JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Herbicide-induced Anthocyanin Accumulation in Transgenic Rice by Expression of Rice *OSB2* Under the Control of Rice *CYP72A21* Promoter

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CYP72A21, a rice cytochrome P450 gene, is induced by chloroacetamide herbicides. *OSB2*, a rice myc-type transcription factor, induces anthocyanin accumulation in rice leaves. To produce plants for biomonitoring by color change, we combined the *CYP72A21* promoter and the *OSB2* gene and introduced them into the rice isogenic line Taichung-65 C^BA (T65), which contains loci C^B and *A* from the rice cultivar Murasakiine. Leaves of the transgenic plants turned red upon treatment with the chloroacetamide herbicides acetochlor, alachlor, and metolachlor. Seedling shoots reddened upon treatment with alachlor or metolachlor at 10 μM, a concentration slightly higher than that used in the field. Anthocyanin content was increased approximately 200% by the treatment. The color changes were consistent with increased shoot expression of *OSB2* and the anthocyanidin synthase gene (*ANS*). This system promises easy detection of rice plant gene expression. Transgenic plants could be used in the future to biomonitor accumulated herbicides.

KEYWORDS: acetochlor; alachlor; biomonitoring; metolachlor; transcription factor

INTRODUCTION

Anthocyanins accumulate in various plant parts as secondary metabolites and create patterns of pigmentation. They are also responsible for a wide range of biological functions such as UV radiation protection, molecular signaling plant-microbe interactions, and plant defense responses (reviewed in refs *1–5*).

Anthocyanins are a major class of flavonoids and are synthesized through several steps, including condensation of three molecules of malonyl−CoA and *p*-coumanyl CoA, oxidation, and hydroxylation. The structural genes for anthocyanin biosynthesis are controlled at the expression level by regulatory genes that influence the intensity and pattern of anthocyanin biosynthesis. In rice (*Oryza sativa* L.), anthocyanin pigmentation in various tissues requires three types of dominant gene: *Chromogen* (*C*), *Activator* (*A*), and tissue-specific regulators for *C* and *A* (*6, 7*). One of the regulators is *Purple* (*P*), and the presence of *P* together with *C* and *A* results in apiculus pigmentation.

The *OSB2* gene has been isolated from the *Purple leaf* (*Pl*) locus, which is involved in the regulation of anthocyanin biosynthesis in rice. *OSB2* encodes a myc-type basic helix–loop–helix transcriptional factor and is markedly similar to the maize *R/B* genes (*8, 9*). Transient complementation assay has shown that *OSB2* induces anthocyanin synthesis in rice (*8*). For the *C* locus of rice, an R2R3-MYB-type regulatory gene, *OsC1*, which shows homology with the maize *R* and *C1* family, has also been cloned (*10–12*). For the *A* locus of rice, the dihydroflavonol-4-reductase (*DFR*) gene was recently cloned (*13*).

The isogenic line Taichung-65 C^BA, contains the loci C^B and *A* derived from the original rice variety Murasakiine in a Taichung 65 genetic background. This particular isogenic line transformed with *OSB2* can accumulate anthocyanin by the expression of *OSB2*. The transgenic rice plants exhibit a red color phenotype, the intensity of which depends on the level of expression of *OSB2* (*14*). If *OSB2*, used as a reporter gene, were to be combined with an appropriate promoter, then the transgenic plants could be used as an easy biomonitoring system to detect chemicals in the environment by simple color change.

The cytochrome monooxygenase P450 (CYP) family is among the biggest enzyme families in plants. CYP enzymes insert one atom of oxygen into hydrophobic molecules to make them more hydrophilic (*15*). Genome sequence analysis has revealed that rice (*O. sativa* L.) genomes include 356 P450 genes and 135 pseudo-P450 genes (*16*). Some P450 species are involved in secondary metabolic processes, such as hormone, steroid, and terpenoid synthesis, and others are involved in xenobiotic metabolic processes such as herbicide tolerance.

