Cloning and characterization of *OSB1* gene controlling anthocyanin biosynthesis from Thai black rice

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ABSTRACT

OSB1 gene encodes *myc*-type basic helix-loop-helix (bHLH) transcription factor which controls expression of several structural genes involving in anthocyanin biosynthesis in rice. In this study, the expression of *OSB1* gene was investigated in young leaves and developing seeds of six rice varieties, including white (Taichung 65 and Sasanishiki), red (Sang Yod and Hom Mali Dang) and black (Khum and Lerm Poa), by RT-PCR. The *OSB1* gene was expressed in both leaves and seeds of all six rice varieties. The full-length coding sequences of *OSB1* genes were isolated from young leaves of white rice Sasanishiki and black rice Khum. Nucleotide sequence analysis revealed that Khum and Sasanishiki had open reading frames (ORF) of 1,767 and 1,725 bp, respectively which were 99–100% and 100% identical with *OSB1* genes in black and white rice reported in GenBank database, respectively. It was found that *OSB1* gene from white rice showed a 2-bp insertion in the 7th exon that caused frameshift mutation and premature termination, leading to the truncation of 14 amino acids at C-terminus of the regulatory protein. Moreover, the amino acid substitution of T64M was found in white rice OSB1 protein, affecting the conserved N-terminal interacting domain, probably causing the non-function of the *OSB1* gene in white rice. The *OSB1* gene from black rice was active in anthocyanin biosynthesis, suggesting this gene might play an important role in anthocyanin pigmentation, especially in seeds. The cloned *OSB1* genes will be further analyzed for gene functions to understand the regulation of anthocyanin biosynthesis in colored rice.

Keywords: anthocyanin biosynthesis; *OSB1* gene; *Oryza sativa*; bHLH regulatory proteins

INTRODUCTION

Anthocyanins are pigments which are classified as a major class of flavonoids. Accumulation of anthocyanin is found in various plant parts displaying red and purple color phenotype. Anthocyanins serve as antioxidants and have several biological functions which are attraction of insects and birds for pollination and protection of plants against UV light, pathogens and insects (Harborne and Williams, 2000; Schijlen *et al*., 2004). Anthocyanins have received considerable attention due to their beneficial health effects, including inhibition of cell proliferation and significant properties of being antimutagenic, antimicrobial, anti-inflammatory, antioxidant and antihypertensive (Akihisa *et al*., 2003; Parejo *et al*., 2004; Shen *et al*., 2009; Seo *et al*., 2011).

The anthocyanin biosynthesis has been extensively studied in maize. There are two major classes of genes involved in anthocyanin biosynthesis which are regulatory and structural genes. The regulatory genes encoding transcription factors that function in the regulation of anthocyanin biosynthesis in plants are classified into two families, *R/B* and *C1/Pl*. The members of *R/B* gene family encode typical basic helix-loop-helix (bHLH) *myc*-type protein (Chandler *et al*., 1989; Ludwig and Wessler, 1990) On the other hand, the member of *C1/Pl* gene family encode *myb*-type R2R3 regulatory protein (Paz-Ares *et al*., 1987; Cone *et al*., 1993;). The interaction of *R/B* and *C1/Pl* genes controls anthocyanin biosynthesis to accumulate pigments in a tissue-specific fashion. The structural genes encode the enzymes catalyzing several steps in anthocyanin biosynthesis pathway. The *R/B* and *C1/Pl* genes coordinately control anthocyanin biosynthesis in various plant tissues by activation of structural genes (Goff *et al*., 1990; Roth *et al*., 1991; Tuerck and Fromm, 1994; Bodeau and Walbot, 1996).

In rice, the *R/B* genes were identified as *Ra1* (formerly *Ra*), *Ra2* and *Rb* (Hu *et al*., 1996; Hu *et al*., 2000), *OSB1* and *OSB2* (Sakamoto *et al*., 2001) while the *C1/Pl* gene was isolated as *OsC1* (Saitoh *et al*., 2004). The functional alleles of the *R/B* and *C1/Pl* gene families may be required, in some cases, for transcriptional activation of structural genes.

