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Overexpression of *OSB2* gene in transgenic rice up-regulated expression of structural genes in anthocyanin biosynthesis pathway

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ABSTRACT

Anthocyanin biosynthesis is regulated by regulatory genes encoding transcription factors which control expression of several structural genes in the anthocyanin pathway. The *OSB2* gene encoding a Myc-type basic-helix-loop-helix (bHLH) transcription factor which regulates anthocyanin biosynthesis was previously cloned from a Thai black rice variety Khum. In this study, the cloned *OSB2* gene was transformed into two white rice varieties Nipponbare and Taichung 65 using *Agrobacterium* to study the regulation of anthocyanin biosynthesis in rice. The semi-quantitative RT-PCR analysis revealed that transgenic rice plants overexpressing the *OSB2* gene up-regulated the expression of some structural genes in anthocyanin biosynthesis including early biosynthesis gene (*EBG*) i.e. *F3H* (flavanone 3-hydroxylase) and also late biosynthesis genes (*LBGs*) i.e. *DFR* (dihydroflavonol 4-reductase) and *ANS* (anthocyanidin synthase). The present results support the other evidences that the regulatory mechanism of anthocyanin biosynthesis in rice may be different from other plants in which *EBGs* and *LBGs* were regulated separately. The results provide the understanding of functions of *OSB2* gene in regulation of anthocyanin biosynthesis pathway in rice and will be useful to apply this gene as a marker gene for the improvement of rice varieties.

Keywords: anthocyanin biosynthesis; *OSB2* gene; *Oryza sativa*; transformation; *Agrobacterium*

INTRODUCTION

Anthocyanins are water-soluble pigments which are classified as a major class of flavonoids. Anthocyanins accumulate in various parts of plants as secondary metabolites and are red and purple in color. They serve as antioxidants and play an important role in a wide range of biological functions including attraction of insects and birds for pollination and protection of plants against UV radiation, microbial pathogens and insect attack (Harborne and Williams, 2000; Schijlen *et al*., 2004). Anthocyanins have received much attention because of their beneficial characteristics for human health. Their properties include inhibition of cell proliferation and being antimutagenic, antimicrobial, anti-inflammatory, antioxidant and antihypertensive (Shen *et al*., 2009; Seo *et al*., 2011).

Anthocyanin biosynthesis involves two groups of genes which are regulatory and structural genes. The regulatory genes encoding transcription factors are classified into two major types in plants that

include the *R/B* gene family encoding Myc basic-helix-loop-helix (bHLH) proteins and *C1/PI* gene family encoding Myb-type R2R3 proteins (Geekiyanage *et al*., 2007). The structural genes encode the enzymes that catalyze several steps in anthocyanin biosynthesis pathway. Expression levels of structural genes are controlled by regulatory genes that influence the intensity and pattern of anthocyanin biosynthesis.

In rice (*Oryza sativa* L.), the regulatory genes namely *OSB1* and *OSB2* have been identified. These genes have been isolated from the *PI* locus of rice on chromosome 4 and encode Myc-type bHLH transcription factors which are similar to the maize *R/B* genes. Transient expression assay showed that *OSB2* gene could induce anthocyanin biosynthesis in rice (Sakamoto *et al*., 2001). In previous report, the *OSB2* gene was not expressed in seedlings of non-pigmented *japonica* rice cultivars (Shih *et al*., 2008). The recent study has shown that the expression of *OSB2* gene was found only in pigmented rice varieties (Inta *et al*., 2013), suggesting that no anthocyanin accumulation in non-pigmented rice may be due to lack of *OSB2* expression.

The structural genes in anthocyanin biosynthesis can be divided into two groups composed of early biosynthesis genes (*EBGs*) and late biosynthesis genes (*LBGs*). The *EBGs* include the major genes encoding chalcone synthase (CHS), chalcone isomerase (CHI) and flavanone 3-hydroxylase (F3H) and the *LBGs* include the important genes encoding dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS) (Schijlen *et al*., 2004). In dicot plants, expression of *EBGs* and *LBGs* are controlled separately (Quattrocchio *et al*., 2006). In pigmented rice, the *OSB2* gene activated some structural genes which were *F3H* (*EBG*), *DFR* and *ANS* (*LBGs*), suggesting probably a different regulatory mechanism of anthocyanin biosynthesis in rice (Shih *et al*., 2008). The relationship between transcription factors encoded by regulatory genes and the structural genes of anthocyanin biosynthesis in rice remains to be investigated.

