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Chemically Induced Expression of Rice *OSB2* under the Control of the *OsPR1.1* Promoter Confers Increased Anthocyanin Accumulation in Transgenic Rice

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Anthocyanin pigmentation provides an excellent system with which to study the regulation of gene expression in higher plants. In this study, *OsPR1.1* promoter was isolated and the promoter activity was monitored using a reporter gene *OSB2*, which encodes a transcription factor for anthocyanin synthesis in rice plants. We introduced *PR::OSB2* plasmid into an isogenic Taichung 65, no. 99-962 T-65 C^BA B_9F_5 (T65 C^BA), rice line (*Oryza sativa* L.) and found that the transgenic rice plants exhibited anthocyanin accumulation by the induced expression of *OSB2* after chemical treatments with methyl jasmonate (MeJA) and 2,6-dichloroisonicotinic acid (DCINA). The shoots of the *PR::OSB2* transgenic rice plants changed color to red after application of the chemicals accompanying with the increased anthocyanin content to approximately 5-fold by MeJA and 2-fold by DCINA, respectively. The anthocyanin accumulation was consistent with the increase of the expression of *OSB2* and anthocyanidin synthase (*ANS*). This color change system could provide a useful and easy way to produce transgenic plants for monitoring of chemicals in the environment.

KEYWORDS: Anthocyanidin synthase; biomonitoring; 2,6-dichloroisonicotinic acid (DCINA); methyl jasmonate; *Oryza sativa*; phenylalanine ammonia lyase

INTRODUCTION

Anthocyanins are a major class of flavonoids that accumulate in a variety of plants, including rice (*Oryza sativa* L.). Anthocyanins are responsible for pigmentation patterns and perform a wide range of biological functions that include protecting plants against UV radiation, acting as signal molecules in plant−microbe interactions, and enhancing defense responses (*1*−*3*). The transcriptional regulators for anthocyanin biosynthesis include members of protein-conserved WD40 repeats and R2R3-MYB domain and basic helix−loop−helix (bHLH) domains (*4*). In maize, for example, these genes are *C1/P* (R2R3-MYB) (*5*), *R/B* (bHLH) families (*6*), and *pac1* (WD40) (*7*). Expression of the structural genes encoding the enzymes involved in anthocyanin biosynthesis is thought to be determined by combination of the WD40 protein and transcription factors with R2R3-MYB or bHLH and their interactions. Activation of the anthocyanin pathway in a tissue-specific manner depends on the interaction of those genes.

In rice, anthocyanin pigmentation of various tissues requires three types of dominant genes: Chromogen (*C*), Activator (*A*), and tissue-specific regulators for *C* and *A* (*8*). The presence of Purple (*P*) together with *C* and *A* results in apiculus pigmentation. Many of the regulatory genes for anthocyanin pigmentation in rice are located on chromosome 4—examples are *Pl*, Pl^w, *P*, *Pr*, and *Pin-1* (*9*). *Purple leaf* (*Pl*) has been shown to control pigmentation through tissue-specific accumulation of anthocyanins. Reddy et al. (*10*) identified cyanidin as the major anthocyanidin in rice and peonidin as a minor anthocyanidin. They showed that UV-B stimulated phenylalanine ammonia lyase (PAL) activity and anthocyanin accumulation, suggesting that UV-B irradiation activates Pl^w, resulting in anthocyanin induction.

OSB1 and *OSB2* (accession no. AB021080) genes have recently been isolated in the *Pl* locus of rice. These genes encode myc type bHLH transcription factors with significant similarity to the maize *R/B* genes that regulate anthocyanin biosynthesis (*11*). A transient complementation assay shows that the *OSB1* or *OSB2* can induce the anthocyanin pathway in rice (*11*). Other

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regulatory rice genes homologous to the maize *R* and *C1* family have also been cloned (*12*−*14*).

Plants have a defense system against diseases caused by viruses, bacteria, and fungi—a protective response referred to as gene-for-gene resistance (*15*). Triggered by pathogen attack, this defense system can include the hypersensitive response; the expression of pathogenesis-related (PR) genes; the production of low molecular weight secondary metabolites, such as antimicrobial phytoalexins; or cell wall rigidification (*16*, *17*). A plant-growth regulator, jasmonic acid (JA), is a signal molecule of the defense pathway against pathogens such as *Botritis cinerea* and *Pythium mastophorum* (Drechs.) (*18*) by activating defense responses including the production of basic PR proteins in tobacco (*Nicotiana tabacum* L.) and *Arabidopsis thaliana*. A dominant group of PR proteins induced by pathogens, JA and salicylic acid (SA) are in the PR1 class, which functions as signals of plant resistance (*19*).

