1, 6 and 24 h, and infection with *X. oryzae pv. oryzae* at 4 and 8 dpi. Specific primers for each gene are described in Table S1. The relative transcript levels were calculated by using the rice *actin* gene (accession; AK060893) as an internal control. The results shown are mean values ± SD of three independent analyses except for the infection with *M. grisea* (more than four analyses). The *numbers above the columns* indicate averages of the values of qRT-PCR

absolute level of each transcript could not be estimated by the results from RNA gel blot analysis (Sasaki et al. 2004). We determined the accumulated transcripts in root, flowers and upper leaf blades at the 4 to 5-leaf stage of healthy Nipponbare rice. Induced levels of transcripts were also determined in the leaf blades after infection with rice blast or *X. oryzae pv. oryzae*, the treatment with or without 100 μM MeJA and wounding. Accumulation of the *R2329* transcript was 20-fold higher in roots, 9-fold higher in flowers and more than 10-fold higher in healthy leaf blades. These results indicated that the transcript levels of *R2329* were clearly higher than those of *R2184* in consistent with the promoter activity (Figs. 1C, D, 4A, B).

The *R2329* transcript was high in roots and leaf blades after blast fungus-infection in both compatible and incompatible hosts (Fig. 5A), confirming the previous result of RNA gel blot analysis. The results in Fig. 5A confirmed the wound-induced expression of *R2329*, and indicated that the transcript level after the fungal-infection was tenfold higher than that after wounding. Furthermore, we found that the *R2329* transcript accumulated after the compatible infection with bacterial blight caused by *X. oryzae* at 8 dpi. In *R2184*, the accumulation of the transcript was constitutively high in roots. The transcript was increased in the leaf blades after the blast fungus-infection in compatible interaction and markedly by MeJA treatment (Fig. 5B).

Effect of 5′-deletions of *P2329a* and *P2184a* on their promoter activities

To analyze the characteristics of the promoters, successive 5′-promoter deletions were prepared and designated as *P2329a* (−1798), *P2329c* (−748) and *P2329e* (−156) (Fig. 6A), and *P2184a* (−1975), *P2184c* (−548) and *P2184e* (−172) (Fig. 6B). These promoter deletions were fused upstream of the *GUS* gene to produce binary vectors, and introduced into rice plants via the *Agrobacterium*-infection method. After the insertion of the transgenes was confirmed by genomic PCR, T_0 transgenic plants containing the *GUS*-fusion genes, designated as *P2329c*, *P2329e*, *P2184c* and *P2184e* plants, were subjected to GUS analysis together with *P2329a* and *P2184a* plants. The results clearly indicated that the important region responsive to both wounding and the fungal-infection was positioned in the region between −1798 and −748 in *P2329a* (Fig. 6C), and the region responsive to both MeJA treatment and the fungal-infection was present in the region between −1975 and −548 in *P2184a* (Fig. 6D).

Putative *cis*-regulatory elements in the two promoters were analyzed by signal scan search (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html) and representative stress responsive *cis*-elements are listed in Table 1. Both *R2329* (−1798/−748) and *R2184* (−1975/−548) promoters