The *Purple leaf* (*Pl)* locus of rice affects anthocyanin pigmentation in various tissues and plays a role in regulation of anthocyanin biosynthesis similar to the maize *R/B* family. The Pl^{w} allele is on a *Pl* locus in the nearly isogenic line Taichung 65-Plw (T65-Plw) generated by using *japonica* rice line Taichung 65 (T65) as a recurrent parent. Two rice genes, namely *OSB1* and *OSB2*, which are located on Pl^{w} locus on chromosome 4 and encode *myc*-type bHLH transcription factors controlling anthocyanin biosynthesis, were identified from T65-Plw (Sakamoto *et al*., 2001). The *OSB1* and *OSB2* genes are homologous to maize *B-Peru* gene. The *OSB1* gene is an allele of rice *Ra1* gene reported by Hu *et al*. (1996).

The expression *OSB2* genes was restricted to black rice, including purple T65-Plw (Shih *et al*., 2008) and Thai black rice varieties (Inta *et al*., 2013). We previously cloned the full-length *OSB2* gene from Thai black rice Khum (Inta *et al*., 2013) and found that this gene could up-regulated the expression of structural genes involved in anthocyanin biosynthesis in rice (Sakulsingharoj *et al*., 2014).

The *OSB1* genes were expressed in white T65 and purple T65-Plw (Shih *et al*., 2008). The sequences of *OSB1* gene were different between white and colored rice. The 2-bp addition in *OSB1* gene was found in white rice varieties, causing a frameshift at the C-terminus and premature termination of regulatory protein. The *OSB1* gene in black rice was functional in anthocyanin pigmentation while the inactive *OSB1* gene with a 2-bp addition in red and white rice showed the absence of anthocyanin biosynthesis (Wang and Shu, 2007; Lim and Ha, 2013).

In this study, we investigated the expression of *OSB1* gene in rice varieties with white, red and black (dark purple) pericarp colors. Sequencing analysis revealed the differences of nucleotide and amino acid sequences of the *OSB1* genes between white and black rice. Our finding suggested that the *OSB1* gene might play an important role in regulation of anthocyanin pigmentation in rice pericarp.

MATERIALS AND METHODS

Isolation of total RNA from rice leaves and seeds

Six rice varieties were used in the experiment. There were white rice varieties, including Taichung 65 and Sasanishiki, red rice varieties, including Sang Yod and Hom Mali Dang, and black rice varieties, Khum (collected from Nong-Tao-Kham village, Sansai district, Chiang Mai, Thailand) and Lerm Poa (provided by Maejo University). Total RNA was extracted from young leaves of 2-week-old rice seedlings and developing rice seeds (about 15-day after flowering) using the TRIzol method (Life Technologies, USA). The extracted RNA was treated with DNase I (New England Biolabs, UK) at 37 ºC for 10 min to remove contaminated DNA.

Expression analysis of *OSB1* gene by RT-PCR

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, about 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 ºC for 5 min, reverse transcription at 50 ºC for 50 min, and reaction termination at 85 ºC for 5 min. Gene-specific primers were designed from coding regions of *OSB1* gene (AB021079), OB1_F: 5´-GGATGGTCTCCTTGGACTGA-3´ and OB1_R: 5´-GGGTGGCAGATTCATCACTT-3´. This primer pair spanned the 7th intron of *OSB1* gene to avoid co-amplification of genomic DNA, giving the expected RT-PCR products of about 360 bp. Amplification of target cDNA was performed with GeNei™ Red dye PCR master mix (Merck, USA). The PCR profile was 95 ºC for 5 min, 35 cycles at 95 ºC for 1 min, 55 ºC for 1 min and 72 ºC for 1 min, and 7 min at 72 ºC for the final extension. Aliquots of PCR products were analyzed on a 1% (w/v) agarose gel by electrophoresis.