The expression of a rice cytochrome P450, *CYP72A21* (AB237166), is chemically inducible in rice seedlings treated with a mixture of chemicals (100 μM chlorotoluron, 2,4-dichlorophenoxyacetic acid (2,4-D), phenobarbital, salicylic acid, and naphthalic anhydride (*17*). Wang et al. (*18*) reported that

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10.1021/jf071575j CCC: $40.75
Published on Web 01/25/2008

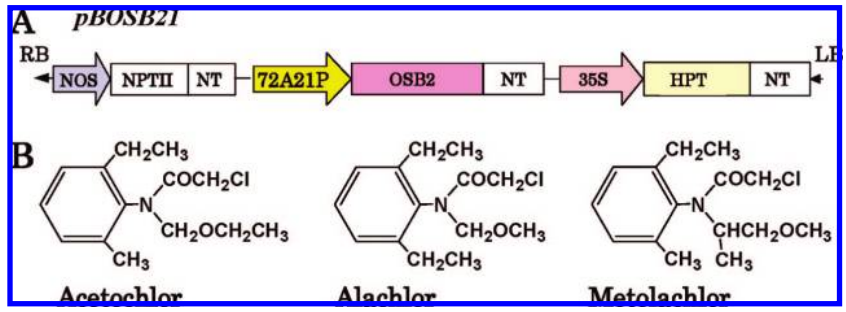

Figure 1. Vector construction and chemicals used for induction of gene expression in pBOSB21 rice plants. (**A**) T-DNA regions of the expression plasmid *pBOSB21*. Rice *OSB2* was expressed under the control of the rice *CYP72A21* promoter. RB, right border; LB, left border; NOS, nopaline synthase promoter; NPTII, neomycin phosphotransferase II; NT, nopaline synthase terminator; 35S, cauliflower mosaic virus (*CaMV*) 35S promoter; HPT, hygromycin B phosphotransferase; 72A21P, rice *CYP72A21* promoter region (2 kbp). (**B**) Chemical structures of the chloroacetamide herbicides acetochlor, alachlor, and metolachlor.

CYP72A21 is highly transcribed in a tissue-specific manner after heading, and they hypothesized that it is involved in seed maturation and substance accumulation.

We have previously reported that the isolation of *CYP72A21* promoter and the promoter activity using β-glucuronidase (GUS) as a reporter. The GUS induction by various chemicals including auxins, other plant hormones, and several gropes of herbicides, has been observed (*17*). Thus, *CYP72A21* expression can be induced by herbicides, and the promoter is revealed to be useful for its chemical responsiveness.

Here, we combined the promoter region of *CYP72A21* with *OSB2* as a reporter gene, and then we introduced the transgene into Taichung-65 C^BA rice plants. In the leaves of the transgenic (pBOSB21) rice plants, an easily detectable color change was induced by chemicals. We analyzed anthocyanin pigmentation in response to treatment with the herbicides that induced GUS expression in the former study (*17*). This work is the basic research for the usage of transgenic plants that can be used for monitoring environmental changes. We discuss the possibility of utilizing these transgenic plants for color change biomonitoring in the field.

MATERIALS AND METHODS

Vector Construction. The promoter region of *CYP72A21* was obtained by PCR amplification from rice (*Oryza sativa* L. Nipponbare) genome, as described previously (*17*). The GUS region of the *pBI101-Hm* expression vector was removed, and a 1751-bp fragment of *OSB2* cDNA (AB021080) was inserted. Then, the cloned *CYP72A21* promoter region was digested by *XbaI* and *Hind*III and inserted into *pBI101-Hm* to construct *pBOSB21* (**Figure 1**).

Plant Materials and Transformation. The rice isogenic line Taichung 65, No. 99–962 T-65 C^BA, BC_9F_5, which contains the loci C^B and *A* derived from the rice original variety Murasakiine, was used for transformation of *pBOSB21*. The expression plasmids were introduced into *Agrobacterium tumefaciens* strain EHA101 by electroporation using an Electro Cell Manipulator 600 (BTX, Holliston, Massachusetts, USA); this was followed by *Agrobacterium*-mediated transformation (*19*). T_0 plants regenerated on Murashige and Skoog (MS) medium (*20*) containing 50 mg L^{-1} hygromycin were analyzed by PCR using rice *OSB2*-specific primers (*OSB2* sense 5′-ATGGCATCTGCTCCTCCAGTTC-3′, *OSB2* antisense 5′-CTATCTGCAGACTGAGCATTGC-3′). DNA preparation for PCR and PCR amplification were performed as previously reported (*21*). The PCR profile was 94 °C for 5 min; 30 cycles of 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 in 1% agarose gels.

Transgenic plants (pBOSB21 rice plants) were grown in a greenhouse, and their seeds (T_1) were harvested. Seedlings were selected on the basis of anthocyanin accumulation after 24 h of treatment with 100 μM herbicides. The representative selected lines #14, #15, #22, #42, and #45 were used for further experiments.