In rice, PR proteins are induced by pathogen attack or by chemical treatment with JA and 2,6-dichloroisonicotinic acid (DCINA, an SA mimic) in both mRNA and protein levels (*20*, *21*). Rice *PR1* cDNA (*OsPR1.1*, accession no. AU163470) has been isolated based on homology with tobacco *PR1a* genes (*22*). The *OsPR1.1* was constitutively expressed in roots, coleoptile, and panicle, and its expression was increased by pathogen infection. Agrawal et al. (*23*) cloned the *PR1a* gene from a leaf of a JA-treated rice seedling and reported that the expression was induced by cut, JA, SA, 3-indoleacetic acid, gibberellin, and ethylene.

DCINA has been found to protect plants from a number of pathogens. Its broad-spectrum activity, conferring protection against viral, bacterial, and fungal diseases, strongly suggests an indirect mode of action via activation of the plant's defense mechanisms. DCINA induces pathogenesis-related genes in mRNA and protein levels, which in turn activate the strong defense mechanism. DCINA also induces endogenous JA in the leaves of rice plants (*20*)

As the *OsPR1.1* gene expression can be readily induced by various chemicals, its promoter is a useful tool to detect chemical responses of plants. *OSB2*, a transcription factor of anthocyanin synthesis, is a useful indicator when regulated by the inducible promoter like that of *OsPR1.1*. In this study, to develop an easy detection system of chemicals, we isolated the promoter region of *OsPR1.1* gene as a chemical responsive promoter and combined it with the *OSB2* gene. Green fluorescent protein gene and β-glucuronidase gene (GUS) are often used as reporter genes for detection systems based on transgenic plants. However, these systems require some experimental procedures in laboratory. On the other hand, the coloring by anthocyanin pigmentation is suitable for a simple monitoring system of chemicals in the field. Here, we report that *PR::OSB2* transgenic rice plants changed the color in leaf and stem to red by accumulated anthocyanins when exposed to various chemicals, indicating that this system is useful to detect the existence of chemicals by the naked eye.

MATERIALS AND METHODS

Cloning the Rice Pathogen-Related Gene 1.1 (*OsPR1.1*) Promoter Region. The promoter region of the *OsPR1.1* gene was obtained through polymerase chain reaction (PCR) amplification from the Nipponbare genome, using GenomeWalker (Invitrogen, CA) kits in accordance with the manufacturer's instructions. The *OsPR1.1* antisense primer, 5′-CGAGCACCAGCAAGCAGCAGGATAACCT-3′, and the GenomeWalker Stu I primer were used for amplification by PCR. The amplified DNA fragment was cloned to the pCR2.1-TOPO vector (Invitrogen) and was sequenced.

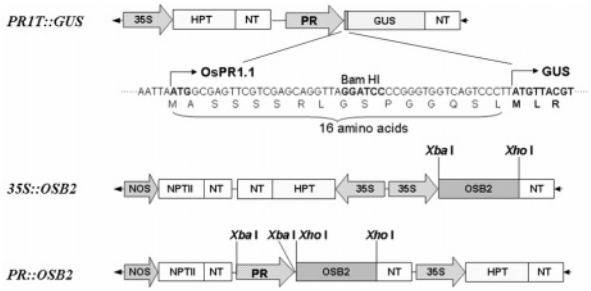

Figure 1. T-DNA regions of the expression plasmids *PR1T::GUS*, *35S::OSB2*, and *PR::OSB2*. *PR1T::GUS* rice plants express a fused GUS protein with 16 amino acids at the N-terminal region of the OsPR1.1 protein. The *OSB2* gene was expressed constitutively in *35S::OSB* rice plants and was controlled by the *OsPR1.1* promoter in *PR::OSB2* rice plants. NOS, nopaline synthase promoter; NPTII, neomycin phosphotransferase II; NT, nopaline synthase terminator; 35S, cauliflower mosaic virus (CaMV) 35S promoter; HPT, hygromycin B phosphotransferase; GUS, β-glucuronidase; *OSB2*, the *OSB2* rice gene; PR, the *OsPR1.1* promoter region.