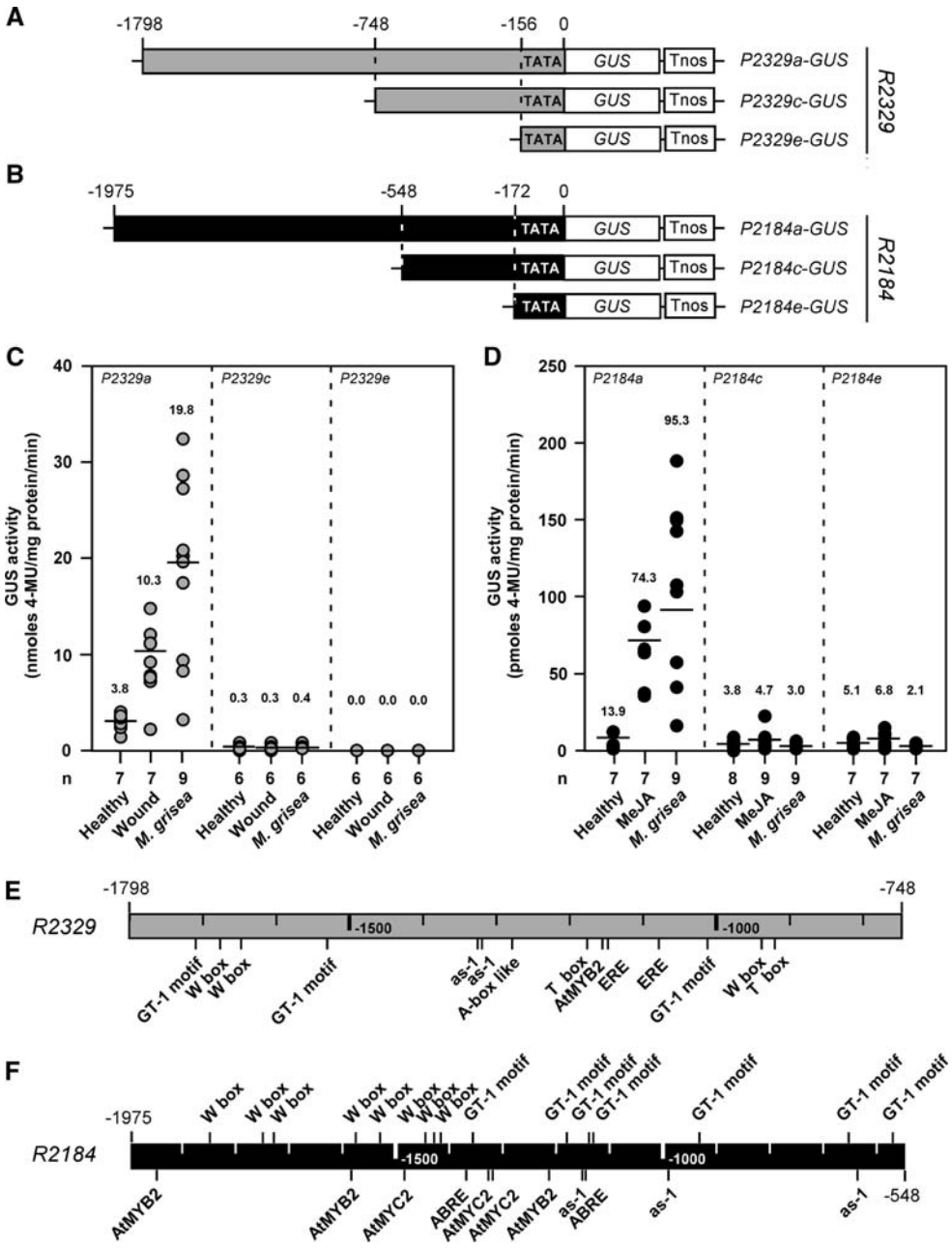

Fig. 6 Stress-inducible GUS activity in transgenic rice plants carrying 5′-deleted *R2329* or *R2184* promoters. **A** *GUS*-fusion gene constructs for analysis of the 5′-deleted *R2329* promoter. The promoters were designated as *P2329a* (−1798), *P2329c* (−748) and *P2329e* (−156), and fused to a *GUS* reporter gene. **B** *GUS*-fusion gene constructs for the analysis of 5′-deleted *R2184* promoter. The promoters were designated as *P2184a* (−1975), *P2184c* (−548) and *P2184e* (−172), and fused to a *GUS* reporter gene. **C** GUS activity of plants carrying 5′-deleted *R2329* promoters was measured before (healthy) and 2 days after wounding or infection with *M. grisea* at 5 dpi in leaf blades of transgenic plants. **D** GUS activity of plants carrying 5′-deleted *R2184* promoters was measured before (healthy) and 2 days after the treatment with 50 μM MeJA or infection with *M. grisea* at 5 dpi in leaf blades of transgenic plants. The *bars* and the *numbers of above the columns* indicate averages of the GUS activities of transgenic plants. Stress-responsive *cis*-elements in Table 1 were positioned on regulatory region of *R2329* (**E**) and *R2184* (**F**) promoters

contain *cis*-elements responsive to pathogen-infection, such as as-1 (activation sequence-1, Strompen et al. 1998) and GT-1 motif (Park et al. 2004), but ERE (ethylene responsive element, Itzhaki et al. 1994) and T-box (Allen et al. 1996) were found only in the *R2329* promoter, and abscisic acid (ABA)-responsive *cis*-elements, such as ABRE (ABA responsive element, Hattori et al. 1995), MYB and MYC recognition sequence (Abe et al. 2003), are present predominantly in *R2184* promoter. Interestingly, 18 and 14 *cis*-elements for expression in roots (Elmayan and Tepfer 1995), were found in *P2329a* and *P2184a*, respectively (Table 1), suggesting that the strong GUS activity in roots would be derived from these motifs.