Cloning of full-length coding sequence of *OSB1* gene

To isolate the full-length coding sequence of *OSB1* gene from rice varieties Sasanishiki and Khum, cDNA was used as template for PCR using primers specific to complete coding sequence of *OSB1* gene (AB021079), OSB1cds_F: 5´-ATGGA AGAGACCCCTCTGCCATC-3´ and OSB1cds_R: 5´-CTAGCTAGCTAGCTTGCTATAGCTTTCC-3´. The amplification was performed with GoTaq® PCR master mix (Promega, USA). The PCR profile was 95 ºC for 5 min, 40 cycles at 95 ºC for 1 min and 68 ºC for 5 min, and 10 min at 68 ºC for the final extension. Aliquots of PCR products were analyzed on a 1% (w/v) agarose gel by electrophoresis. The expected PCR product was about 1,800 bp.

The amplified fragments were cloned into the pGEM-T Easy vector (Promega, USA). The recombinant vectors were transformed into competent cells of *E. coli* DH5α. The recombinant clones were selected by blue/white screening, rapid size screening and restriction enzyme digestion. Then, the selected recombinant plasmids were subjected to sequencing analysis by 1st BASE (Malaysia).

Analysis of nucleotide and amino acid sequences of *OSB1* gene

The nucleotide and amino acid sequences of the cloned *OSB1* genes from white (Sasanishiki) and black (Khum) rice varieties were analyzed and compared with *OSB1* gene reported in GenBank. Multiple sequence alignments were performed using ClustalX 1.83. The motifs of amino acid sequences were analyzed by Pfam (http://pfam.xfam.org/).

RESULTS AND DISCUSSION

Expression of *OSB1* gene in rice leaves and seeds

Expression of *OSB1* gene was analyzed in six rice varieties, including each two of white, red and black rice. The cDNA prepared from total RNA of young leaves and developing seeds of rice was subjected to RT-PCR analysis. The results showed that the expected 360-bp fragments were amplified in all rice samples, suggesting the expression of *OSB1* gene in white (Taichung 65 and Sasanishiki), red (Sang Yod and Hom Mali Dang) and black rice (Khum and Lerm Poa) (Figure 1). In addition, *OSB1* expression was found in both leaves and seeds of all rice varieties analyzed.

The *Purple leaf* (*Pl*) locus on chromosome 4 of rice is necessary for anthocyanin accumulation in leaves and shoots. Pl^{w} allele in isogenic line Taichung 65-Plw (T65-Plw) includes two genes, *OSB1* and *OSB2*. Expression analysis of *OSB2* was found not only in colored rice leaves of *japonica* T65-Plw (Sakamoto *et al*., 2001; Shih *et al*., 2008) but also in leaves and seeds of *indica* Thai black rice varieties Lerm Poa, Hom Nil and Khum (Inta *et al*., 2013).

However, expression of *OSB1* was previously found in leaves of both white and colored rice (Shih *et al*., 2008). In this study, we found the *OSB1* expression in leaves, corresponding to the finding of Shih *et al*. (2008) and also in seeds of white and colored rice. These results suggested that *OSB1* gene might also be important for anthocyanin accumulation in seeds.