Vector Construction. *PR::OSB2*. The GUS region of the pBI101-Hm expression vector was removed, and a 1751 bp fragment of the *OSB2* gene cDNA was inserted with the pBI101-Hm vector plasmid. The cloned *OsPR1.1* promoter region (accession no. AP008213) was amplified using 5′-TCTAGAGAAATCACAACGTACTCGA-3′ and 5′-TCTAGATAATTGAAGCTAGCTACTAT-3′ by PCR. The amplified 1.9 kb DNA fragment was digested by *Xba* I and inserted to construct *PR::OSB2* (**Figure 1**).

35S::OSB2. The positive control vector (**Figure 1**) was constructed by insertion of an *OSB2* cDNA fragment at the *Xba* I−*Xho* I site of the pMSH2 expression vector (kindly provided by Dr. T. Kawasaki of the Nara Institute of Science and Technology).

PR1T::GUS. The cloned *OsPR1.1* promoter region was amplified by PCR using 5′-GCGGCCGCCTCGAGAAATCACAACGTACTCGATAGA-3′ and 5′-GGATCCTAACCTGCTCGACGAACTCGCCAT-3′. The amplified fragments were digested by *Not* I and *Bam* HI and inserted into the pTH2 vector (*24*) to construct the *PR1T::GUS* plasmid (**Figure 1**). The *PR1T::GUS* plasmid contained 1919 bp at the promoter and the coding regions for 16 amino acids at the N-terminal of the *OsPR1.1* gene, to express a fused GUS protein effectively.

Plant Materials and Transformation. *O. sativa* ssp. japonica cv. Nipponbare was used for the transformation of *PR1T::GUS*. An *O. sativa* japonica isogenic line of Taichung 65, no. 99-962 T-65 C^BA B_9F_5 (T65 C^BA), which contains loci C^B and *A* derived from the original variety Murasakiine, was used for the transformation with *PR::OSB2* and *35S::OSB2*.

Expression plasmids were introduced into *Agrobacterium tumefaciens* strain EHA101 by electroporation using a BTX Electro Cell Manipulator 600 (BTX, Holliston, MA). This was followed with *Agrobacterium*-mediated transformation (*25*). Hygromycin-resistant T_0 plants were analyzed by PCR using hygromycin B phosphotransferase-specific primers (5′-ATGTGTATCACTGGCAAACTG-3′ and 5′-CTATCGGCGAGTACTTCTAC-3′). The DNA preparation for PCR and PCR amplification was performed as described previously (*26*). The PCR profile was 94 °C for 5 min; 30 cycles at 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 1.5 min; and 5 min at 72 °C for the final extension. The PCR products were analyzed on 1% agarose gels.

Transgenic rice plants were grown in a greenhouse, and their seeds (T_1) were harvested. The *PR1T::GUS*-transformed seedlings were tested by assessing their levels of GUS staining after 24 h of treatment with 100 μM probenazole (PBZ). Two representative plants, lines 4−5 and 4−10, were used for further experiments. The *PR::OSB2*-transformed seedlings were selected by induced anthocyanin accumulation after 24 h of treatment with 100 μM methyl jasmonate (MeJA). The two

representative lines, PR12 and PR16, were grown in a greenhouse, and the T_2 seeds, which seemed to be homozygotes, were used for further experiments.

GUS Assay. In 9 cm Petri dishes, T_2 seeds were embedded in 40 mL of MS solid medium (*27*) containing 50 mg L^{-1} hygromycin. They were incubated at 27 °C for 3 days with 16 h of light daily (photon flux density, 40 μmol m^{-2} s^{-1}). The seedlings were then transferred into 500 mL glass bottles containing 50 mL of MS culture medium. After 4 days of incubation, one of the following four chemicals was added to each bottle and they were incubated for a further 24 h: 100 μM MeJA (Aldrich, WI); 200 μM 3-allyloxy-1,2-benzisothiazole-3(2H)-one-1,1-dioxide (PBZ; Wako Pure Chemical, Tokyo); 20 μM benzo-(1,2,3)thiadiazole-7-carbothiic acid S-methyl ester (BTH; Novartis, Switzerland); or 200 μM DCINA (Aldrich). Measurements were taken using the second leaf and a 1 cm length of stem from the basal region from each plant. GUS activity was then measured by fluorimetry using standard methods (*28*). The protein content of the tissue homogenate was measured using the Bradford reagent, in accordance with the manufacturer's instructions (Bio-Rad, CA). Fluorescence was measured on an MTP-100F microplate reader (Corona Electric, Ibaraki, Japan) with 4-methylumbelliferone (4-MU; Sigma) as the standard.