Anthocyanin Analysis. The seeds of pBOSB21 rice plants (T_1) were sterilized, embedded in 40 mL of MS solid medium (*20*) containing 50 mg L^{-1} hygromycin in a 9 cm Petri dish, and incubated at 27 °C for 3 days in the dark. The hygromycin-resistant seedlings were transplanted on a stainless steel basket (2.5 × 2.5 × 0.6 cm) in a glass bottle (9 cm diameter × 13 cm high) containing 50 mL of MS medium. After the seedlings were incubated for 4 days in the dark to make the shoots white. Then, a 100 μM solution of acetochlor, metolachlor (**Figure 1B**), esprocarb, trifluralin, 2,4-dichlorophenoxyacetic acid (2,4-D), or 4-chloro-o-tolyloxyacetic acid (MCPA) containing 0.1% (v/v) ethanol (final concentration) was added to each bottle, which was then incubated for 3 days at 27 °C under 16 h of light daily (photon flux density 40 μmol m^{-2} s^{-1}). The negative control was 0.1% (v/v) ethanol. Shoots without mesocotyls were weighed and cut into small pieces. Anthocyanins were thoroughly extracted in 1 mL of concentrated HCl and methanol (1:99, v/v) for 48 h at 4 °C; the absorbance was then measured at a wavelength of 530 nm by a spectrometer (DU 640, Beckman Instruments, Inc., Fullerton, California, USA). As for chloroacetamide herbicides, *pBOSB21* rice plants were treated for 6 days with a 10 μM alachlor or metolachlor solution containing 0.1% (v/v) ethanol, a slightly higher concentration of these chemicals than is used in the field (alachlor, 5–10 μM; metolachlor, 3–6 μM) (*22*). The anthocyanin contents were measured as described above.

Reverse Transcription−Polymerase Chain Reaction (RT-PCR) Assay. Rice seedlings treated with 10 μM alachlor as described in the anthocyanin assay were separated into three parts: leaves; whole aerial parts without stem base, stem base; 1.5 cm segments of the basal region of the seedlings; and roots. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen K.K., Tokyo, Japan). 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) Ver 2.1 (Takara Bio, Tokyo, Japan) in accordance with the manufacturer's instructions. Total RNA (0.5 μg) was used as the template for the 20 μL RT reaction. The RT profile was as follows: 30 °C for preincubation for 10 min, 50 °C for 60 min for reverse transcription, and 99 °C for 5 min for denaturation. Four microliter aliquots of cDNA solution were amplified with 20 μL of PCR mixture using the following pairs of *OSB2*-, anthocyanidin synthase (*ANS*)-, or phenylalanine ammonia-lyase (*PAL*)-specific primers: *OSB2* sense, 5′-ATGGCATCTGCTCCTCCAGTTC-3′; *OSB2* antisense, 5′-CTATCTGCAGACTGAGCATTGC-3′; *ANS* sense, 5′-TGTTCAAGAAGCTCAAGGATCA-3′; *ANS* antisense, 5′-TGGTGACACATTTATAGCAAATC-3′; *PAL* sense, 5′-GCTCTCGGCGGTGTTCTGCGA-3′; *PAL* antisense, 5′-GAGGTACTGGAGCTCAGAGCTG-3′.

The PCR profile was 94 °C for 5 min; 28 cycles of 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

Table 1. Anthocyanin Accumulation Induced in pBOSB21 Rice Plants by Herbicides

herbicide	chemical family	anthocyanin A_{530} g^{-1} (F.W.)
ethanol (control)		22.9 ± 3.2
trifluralin	dinitroanilines	30.7 ± 12.5
esprocarb	thiocarbamates	26.1 ± 10.7
2,4-D	phenoxy carboxylic acids	12.8 ± 5.7
MCPA	phenoxy carboxylic acids	12.9 ± 5.8
acetochlor	chloroacetamides	40.9 ± 9.2
metolachlor	chloroacetamides	84.6 ± 24.4

The seedlings of pBOSB21 rice plants were treated with 100 μM of each herbicide solution containing 0.1% (v/v) ethanol for 3 days. The accumulated anthocyanin was measured as described in the Materials and Methods section. More than 5 lines of pBOSB21 rice plants were tested. Values are presented as means ± S.E.

extension. Four-microliter aliquots of PCR products were analyzed in 1.5% agarose gels.