Discussion

We characterized here the expression profiles of two rice *POX* genes, *R2329* and *R2184*, using transgenic rice plants with *POX* promoter::*GUS*-fusion genes. The activity of the reporter GUS was found constitutively in specific tissues and induced after various biotic and abiotic stress-treatments including infection with rice blast fungus and bacterial blight. The levels of GUS activity in transgenic rice plants containing *P2329a*::*GUS* or *P2184a*::*GUS* were at the understandable levels compared with the previous results by RNA gel blot analysis (Sasaki et al. 2004) and qRT-PCR analysis (Fig. 5), indicating the promoter::*GUS*

Table 1 *Cis*-elements in *R2329* (−1798/−748) and *R2184* (−1975/−548) promoters

Name of *cis*-element	Sequences of *cis*-element[a]	Number of element		Response or localization	Reference
		R2329	*R2184*		
ABRE	ACGTG	0	3	ABA	Hattori et al. (1995)
as-1	TGACG	2	3	Pathogen, SA	Strompen et al. (1998)
AtMYB2[b]	WAACCA	1	3	Dehydration	Abe et al. (2003)
AtMYC2[b]	CAYRTG	0	3	ABA	Abe et al. (2003)
ERE	ATTTCAAA	2	0	Ethylene	Itzhaki et al. (1994)
GT-1 motif	GAAAAA	4	8	Pathogen, salt	Park et al. (2004)
W box	TGACY	3	8	Wound	Nishiuchi et al. (2004)
Amy box[c]	TAACARA	2	3	Seed	Huang et al. (1990)
GARE[c]	TAACAAR	2	2	GA, seed	Ogawa et al. (2003)
RY repeat[c]	CATGCA	4	3	Seed	Monke et al. (2004)
root motif[c]	ATATT	18	14	Root	Elmayan and Tepfer (1995)
T-box	TTWTWTTWTT	2	0	S/MAR, express	Allen et al. (1996)

[a] W, R, Y and N means A or T, A or G, C or T and all nucleotides, respectively

[b] There is not a specific name of the *cis*-element, and a name of the transcription factor which recognizes the indicated sequence is shown

[c] Search was performed using full length promoter regions

analyzing system accurately reflected the characteristic expression of the *R2329* and *R2184* genes. The two promoters have both common and different characteristics. Analysis of basal and induced expression of both promoters indicated the importance of the two *POX* genes in self-defense systems to various stresses. The results obtained from the study are summarized in the following five points.

1. Basal GUS signal in both *P2329a* and *P2184a* plants was found predominantly in vascular bundles (Fig. 2B-b-2, C-b-4, C-c-3, C-c-4), exodermis or endodermis in roots (Fig. 2D-b-1, D-b-2, D-c-1, D-c-2). In *P2329a* plants, strong GUS staining was shown in the outer parts of the plant body such as epidermises (Fig. 2B-b-2, C-b-3, C-b-4). POXs are involved in lignification and suberization (Dean and Kolattukudy 1976; Quiroga et al. 2000), which would contribute to self-defense via structural reinforcement of plants upon exposure to biotic and abiotic stresses (for review see Collinge and Slusarenko 1987). Lignin and suberin were reported to accumulate in vascular bundles, exodermis and endodermis (for review see Hose et al. 2001; Ito et al. 2000), then constitutive expression of *R2329* and *R2184* in such tissues would be useful for preparing the defensive situation for prospected pathogen-attack or environmental stresses. In roots, casparian bands are composed of lignin and suberin localized in the primary walls of exodermis and endodermis, possibly participating in the resistance to various environmental stresses (for review see Hose et al. 2001). Recently, it has been shown that the blast fungus can infect roots of rice plants (Sesma and Osbourn 2004). Constitutive expression of *R2329* and *R2184* in roots would be of advantage to protect from such type of the infection.
2. Blast fungus-infection clearly induced GUS activity in both *P2329a* and *P2184a* plants when analyzed by a sensitive fluorometric method (Fig. 4A, B). By histochemical analysis, we found specific GUS staining around the expanding lesions in blast fungus-infected *P2329a* leaves (Fig. 4D). The staining was not found in leaf blades of *P2184a* plants, probably because of the lower activity of the promoter even after induced expression. In leaf blades of *P2329a* plants, no clear disease symptom was found at 3 dpi (Fig. 4D-a), but many GUS positive signals were detected (Fig. 4D-e). The GUS positive regions were possibly the sites of fungus-infection (Fig. 4D-e), because such signals were not found in mock-inoculated leaves and negative control plants (data not shown). The small lesions may develop to whitish enlarged lesions after two or more days (Fig. 4D-e, D-f), indicating that *P2329a* has already responded to blast fungus-invasion at very early infection stage. At 5 dpi, the GUS-stained areas were clearly detected around the expanding lesions, and the findings from cross-sections showed that the area stained by blue dye was distributed broadly to mesophyll tissue, which were surrounded by or neighbored on necrotic lesions (Fig. 4D-k to D-o). These characteristics of *P2329a* indicate a predicted role of the *R2329* gene for the defense against blast fungus-infection. The data shown in Fig. 5A also suggested the defensive role of *R2329* to infection with *X. oryzae* for bacterial blight disease.
3. Basal *P2329a* activity in various rice organs was clearly higher than the *P2184a* activity, for example 100-fold or more in healthy roots, leaf sheathes and leaf blades (Fig. 1C, D). A possible explanation would be the fact that *P2329a* contains two sets of S/MAR (scaffold/matrix-associated region)-characteristic T-box