The aim of this study was to examine the functions of the *OSB2* gene in the regulation of anthocyanin biosynthesis pathway in rice. The *OSB2* gene was cloned from black rice variety Khum and transformed to white rice varieties Nipponbare and Taichung 65. The transgenic rice plants overexpressing *OSB2* gene showed no anthocyanin accumulation in leaves and stems; however, up-regulation of structural gene expression was detected.

MATERIALS AND METHODS

Construction of binary vector for rice transformation

The *OSB2* gene of 1,365 bp isolated from cDNA prepared from total RNA of young leaves of black rice variety Khum (collected from Nong-Tao-Kham village, Sansai district, Chiang Mai, Thailand) was revealed by our previous report (Inta *et al*., 2013). The *OSB2* gene was cloned in pGEM-T Easy (Promega, USA) and digested by *Eco*RI. Then the *Eco*RI-cut *OSB2* gene was blunt ended by using End-It™ DNA End-Repair Kit (Epicentre, USA) and inserted into p2CA (pUC19 containing a dual *35s* promoter and a *nos* terminator) to construct the cassette of *OSB2* gene under the control of a dual *35s* promoter and a *nos* terminator. The gene cassette (dual *35s*P::*OSB2*::T*nos*) was cloned into a binary vector pCAMBIA1305.1 (Cambia, Australia) at *Hin*dIII/*Eco*RI sites to construct pPI01_B2S which contained hygromycin phosphotransferase (*hptII*) as a selectable marker gene and β-glucuronidase (*GUSPlus*) as a reporter gene (Figure 1). The pPI01_B2S was introduced into *Agrobacterium tumefaciens* strain AGL1 to be used for transformation of rice.

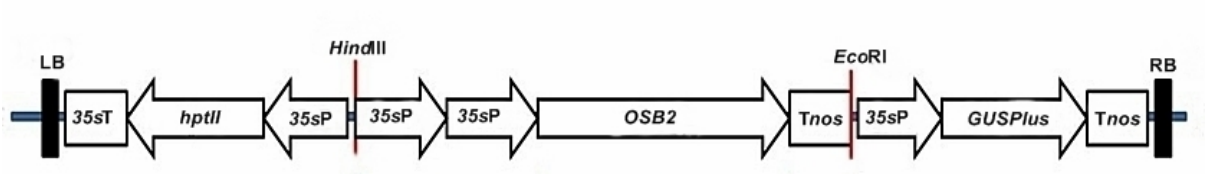

Figure 1 Schematic presentation of T-DNA region of pPI01_B2S plasmid harboring the *OSB2* gene expression cassette which was cloned in the binary vector pCAMBIA1305.1. LB: left border, RB: right border, *35s*P: *35s* Promoter, T*nos*: *nos* terminator, *35s*T: *35s* terminator, *hptII*: hygromycin phosphotransferase gene, *GUSPlus*: β-glucuronidase gene. *Hin*dIII and *Eco*RI restriction sites were marked.

***Agrobacterium*-mediated transformation of rice**

Japonica rice varieties Nipponbare and Taichung 65 were used for transformation of pPI01_B2S. Mature rice seeds were used for callus induction for 4 weeks (Toki, 1997). The scutellum-derived embryogenic calli from mature rice seeds were subcultured using fresh induction medium for 3–5 days. The subcultured calli were infected with *A. tumefaciens* strain AGL1 harboring pPI01_B2S and then co-cultivated for 3 days. After 3-day co-cultivation, the calli were washed to remove excess bacteria and then cultured on selection medium supplemented with 15 mg/l hygromycin and 150 mg/l timentin for 2 weeks. Following the 1st selection, the calli were transferred to the 2nd selection medium supplemented with 30 mg/l hygromycin and 150 mg/l timentin and cultured for 2 weeks.