Anthocyanin Analysis. Using 9 cm Petri dishes, six T_2 seeds were embedded in each dish in 40 mL of MS solid medium containing 50 mg L^{-1} hygromycin. They were incubated in the dark at 27 °C. After 7 days, either 100 μM MeJA or 200 μM DCINA solution containing 0.1% ethanol was added to each dish (final concentration) and the dishes were incubated under continuous light (photon flux density, 40 μmol m^{-2} s^{-1}) for another 24 h. For the negative control, 0.1% ethanol (v/v) was used. The shoots above the mesocotyl were weighed and cut into small pieces, and their anthocyanins were thoroughly extracted using 1 mL of hydrochloric acid and methanol (1:99, v/v) at 4 °C for 48 h, then absorbance was measured at 530 nm using a spectrometer (DU 640, Beckman Instruments, Inc., CA).

Reverse Transcription-PCR (RT-PCR) Assay. The rice seedlings were treated with MeJA or DCINA, as described for the anthocyanin analysis. They were separated into two parts, shoots and roots, and total RNA was extracted from each part using the RNeasy Plant Mini Kit (Qiagen KK, Tokyo). The RNA was treated with DNase I at 37 °C for 60 min to remove contaminated DNA. RT-PCR was performed using an RNA PCR Kit (AMV) Version 2.1 (TakaraBio, Tokyo) according to the manufacturer's instructions. For the template, 1 μg of total RNA was used for a 20 μL RT reaction. The RT profile was as follows: preincubation, 30 °C for 10 min; reverse transcription, 50 °C for 60 min; and denaturation, 99 °C for 5 min. Aliquots of cDNA solution (4 μL) were amplified in a 20 μL PCR reaction using the pairs of *OsPR1.1*, *OSB2*, anthocyanidin synthase (ANS), or rice phenylalanine ammonia-lyase (PAL)-specific primers listed below. The PCR profile was 94 °C for 5 min; 25−33 cycles at 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 90 s; and 5 min at 72 °C for the final extension. Aliquots of PCR products (4 μL) were analyzed by electrophoresis on 1.5% agarose gels.

Primer Pairs. *OsPR1.1* sense, 5′-GTCTTCATCACCTGCAAC-TACTC-3′; *OsPR1.1* antisense, 5′-ACTAAGCAAATACGGCTGA-CAGT-3′; *OSB2* sense, 5′-ATGGCATCTGCTCCTCCAGTTC-3′; *OSB2* antisense, 5′-CTATCTGCAGACTGAGCATTGC-3′; *ANS* sense, 5′-TGTTCAAGAAGCTCAAGGATCA-3′; *ANS* antisense, 5′-TGGTGA-CACATTTATAGCAAATC-3′; *PAL* sense, 5′-GCTCTCGGCGGT-GTTCTGCGA-3′; *PAL* antisense, 5′-GAGGTACTGGAGCTCAGAGCTG-3′. Accession nos.: *OSB2*, AB021080; *OsPR1.1*, AU163470; *OsPR1.1* promoter region, AP008213.

RESULTS

Induction of the GUS Gene under Control of the *OsPR1.1* Promoter. We transformed rice plants (*O. sativa* L. cv. Nipponbare) using the *PR1T::GUS* plasmid, which is supposed to express GUS gene effectively by *OsPR1.1* promoter (**Figure 1**). In the transgenic rice plants harboring *PR1T::GUS*, the expression level of the GUS gene was increased through treatment with chemicals including MeJA, BTH, DCINA, and PBZ (**Figure 2**); those chemicals are used as herbicide antidotes, or "safeners", to induce the pathogen-related response in plants. GUS activity showed a 1.2−2-fold increase in the stem and a 2−3-fold increase in the leaves. Of the chemicals tested, DCINA and MeJA provoked the greatest increase of GUS activity. We confirmed that the *OsPR1.1* promoter affects the expression of the GUS reporter gene in response to treatment with these chemicals.