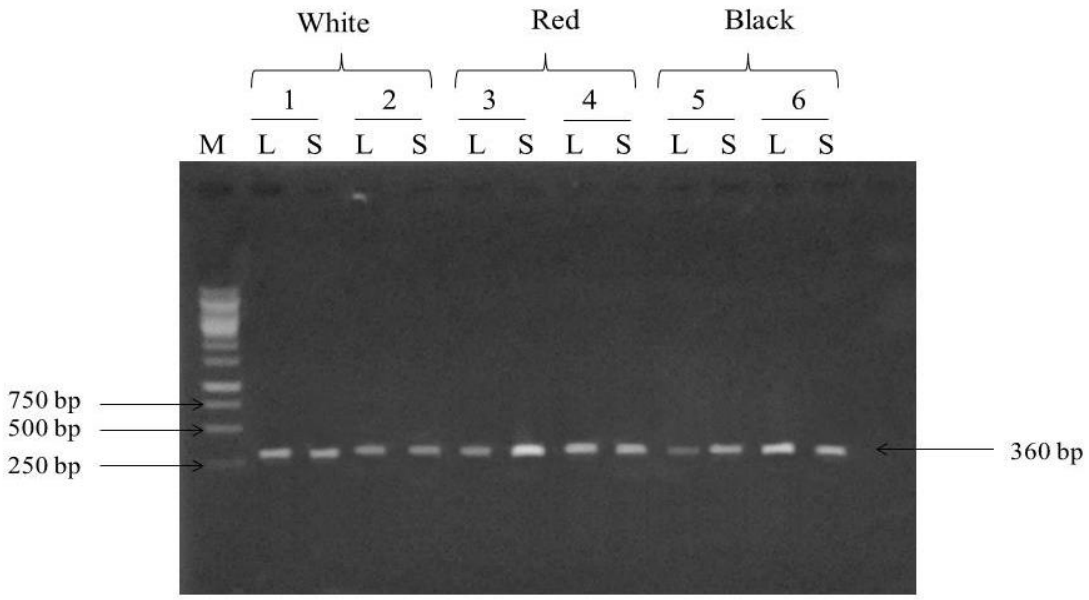


Figure 1 Expression analysis of *OSB1* gene in leaves (L) and seeds (S) of six rice varieties. White pericarp rice varieties were Taichung 65 and Sasanishiki. Red pericarp rice varieties were Sang Yod and Hom Mali Dang. Black pericarp rice varieties were Khum and Lerm Poa. Lane M, GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, USA).

Identification and cloning of full-length coding sequence of rice *OSB1* gene

To isolate and clone the *OSB1* gene, cDNA prepared from young leaves of rice varieties was subjected to PCR using primers specific for full-length coding sequence of rice *OSB1* gene. We obtained the expected about 1800-bp of amplified fragments from cDNAs of white rice Sasanishiki and black rice Khum (Figure 2).