(Table 1) and A-box (AATAAAYAAA)-like elements (Allen et al. 1996) (data not shown), while *P2184a* does not have these elements (Table 1). S/MAR regions reportedly confer 140-hold higher transgene expression (Allen et al. 1996), and would also be important for *P2329a* activity. The region around the T-boxes and A-box like elements in the *R2329* promoter (−985/−1371) share about 80% of A and T nucleotides (AT-rich region), and that is also characteristic of MARs (Slatter et al. 1991). The 4-kb promoter regions of both *R2329* and *R2184* contain four and one T-boxes, respectively (data not shown), but the *R2184* promoter does not contain A-box like element nor A + T rich region even in the 4-kb region. Then, the putative MARs on *P2329a* promoter may enhance the promoter activity (Figs. 1C, 4A, 6C) and expression level of endogenous *R2329* (Fig. 5A), while *R2184* gene may not be neighbored on MAR.

4. The kinds of stress-responsive *cis*-elements in *P2329a* are likely similar to that in *P2184a*, except for ethylene responsive EREs in *P2329a* and ABA-responsive elements in *P2184a* (Table 1, Fig. 6E, F). We think that not all *cis*-elements in *POX* promoters (Table 1) could function in stress responses, but some specific biotic or abiotic stress-responsive *cis*-elements would complexly function in rice plants. For example, the ERE in *P2329a* seems to be non-functional as an ethylene responsive element, because the *R2329* transcript does not respond to the treatment with ethylene (Sasaki et al. 2004). Also, *P2329a* and *P2184a* contain a SA-responsive element *as-1*, but the two *POXs* did not respond to the SA-treatment (Sasaki et al. 2004), and further, wound-unresponsive *P2184a* contains eight wound-responsive W-boxes (Nishiuchi et al. 2004), and wound-responsive *P2329a* contains only three W-boxes (Table 1). Therefore, further precise analysis of the 5′-deleted promoters and mutation of each *cis*-element would be necessary to understand the mechanism of *POX* promoter expression after biotic and abiotic stresses.
5. *R2184* gene responds to the treatment with MeJA (Figs. 4B, 5B), but *R2184* promoter does not contain known JA-responsive elements, such as JERE (Menke et al. 1999), JAMYC binding element (Boter et al. 2004) and 13-bp motif (Takeda et al. 1999). Therefore, a novel JA-responsive *cis*-element could be found in *R2184* promoter. In higher plants, JA is one of the important defense signal compounds, but the contribution to the resistance against blast-fungus infection in rice plants is not clear. Schweizer et al. (1997) have been shown that infection of rice plant with *M. grisea* did not induce enhanced accumulation of JA in compatible hosts. According to the result, the possible MeJA-responsive *cis*-element in *P2184a* could not work after blast fungus infection in the compatible rice, but it may contribute for other stresses, which induce JA accumulation in rice.

The results obtained here would be useful for understanding the characteristics of not only *POX* promoters but also other stress-responsive genes especially in monocotyledonous plants. Inducible promoters are suitable for effective expression of foreign genes locally at the targeting regions, contributing the basal research and applied biology such as generation of model or useful transgenic plants. Such useful promoters have been poorly studied and developed for general use especially for rice plants. The precise promoter analysis on *P2329a* and *P2184a* described here would be useful for their use for blast fungus- and bacterial leaf blight-inducible promoters.

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