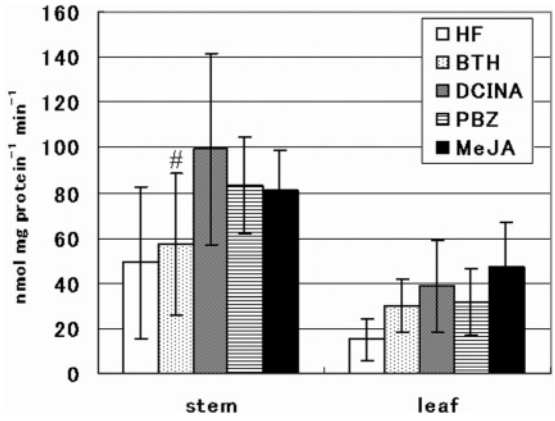

Figure 2. GUS activity of *PR1T::GUS* rice seedlings treated with various chemicals. *PR1T::GUS* rice seedlings were treated with chemicals for 24 h at 27 °C. The final concentrations of the chemicals are described in the Materials and Methods. GUS activity was measured by 4-MU fluorescence, using the second leaf and 1 cm of stem from the basal region. HF, hormone-free medium without any chemicals (control). The values are the means of three plants from each of the two lines ($n = 6$) ± SD ($p < 0.05$ by *t*-test; #, no significant difference).

Induction of Anthocyanin Synthesis in *PR::OSB2* Rice Plants. For *PR::OSB2* rice transformation, we used an isogenic line of the rice variety Taichung 65, no. 99-962 T65 C^BA B_9F_5 (T65 C^BA), which contains loci C^B and *A* from the rice variety Murasakiine. Because it has been reported that anthocyanin pigmentation occurs by the expression of *OSB2* gene in this particular isogenic line (*11*), we transformed this isogenic line using a *PR::OSB2* plasmid (**Figure 1**), which expresses the *OSB2* gene under the control of the *OsPR1.1* promoter. The shoots of *PR::OSB2* rice (PR12 and PR16) grown in the dark were white. Then, we treated the rice seedlings with chemicals and observed the coloration after 24 h. The shoots of *PR::OSB2* rice turned red by treatment with MeJA or DCINA solution containing 0.1% ethanol (**Figure 3**). In contrast, the shoots were pale green when treated with 0.1% ethanol (control). In the case of nontransgenic rice plants, however, the shoots were pale green, with only a slight red coloration after treatment with MeJA. There was no color change in the nontransgenic rice plants under DCINA treatment. The positive control, *35S::OSB2* rice plants that express the *OSB2* gene under the control of CaMV 35S promoter, showed red shoots with or without the chemical treatments.

After MeJA treatment, the anthocyanin content in the aerial parts of the plant increased 5.9-fold in the PR12 shoots and 4.6-fold in the PR16 shoots of the *PR::OSB2* rice plants (**Figure 4A**). Under DCINA treatment, anthocyanin increased 1.6-fold in the PR12 shoots and 2.2-fold in the PR16 shoots (**Figure 4B**). Interestingly, the anthocyanin content increased 2.6-fold in *35S::OSB2* rice plants under MeJA treatment, whereas it decreased by 30% under DCINA treatment.

Expression of the *OSB2* Gene by RT-PCR. We assumed that the differences in observed color between the transgenic

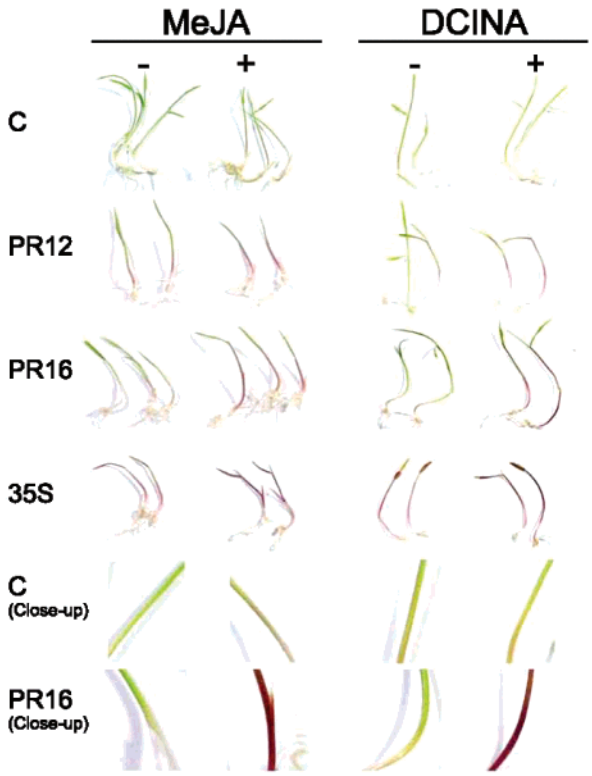

Figure 3. Color changes in *PR::OSB2* rice plants caused by accumulation of anthocyanin. Rice seedlings were treated with 100 μM MeJA (left panel) or 200 μM DCINA solution containing 0.1% ethanol (right panel) for 24 h. Ethanol (0.1%) was used as the negative control. The anthocyanin content was increased, and the shoots of the seedlings turned red as a result of the chemical treatments. C, nontransgenic T65 C^BA (control); PR12 and PR16, *PR::OSB2* rice plants; and 35S, *35S::OSB2* rice plants.