After 2 cycles of selection, the surviving calli were transferred to regeneration medium supplemented with 30 mg/l hygromycin and 150 mg/l timentin and cultured at 28 ± 2 ^{o}C with a 16 h photoperiod and later subcultured every two weeks until the calli were regenerated to plantlets. Then, the plantlets were transferred to half-strength MS medium for root formation (Sakulsingharoj *et al.*, 2014).

GUS assay

After 3 days of co-cultivation, some calli were washed to remove excess *Agrobacterium* then incubated in X-Gluc solution at 37 ^{o}C for 1 hour to overnight (Jefferson, 1987). The transient GUS expression in the calli was examined and compared with that in the untransformed calli. For the transformed plants, leaf segments were cut and immersed in X-Gluc solution followed by incubation at 37 ^{o}C for 1 hour to overnight. After staining, the leaf segments were bleached out in 95 % ethanol to remove chlorophyll and then GUS expression was investigated.

PCR analysis of transformed plants

Genomic DNA was extracted from young leaves of transformed rice plants and untransformed control plants using modified CTAB method (Hwang and Kim, 2000). The extracted DNA was subjected to PCR methods using primers specific to *OSB2* gene. The primers were OSB2cdsF: 5'-ATGGCATCT GCTCCTCCAGTTCAGG-3' and OSB2cdsR: 5'-TTA CGGCGCCTTCCCCTGTCC-3'. The PCR amplification was carried out in a 20 µl reaction mixture containing GoTaq® Green Master Mix (Promega, USA), 100 ng of DNA and 0.5 µM of each primer. The PCR profile was initially at 95 ^{o}C for 3 min, followed by 35 cycles at 95 ^{o}C for 1 min, 68 ^{o}C for 1 min and 72 ^{o}C for 1.5 min and 5 min at 72 ^{o}C for final extension. The expected PCR products of 1,356 bp were analyzed on a 1 % (w/v) agarose gel by electrophoresis.

Expression analysis by semi-quantitative RT-PCR

Total RNA was extracted from young leaves of 2-week-old rice seedlings using the TRIzol method (Life Technologies, USA). The extracted RNA was treated with DNase I (New England Biolabs, UK) at 37 °C for 10 min to remove contaminated DNA. The DNase I – treated RNA samples were reverse transcribed by Superscript III first-strand synthesis system (Life Technologies, USA) according to the manufacturer's instructions. For the template, 1 μg of total RNA was used in a 10 μl Reverse Transcription (RT) reaction. The RT profile was as follows: denaturation and annealing of oligo (dT) at 65 °C for 5 min, reverse transcription at 50 °C for 50 min, and reaction termination at 85 °C for 5 min.

Gene-specific primers were designed from coding regions of different rice anthocyanin structural genes. A list of primers was shown in Table 1. Amplification of target cDNA was performed with GoTaq® Green Master Mix. The PCR profile was 95 °C for 3 min, 27 cycles at 95 °C for 1 min, 52-56 °C for 1 min and 72 °C for 1 min, and 5 min at 72 °C for the final extension. Aliquots of PCR products were analyzed on a 1 % (w/v) agarose gel by electrophoresis. For semi-quantitative RT-PCR assays, the total amount of cDNA in samples was standardized after the amount of actin mRNA was evaluated with an *OsActin* primer pair (Table 1).

Table 1 Primers used in semi-quantitative RT-PCR analysis for amplification of anthocyanin structural genes.

Genes	Primer names and sequences (5′→3′)
OsCHS	OCHSF: CGGACTGGAACTCCATCTTC
	OCHSR: TAAAAGATGACGTGTGGCGTA
OsCHI	OCHIF: TCCATCCTCTTCACCCACTC
	OCHIR: TGTCAAACACGAGGGCAGTA
OsF3H	OsF3HF: GAGCAATGGGAGGTTCAAGA
	OsF3HR*:* CTTCGATTTTCGACGGAAGA
OsDFR	ODFRF: CGGGTTCAGGTTCAGGTACA
	ODFRR: TGAAACCGGAGGGAGTAAC
OsANS	OANSF: GAAGAGGGAGTGGGAGGACT
	OANSR: CAGAAGACGACCCAGGAGAG
OsActin	OsactinF: TGATGCGCCCAGGGCTGTCT
	OsactinR: CGATTGGCCTTGGGGTTGAG