In situ Hybridization. Seven-day-old rice seedlings (*O. sativa* L. cv. Nipponbare) grown on a stainless basket in a glass bottle, as for anthocyanin analysis, were treated for 24 h with a 10 μM alachlor solution containing 0.1% (v/v) ethanol and then fixed in 4% (w/v) paraformaldehyde and 0.25% (w/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.4) overnight at 4 °C. The seedlings were then dehydrated through a graded ethanol series and a *t*-butanol series (*23*) and finally embedded in Paraplast Plus (Oxford, St. Louis, Missouri, USA). Microtome sections (10 μm thick) were mounted on glass slides treated with silane. Hybridization and immunological detection of the hybridized probes using digoxigenin-labeled RNA produced from the *CYP72A21* cDNA were performed in accordance with the method of Kouchi and Hata (*24*).

RESULTS

Anthocyanin Accumulation in pBOSB21 Rice Plants. The seedlings of pBOSB21 rice plants were treated with various herbicides at 100 μM (**Table 1**). When trifluralin or esprocarb was applied, the amount of anthocyanin was slightly higher than control treated with 0.1% ethanol. In these cases, the intensity of red color was different by the individual plant, and partial accumulation of anthocyanin was occasionally observed. When auxins 2,4-D or MCPA was applied, the amount of anthocyanin was lower than the control. On the other hand, when chloroacetamide herbicide acetochlor or metolachlor was applied, the aerial parts of the plants became red, apparently and reproducibly. The amount of anthocyanin in the aerial parts of seedlings treated with 100 μM of acetochlor or metolachlor was approximately doubled or quadrupled on average, respectively (**Table 1**).

In the next step, pBOSB21 rice plants treated with 10 μM alachlor or metolachlor showed a clear redness of the shoots, indicating that both chemicals induced anthocyanin synthesis in this lower concentration (**Figure 2**, alachlor). The color change was observed not only in the stem but also in the leaves. A representative line #42 showed an approximately triple increase of the amounts of anthocyanin when treated with 10 μM of alachlor or metolachlor (**Figure 3**).

Expression of OSB2 in pBOSB21 Rice Plants. In situ hybridization revealed the induced expression of *CYP72A21* in Nipponbare at the base of the leaf along the vascular bundle, after treatment with 10 μM alachlor (**Figure 4**). Thus, endogenous *CYP72A21* was induced in the leaf by treatment with 10 μM alachlor, which is consistent with the increase of *CYP72A21* expression by RT-PCR in response to the treatment with 10 μM alachlor (data not shown).

We analyzed the level of expression of *OSB2* mRNA in pBOSB21 rice plants by RT-PCR (**Figure 5**). In the leaves of

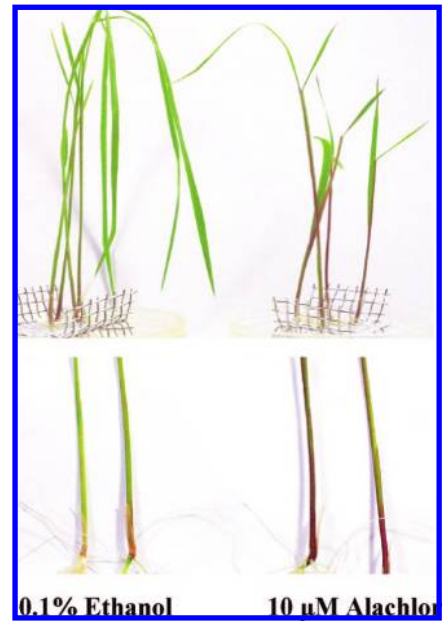

Figure 2. Color change of pBOSB21 rice plants treated with 10 μM of alachlor. (Top panel). Rice seedlings were treated for 6 days with 0.1% (v/v) ethanol (left) or a 10 μM alachlor solution containing 0.1% (v/v) ethanol (right). (Bottom panel) Close-up view of stem of pBOSB21 rice plants treated for 6 days with 0.1% (v/v) ethanol (left) or a 10 μM alachlor solution containing 0.1% (v/v) ethanol (right).