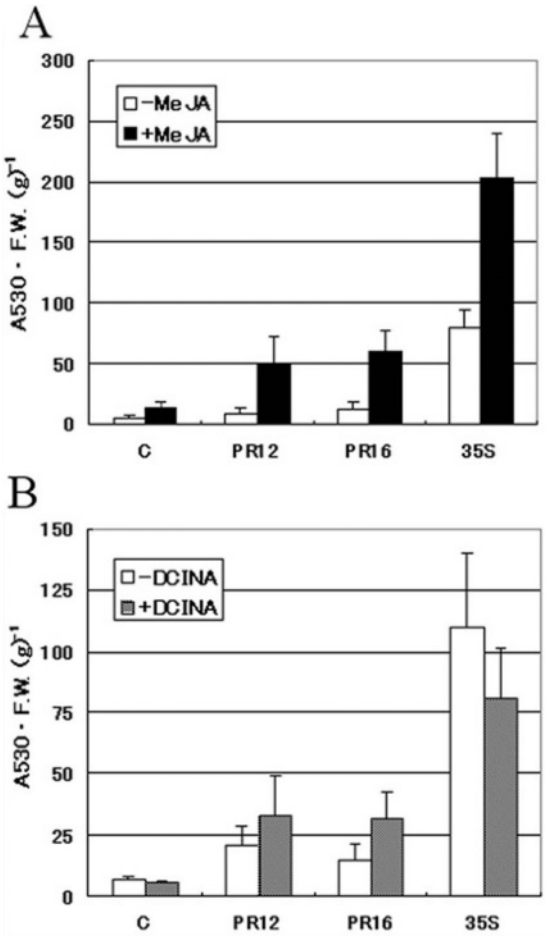

Figure 4. Induction of anthocyanin synthesis in *PR::OSB2* rice plants. Anthocyanin was accumulated in aerial parts of rice plants through treatment with either 100 μM MeJA (**A**) or 200 μM DCINA (**B**) solution containing 0.1% ethanol for 24 h. Ethanol (0.1%) was used as the negative control. The aerial parts of the seedling were used for anthocyanin quantification. Anthocyanin was extracted using 1% HCL–methanol, and the absorbance was measured at 530 nm using a spectrometer. C, nontransgenic T65 C^BA (control); PR12 and PR16, *PR::OSB2* rice plants; and 35S, *35S::OSB2* rice plants. The values are means ± SD ($n > 4$ and $p < 0.05$ by *t*-test).

rice lines were due to differences in the level of *OSB2* gene expression. Accordingly, we examined the mRNA expression by RT-PCR (**Figures 5** and **6**). *OsPR1.1* expression was detected in roots constitutively and was increased slightly by MeJA or DCINA. *OsPR1.1* gene expression was hardly detected in the rice shoots without chemical treatment, but the chemicals increased it to detectable levels. In *PR::OSB2* rice plants, as we expected, expression profile of the introduced *OSB2*, which was controlled by the *OsPR1.1* gene promoter, was similar to that of *OsPR1.1*. Expression of the *OSB2* gene was constitutive in roots, and it increased in both roots and shoots following chemical treatment. Expression of ANS was not detected in the roots of any of the rice plants. The *ANS* expression was detected in the shoots of *PR*::*OSB2* and *35S::OSB2* rice plants and increased with the chemical treatment. The expression patterns of *OSB2* and *ANS* were clearly related to anthocyanin accumulation in *PR::OSB2* and *35S::OSB2* rice plants. The expression of *PAL* was constant in the experiments and thus used as a positive control.

DISCUSSION

We isolated the *OsPR1.1* promoter region, which is thought to be a chemical-responsive promoter. To examine its expression pattern, we constructed a *PR1T::GUS* plasmid, which expresses a fused GUS protein with the 16 amino acids of OsPR1.1 protein at the N-terminal, under control of the *OsPR1.1* promoter, and introduced it into rice plants (cv. Nipponbare). GUS activity in both stem and leaves increased to approximately 2-fold by treatments with MeJA, PBZ, and DCINA. The GUS activity was also detected continuously in roots (data not shown). The *OsPR1.1* gene was expressed constitutively in the rice roots, and the level was increased by treatment with either MeJA or DCINA (**Figures 5** and **6**). In aerial parts, the expression of the *OsPR1.1* gene was very low without chemical treatment, but it had increased after 24 h of chemical treatment. These results indicate that the expression profile of *OsPR1.1* is correctly reflected in the GUS or OSB2 gene expression.