RESULTS

Transformation of *OSB2* gene into calli of rice varieties Nipponbare and Taichung 65

The *OSB2* gene from black rice variety Khum was cloned into binary vector pCAMBIA1305.1 under the control of a dual *35s* promoter and a *nos* terminator to construct plasmid named pPI01_B2S (Figure 1). Plasmid pPI01_B2S was transformed into embryogenic calli derived from mature seeds of Nipponbare and Taichung 65 rice using *A. tumefaciens* strain AGL1. After 3-day co-cultivation, the GUS activity was detected on transformed calli of both rice varieties (Figure 2). The highest percentage of GUS activity observed on transformed calli of Nipponbare and Taichung 65 rice was 90.48 and 100, respectively.

It was found that the surviving calli after 2 cycles of selection for 4 weeks was 96 % and 98.75 % for Nipponbare and Taichung 65 rice, respectively. The surviving calli were transferred to regeneration medium supplemented with 30 mg/l hygromycin and 150 mg/l timentin. After 4 weeks of regeneration, 6.25% of Nipponbare calli showed the green spots which could regenerate into 16 plantlets while 7.59% of Taichung 65 calli showed green spots after regeneration of 2 weeks and turned into 13 plantlets (Figure 3). However, red spots on calli and anthocyanin accumulation on plantlets were not observed.

GUS assay was performed with transformed rice plants. Only 4 out of 6 putative transgenic Nipponbare rice lines showed GUS positive, while GUS expression was also detected in all 7 putative transgenic Taichung 65 rice lines (Figure 4).

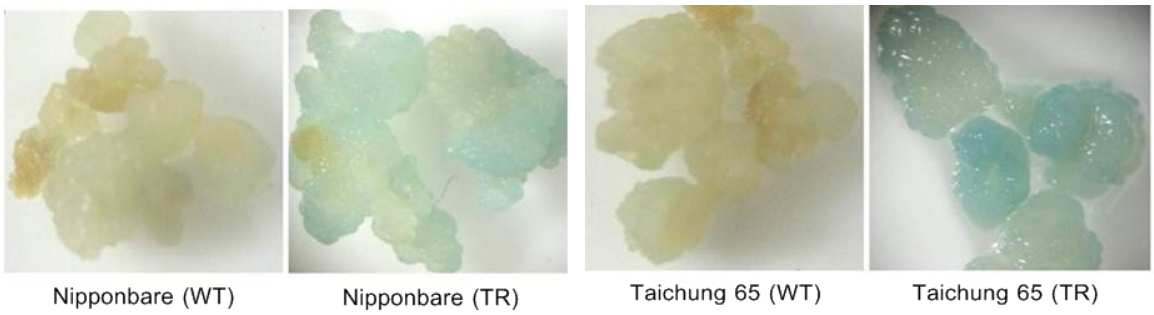

Figure 2 GUS expression in calli of rice varieties Nipponbare and Taichung 65 after 3-day co-cultivation. Transformed callus (TR) and untransformed (control) callus (WT).

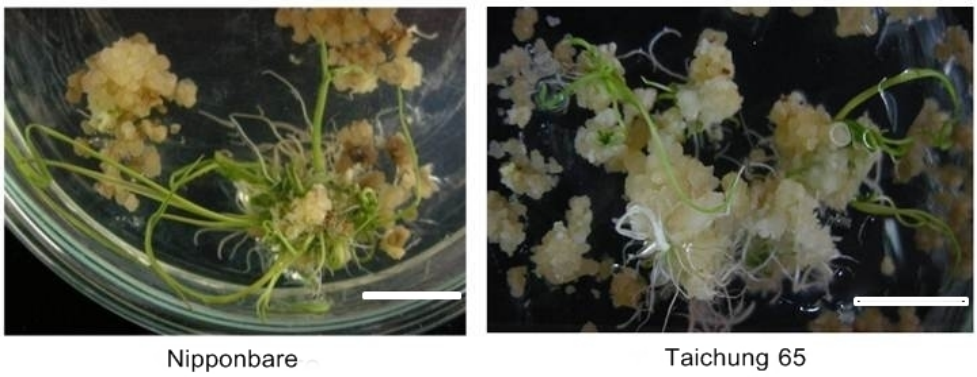

Figure 3 Regeneration of shoots from transformed calli of rice varieties Nipponbare and Taichung 65 cultured on regeneration medium containing 30 mg/l hygromycin and 150 mg/l timentin for 4-6 weeks (subcultured every two weeks). Bar = 1 cm.