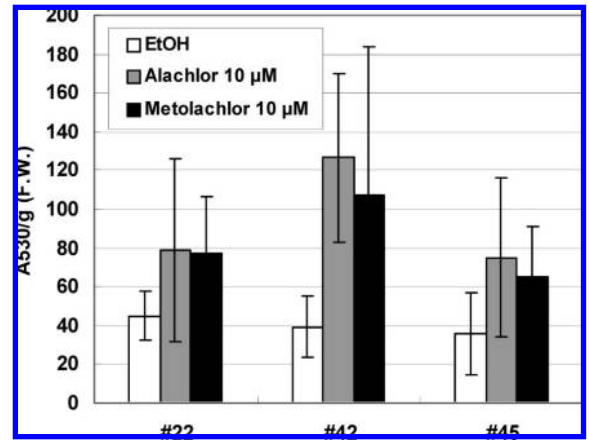

Figure 3. Induction of anthocyanin production in pBOSB21 rice plants treated with 10 μM of herbicides. pBOSB21 rice plants were treated for 6 days with 0.1% (v/v) ethanol or a 10 μM solution of alachlor or metolachlor containing 0.1% (v/v) ethanol. Aerial parts of seedlings were used for anthocyanin quantification. Anthocyanin was extracted in 1% HCL/methanol and measured by absorbance at a wavelength of 530 nm. Values are the means of three independent samples ± SD ($n = 3$).

plants treated with 0.1% (v/v) ethanol, *OSB2* expression was barely detected, and the expression increased to detectable level upon treatment with 10 μM of alachlor. At the stem base of the aerial parts, *OSB2* expression was detectable with 0.1% (v/v) ethanol treatment, and the transcript level increased after treatment with 10 μM alachlor. Thus, as we expected, similar to the result with in situ hybridization with *CYP72A21*, *OSB2* expression was induced by alachlor treatment. *ANS* expression in leaves and stem base (**Figure 5**) was increased in the same manner as for *OSB2* by treatment with alachlor. The expression patterns of *OSB2* and *ANS* were clearly correlated with anthocyanin accumulation in the aerial parts of seedlings (**Figures 3** and **5**). In roots, *OSB2* expression was induced by

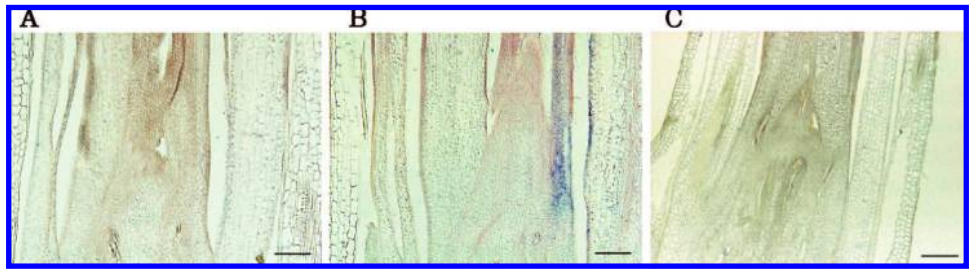

Figure 4. In situ hybridization of *CYP72A21* transcripts in vertical sections of rice seedlings. (**A**) A seedling treated with 0.1% (v/v) ethanol for 24 h and hybridized with *CYP72A21* antisense probe. (**B**) A seedling treated with a 10 μM alachlor solution containing 0.1% (v/v) ethanol and hybridized with *CYP72A21* antisense or (**C**) sense probe. Scale bars represent 100 μm.

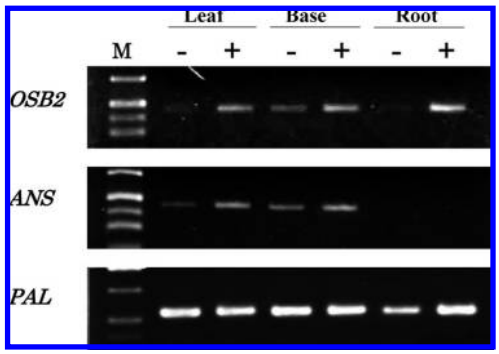

Figure 5. RT-PCR analysis of pBOSB21 transgenic rice plants treated with 10 μM of alachlor. Total RNA was extracted from rice seedlings treated for 6 days with 0.1% (v/v) ethanol (−) or a 10 μM alachlor solution containing 0.1% (v/v) ethanol (+). RT-PCR was performed with *OSB2*-, *ANS*-, and *PAL*-specific primers. *PAL* was used as a positive control. There were 28 PCR cycles. M, DNA size marker.

alachlor treatment, but *ANS* expression was not induced. The expression of *PAL* was consistent under these conditions and was thus used as a positive control.

DISCUSSION

Biomonitoring has been used to monitor environmental contamination and to determine chemical toxicity in the field. One promising biomonitoring method is the use of a transgenic organism containing a reporter gene whose expression is controlled by a contaminant-specific responsive promoter. For example, transgenic plants have been produced for the detection of nuclear pollution (*25*), heavy metals (*26–28*), and phenoxy herbicides such as 2,4-D (*29*). By an anthocyanin-coloring system, transgenic plants can be used to detect specific changes in the environment and to provide responses that can be easily recorded as simple color changes associated with anthocyanin accumulation (*14*).