The *PR::OSB2* plasmid using the *OSB2* gene as a reporter gene was introduced into a T65 C^BA rice line. Transgenic T65 C^BA rice could accumulate anthocyanin in its stem and leaves through the expression of the *OSB2* gene, which is a transcription factor for anthocyanin synthesis. Because the expression level of GUS was not as high as expected in *PR1T::GUS* plants, *OSB2* gene was expressed just under *OsPR1.1* promoter, not as a fused protein in *PR::OSB2* plants. The *PR::OSB2* rice plants exhibited anthocyanin accumulation after treatment by either MeJA or DCINA, which was also confirmed by the accumulation at the mRNA level via RT-PCR analysis. The magnitude of the induction was greater with MeJA than with DCINA.

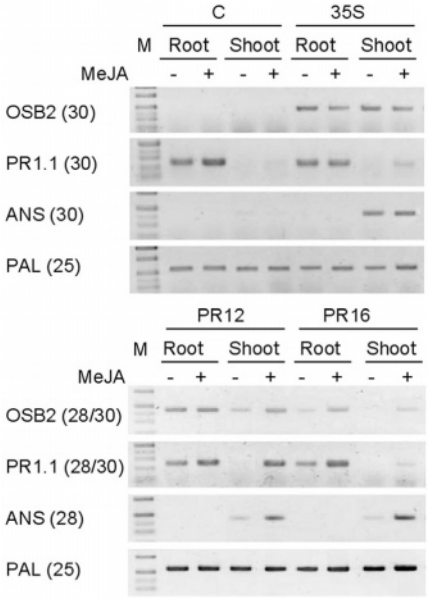

Figure 5. RT-PCR analysis of transgenic rice plants with or without MeJA treatment. Total RNA was extracted from rice seedlings treated for 24 h with 0.1% ethanol (–) or 100 μM MeJA solution containing 0.1% ethanol (+). RT-PCR was performed using *OSB2*-, *OsPR1.1*-, *ANS*-, and *PAL*-specific primers. The numbers of PCR cycles for root and shoot are shown in parentheses; if they differ, they are presented as root/shoot. The expression of *PAL* was constant in the experiments and used as a positive control. M, DNA size marker; C, nontransgenic T65 C^BA (control); PR12 and PR16, *PR::OSB2* rice plants; and 35S, *35S::OSB2* rice plants.

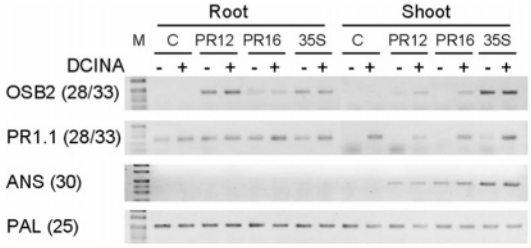

Figure 6. RT-PCR analysis of transgenic rice plants with or without DCINA treatment. Total RNA was extracted from rice seedling treated with 0.1% ethanol (–) or with 200 μM of DCINA solution containing 0.1% ethanol (+) for 24 h. RT-PCR was performed using *OSB2*-, *OsPR1.1*-, *ANS*-, and *PAL*-specific primers. The numbers of PCR cycles for root and shoot are in parentheses; if they differ, they are presented as root/shoot. The expression of *PAL* was constant in the experiments and used as a positive control. M, DNA size marker; C, nontransgenic T65 C^BA (control); PR12 and PR16, *PR::OSB2* rice plants; and 35S, *35S::OSB2* rice plants.

MeJA activated the expression of *OSB2* in *PR::OSB2* rice plants, and the anthocyanin content was increased 4.6- and 5.2-fold in PR12 and 16 lines, respectively. The magnitude of *OsPR1.1* response in *PR::OSB2* rice plants was greater than that in *PR1T::GUS* plants after induction of MeJA, suggesting that transcriptional factors other than *OSB2* gene and structural genes for anthocyanin synthesis may also be induced by MeJA.