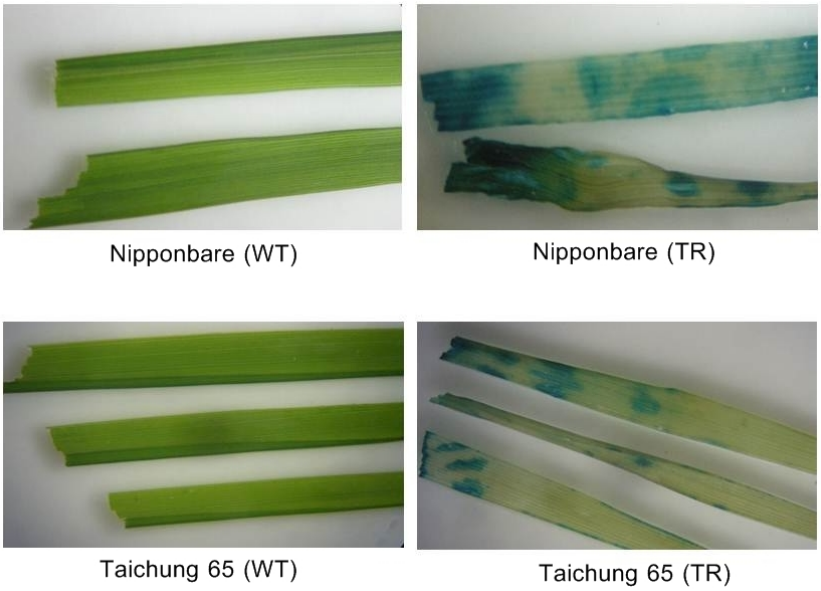

Figure 4 GUS expression on leaf segments of transgenic rice varieties Nipponbare and Taichung 65. TR: transformed rice. WT: wild-type rice used as control.

PCR analysis of *OSB2* gene in putative transgenic rice plants

The transgenic lines with GUS positive were analyzed for the presence of *OSB2* gene in their genome. Two of Nipponbare and 5 of Taichung 65 transgenic lines were analyzed. PCR analysis showed that about 1,300 bp fragments were amplified, suggesting the presence of the introduced *OSB2* gene in all transgenic rice plants (Figure 5). Transgenic rice plants of both varieties were obtained for further analysis.