In our study, *OSB2* was used as the reporter gene and *CYP72A21* was used as a chemical-responsive promoter. Color changes in transgenic pBOSB21 rice plants were observed after treatment with 100 μM of chloroacetamide herbicides (**Table 1**). The anthocyanin content in line #42 was increased approximately 200% by 10 μM alachlor or metolachlor (**Figure 3**). Thus, alachlor and metolachlor certainly act on the *CYP72A21* promoter and induce the expression of *OSB2*, which positively regulates the expression of structural genes for anthocyanin synthesis. The anthocyanin accumulation in pBOSB21 rice plants was induced by a slightly higher concentration of these herbicides than that used in the field (alachlor, 5–10 μM; metolachlor, 3–6 μM) (*22*) and it was easily detectable by the naked eye (**Figure 3**).

Consistent with the accumulation of anthocyanin in pBOSB21 rice plants, the RT-PCR results showed that *OSB2* expression levels were clearly increased in the leaves and the stem base by alachlor treatment (**Figure 5**). The increase of *ANS* expression and anthocyanin accumulation was correlated with that of *OSB2* expression. At the stem base, *OSB2* and *ANS* expression were observed without alachlor treatment, because *CYP72A21* was slightly induced at the stem base by ethanol (data not shown). In the roots, *OSB2* expression was induced as well; however, *ANS* expression was not induced. These results suggested that other regulatory genes were needed to induce *ANS* expression in roots.

Anthocyanin accumulation in pBOSB21 rice plants upon treatment with 2,4-D was not detected, although GUS production has been observed in transgenic plants on treatment with 2,4-D by use of the *CYP72A21* promoter (*17*). This result also indicated that anthocyanin accumulation was regulated by multiple genes, the gene for *C* locus in rice, *OsC1* (*12*), the gene for *A* locus, *DFR* (*13*) and other regulatory genes. Even if the expression of *OSB2* is induced by 2,4-D, other regulatory genes did not seem to be expressed to promote anthocyanin synthesis. Otherwise, 2,4-D treatment probably induced not only *CYP72A21* but also a lot of genes that might inhibit anthocyanin synthesis.

Endogenous *CYP72A21* expression in the shoots has also been increased by trifluralin and esprocarb (*17*). However, only a few plant lines showed slight accumulation of anthocyanin with the treatment of 100 μM trifluralin or esprocarb. It may be because the rice plants were not able to grow normally in the medium containing high concentrations (100 μM) of these herbicides and, consequently, the expression of other regulatory genes for anthocyanin synthesis was very low. Treatment of these herbicides also might induce some other genes, which have inhibitory effects on anthocyanin synthesis, similar to the case of 2,4-D treatment. The relationships between the transcription factor *OSB2* and the structural genes for anthocyanin synthesis in rice are still unknown and remain to be investigated.

In this study, we demonstrated that the pBOSB21 rice plants can be used for the biomonitoring of chloroacetamide herbicides. Further research into specific chemical-responsive promoters and the identification of chemical-responsive elements is needed to increase the accuracy of such biomonitoring. To enhance the accumulation of anthocyanin for producing transgenic plants for biomonitoring, other transcriptional factors such as *OsC1* and *DFR* for anthocyanin pigmentation should be considered. Transgenic rice plants expressing *OSB2* as a reporter gene, with appropriate promoters, would be useful for detecting environmental pollution and chemicals in soils and in the waters of the surrounding environment.

ACKNOWLEDGMENT

The rice isogenic line Taichung 65, No. 99-962 T-65 C^BA, BC_9F_5 was kindly provided by Professor M. Maekawa in Okayama University, Japan. We thank Prof. W. Sakamoto

(Okayama University, Japan) for providing the rice *OSB2* cDNA and Dr. S. Toki (National Institute of Agrobiological Sciences (NIAS), Ibaraki, Japan) for providing the expression vector *pBI101-Hm*.

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Received for review May 30, 2007. Revised manuscript received November 29, 2007. Accepted November 30, 2007. This study was supported by the Program for the Promotion of Basic Research Activities of Innovative Biosciences of the Bio-oriented Technology Research Advancement Institution (BRAIN), Tokyo, Japan.

JF071575J