The *35S::OSB2* rice plants exhibited red leaves, indicating constitutive anthocyanin accumulation. When the anthocyanin content in *35S::OSB2* rice plants was analyzed, it increased 2.6-fold by MeJA treatment, while induction of GUS gene expression in the *35S::GUS* rice plants by MeJA or DCINA was not detected (data not shown). These results indicated that MeJA did not affect the 35S promoter itself and suggested that transcriptional factors other than *OSB2* also play important roles to express the structural genes of anthocyanin synthesis in rice.

DCINA also activated expression of the *OSB2* gene as MeJA did. By 24 h after the treatment, the anthocyanin content had increased 1.6- and 2.2-fold in the *PR::OSB2* rice lines PR12 and PR16, respectively. As Schweizer et al. reported (*20*), 100 ppm of DCINA induced the expression of *PR1* genes at the protein level. Our results also indicated that DCINA induced the expression of *OsPR1.1* after 24 h at the mRNA level. In our experiments, the anthocyanin content decreased in both nontransgenic and *35S::OSB2* rice plants under DCINA treatment, suggesting that DCINA did not activate the expression of the structural genes of anthocyanin synthesis or it could be the side effect of DCINA to inhibit signaling for anthocyanin pigmentation.

The *PR::OSB2* rice plants appeared reddish by 0.1% ethanol (without chemical treatment); thus, OsPR1.1 promoter is leaky and responsive to ethanol. Because the chemical induction by MeJA and DCINA was rather mild (2−6-fold), for stringent control of anthocyanin accumulation, the *OsPR1.1* promoter should be studied in detail to identify chemical-responsive elements.

It has been reported that JA induces the accumulation of *PAL* transcript in a suspension culture of *Rauvolfia canescens* and *Eschscholtzia californica* (*29*) and that of *CHS* transcript in a suspension culture of *Glycine max* (*30*). Reddy et al. (*10*) reported that PAL activity was increased by UV light induction in rice as well. However, in our RT-PCR experiments, no remarkable change in expression of *PAL* was observed. It could be because the light was not as strong as outside conditions to induce *PAL* expression or just simply because the PCR cycle number was too much to detect the difference of expression of *PAL*.

Regulatory genes of anthocyanin synthesis in different species are functionally homologous and are probably derived from common ancestors, whereas the structural genes differ among species and are regulated differently—by distinct regulatory genes (*2*). For example, in maize, *CHS*, *DFR*, and *3GT* are regulated by the regulatory genes R and C1 (*1*). In Snapdragon (*Antirrhinum majus*), *F3H*, *DFR*, *ANS*, and *3GT* are controlled by the *delila* gene (*31*). Similarly, it has been suggested that *OSB2* controls the expression of the structural genes of anthocyanin synthesis in rice, although the relationship between *OSB2* gene and structural genes has not yet been clarified.

As the color changed, at least, the expression of *ANS* was induced by *OSB2*. By the results of RT-PCR, as we expected, *ANS* expression was increased in shoots of seedlings commensurate with *OSB2* expression increase. *ANS* expression was not detected in roots of seedlings, even in *35S::OSB2* rice plants. In this case, again, other transcription factors like OsC1, which was reported as myb type transcription factor to induce rice *DFR* and *ANS* (*32*), are needed to induce the expression of *ANS*. In rice, the relationship between the transcription factors including *OSB2*, *OsC1*, and the structural genes of anthocyanin synthesis remains to be investigated.

Transcription factors play a key role in the genetic metabolic engineering of plants (*33*). Color changes caused by anthocyanin accumulation are easily visible in the field. The color-changed plants would be a good candidate for a chemical-monitoring system. In our experiment, expression of the *OSB2* gene in PR::OSB2 rice plants was responsive to some chemicals including MeJA and DCINA. It was also suggested that transcriptional factors other than *OSB2* also play important roles to express the structural genes of anthocyanin synthesis in rice. Thus, other

transcriptional factors containing R2R3-MYB domain and WD40 protein for anthocyanin pigmentation need to be controlled to produce a better monitoring system. The transgenic plants using appropriate promoters and transcriptional factors could be useful in detecting environmental pollution, by indicating the presence of chemicals in the soil and water of a plant's surrounding environment.

ABBREVIATIONS USED

ANS, anthocyanidin synthase; BTH, benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester; DCINA, 2,6-dichloroisonicotinic acid; JA, jasmonic acid; MeJA, methyl jasmonate; PAL, phenylalanine ammonia lyase; PBZ, probenazole; PR, pathogen related; RT-PCR, reverse transcription polymerase chain reaction; SA, salicylic acid; T65 C^BA, isogenic line of the rice variety Taichung 65, no. 99-962 T-65 C^BA B_9F_5.

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