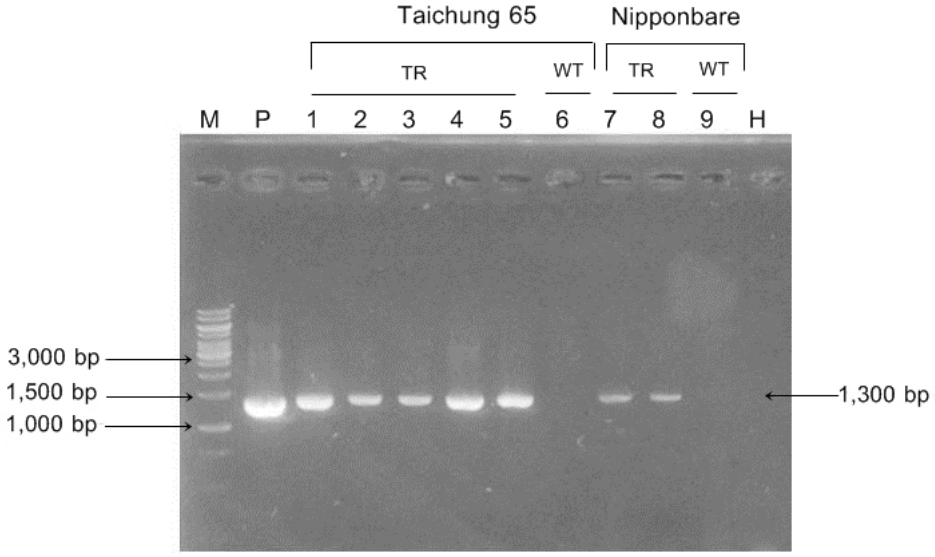

Figure 5 PCR analysis of transgenic plants of rice varieties Nipponbare and Taichung 65 using primers specific to *OSB2* gene. Lane M: 1 kb DNA Ladder, Lane P: plasmid pPI01_B2S (positive control), Lanes 1-5: transgenic plants of Taichung 65, Lanes 7-8: transgenic plants of Nipponbare. Lanes 6 and 9: wild-type plants (negative control) of Taichung 65 and Nipponbare, respectively. Lane H: distilled water.

Expression of *OSB2* gene and structural genes in transgenic rice plants

The expression levels of *OSB2* and structural genes were analyzed in both varieties of transgenic rice by semi-quantitative RT-PCR (Figure 6). The expression of *OsActin* gene was consistent in all wild-type and transgenic plants and was used as a reference control. The expression of *OSB2* transcripts was detected in all transgenic rice plants but not in wild-type plants, indicating that the *OSB2* gene introduced into their genome was expressed in transgenic rice. The transgenic Taichung 65 showed higher level of *OSB2* expression than the transgenic Nipponbare rice.

The structural genes involved in anthocyanin biosynthesis, including early anthocyanin biosynthesis genes (*EBGs*) such as *CHS, CHI* and *F3H* as well as late anthocyanin biosynthesis genes (*LBGs*) such as *DFR* and *ANS*, were analyzed. The expression patterns for *EBGs, CHS* and *CHI*, in all transgenic and wild-type plants were similar. However, *F3H* gene expression was detected only in transgenic plants. Transgenic Nipponbare rice showed higher level of *F3H* expression than that of transgenic Taichung 65 rice. The expression patterns of *LBGs* (*DFR* and *ANS*) were correlated with the presence of *OSB2* expression (Figure 6).

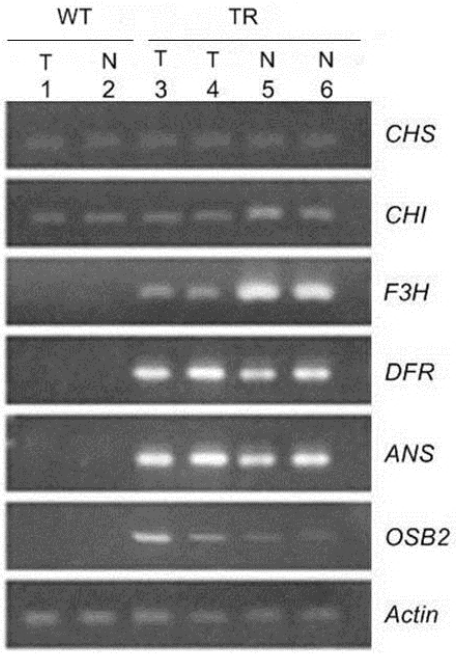

Figure 6 Semi-quantitative RT-PCR analysis of transgenic plants of rice varieties Taichung 65 (T) and Nipponbare (N). Total RNA was extracted from young leaves of 2-week-old seedlings and subjected to RT-PCR using primers specific to *OSB2*, *CHS*, *CHI*, *F3H*, *DFR* and *ANS* genes. Lanes 1 and 2: wild-type plants of Taichung 65 and Nipponbare, respectively. Lanes 3 and 4: transgenic Taichung 65 line 1 and 2. Lanes 5 and 6: transgenic Nipponbare line 1 and 2. *CHS*: chalcone synthase, *CHI*: chalcone isomerase, *F3H*: flavanone 3-hydroxylase, *DFR*: dihydroflavonol 4-reductase, *ANS*: anthocyanidin synthase, *OSB2*; Myc-type bHLH transcription factor gene from black rice variety Khum and *Actin*: rice *Actin* gene (control).

DISCUSSION

Production of transgenic rice transformed with *OSB2* gene

Two white rice varieties were used for transformation of the *OSB2* gene cloned from black rice variety Khum by *Agrobacterium* method. In previous reports (Saika and Toki, 2010; Yara *et al*., 2001), it was found that transformation efficiency in Nipponbare was higher than that of Taichung 65 rice. However, in the present study, transformation efficiency of both rice varieties was similar.

Transgenic rice plants of both varieties were confirmed by PCR analysis for the presence of *OSB2* transgene. The PCR products of about 1,300 bp indicated the expected size of *OSB2* transgene which was different from the 24-kb endogenous *OSB2* gene in rice genome (Sakamoto *et al*., 2001).

In other reports, the rice *OSB2* gene induced anthocyanin accumulation in transgenic rice variety Taichung 65 and could be used as a reporter gene for biomonitoring of chemicals in environment (Hirose *et al*., 2008; Kawahigashi *et al*., 2007). However, the anthocyanin pigmentation was not observed on hygromycin-resistant calli and transgenic plants, suggesting that Taichung 65 lines used in this study may be different from the one used in previous report, so it may lack other functional genes required for anthocyanin biosynthesis.

Overexpression of *OSB2* gene up-regulated structural gene expression

In this study, the *OSB2* expression was not detected in wild-type white rice varieties both Nipponbare and Taichung 65 which was consistent with the previous studies (Inta *et al*., 2013; Shih *et al*., 2008). The semi-quantitative RT-PCR results showed that the *OSB2* overexpression in transgenic rice plants up-regulated the expression of three structural genes including *F3H*, *DFR* and *ANS* which encode the enzymes involved in anthocyanin biosynthesis pathway.

In dicot plants, expression of the early biosynthesis genes (*EBGs*) encoding CHS, CHI and F3H as well as the late biosynthesis genes (*LBGs*) encoding DFR and ANS are controlled separately (Quattrocchio *et al*., 2006). In pigmented rice seedlings, the expression patterns of structural genes of anthocyanin biosynthesis were different from the other plants (Shih *et al*., 2008). The rice Pl^{w} locus which harbors two bHLH genes, *OSB1* and *OSB2*, activated only some structural genes in rice seedlings which were *F3H*, *DFR* and *ANS*. The *F3H* gene classified as an *EBG* in dicot plants was differentially up-regulated with the *LBGs* (*DFR* and *ANS*). Our results were consistent with the previous reports of Shih *et al*. (2008), suggesting that regulatory mechanism for anthocyanin biosynthesis in rice may be different from other dicot plants.

The regulation of anthocyanin biosynthesis in plants requires transcription factors including the *R/B* gene family encoding basic-helix-loop-helix (bHLH) and *C1/Pl* gene family encoding Myb-type R2R3 proteins (Lepiniec *et al*., 2006). In rice, the *R/B* gene family has been identified as *OSB1/OSB2* (Sakamoto *et al*., 2001) and *C1/Pl* gene family as *OSC1* (Saitoh *et al*., 2004). The activation of structural genes requires functional alleles of both *R/B* and *C1/Pl* gene families in some cases (Lim and Ha, 2013). Although transgenic rice plants showed up-regulation of the structural genes by the regulatory *OSB2* gene, anthocyanin pigmentation was not observed in all transgenic plants. In this case, other transcription factors such as OSC1 which is Myb-type transcription factor classified in *C1/Pl* gene family may be needed to induce expression of structural genes.

In conclusion, overexpression of the *OSB2* gene activated three structural genes in anthocyanin

biosynthesis including *OsF3H, OsDFR* and *OsANS* which supported the unique regulatory mechanism of anthocyanin biosynthesis in rice. Further investigation on the molecular regulation of structural genes by transcription factors is required for engineering pigmentation in rice tissues that has no anthocyanin biosynthesis. The complicated regulation mechanism of anthocyanin biosynthesis may require combined interaction of several types of proteins including transcription factors and other enzymes encoded by regulatory and structural genes, respectively. Thus, further study of the function of the *OSB2* gene would be conducted together with other regulatory genes and structural genes in rice plants to gain more understanding of anthocyanin biosynthesis in rice and to be applied as a marker gene for the improvement of rice varieties through molecular breeding and genetic engineering.

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