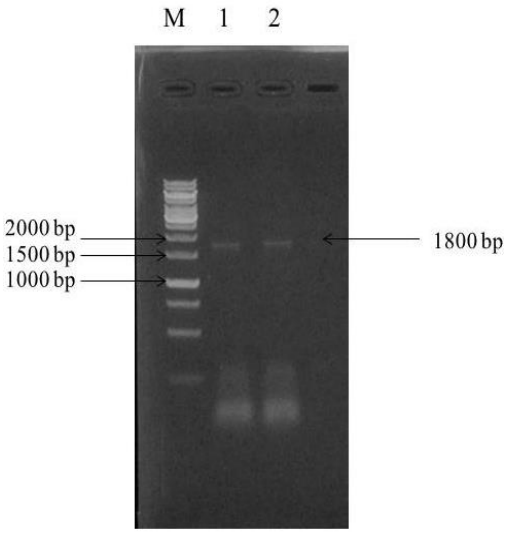


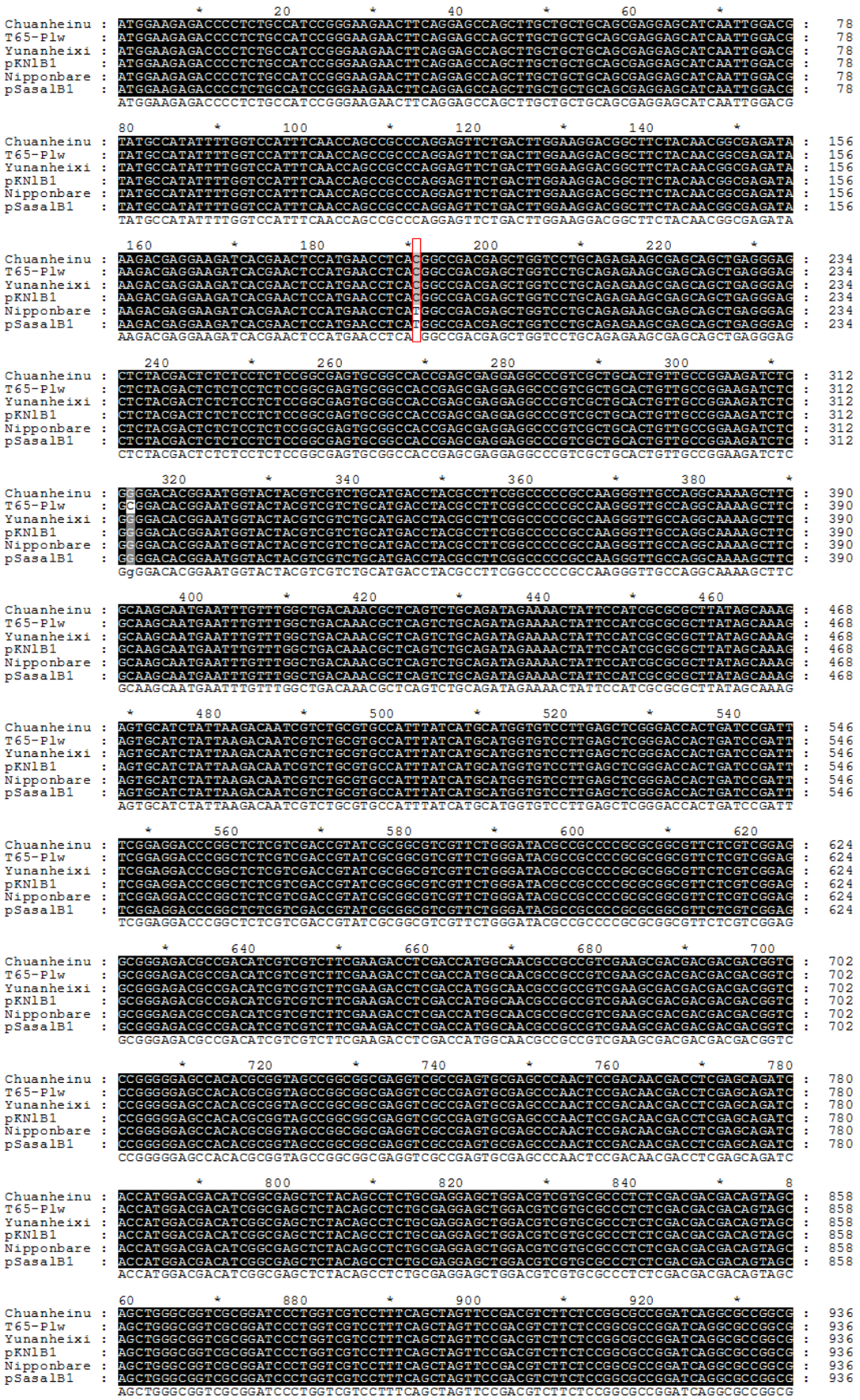
Figure 2 RT-PCR analysis of the full-length coding sequences of *OsB1* gene in white rice Sasanishiki and black rice Khum using cDNA prepared from rice leaves. Lane M, GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, USA). Lane 1–2, Sasanishiki and Khum, respectively.

The *OSB1* gene isolated from leaf cDNA library of colored T65-Plw rice plants by using maize *B-Peru* cDNA as a probe showed high similarity with the *R* gene family of maize (Sakamoto *et al*., 2001). The 2.2-kb *OSB1* cDNA composed of a 1,767 bp open reading frame (ORF) which gave rise to polypeptide containing 588 amino acids (Sakamoto *et al*., 2001). The isolated *OSB1* gene appeared to be allelic to *Ra1* gene isolated from purple rice leaves by screening cDNA library using maize *Lc* cDNA as a probe (Hu *et al*., 1996; Hu *et al*., 2000). Our RT-PCR results showed the expected about 1,800 bp amplified fragment corresponding to the 1,767 bp ORF of the *OSB1* gene isolated from T65-Plw.

Comparison of nucleotide and amino acid sequences of rice *OSB1* genes

Sequencing analysis revealed the cloned *OSB1* genes of black rice Khum and white rice Sasanishiki were 1,767 and 1,768 bp, respectively. When the sequences of *OSB1* genes of black and white rice were compared, the 2-bp addition and 1-bp deletion were found, causing the addition of 1 bp in the full-length coding sequence (1,768 bp) of white rice (data not shown). The full-length coding sequences of *OSB1* genes were then analyzed for the region of open reading frame (ORF). Sequencing of the cloned *OSB1* gene from black rice Khum showed the ORF of 1,767 bp. The nucleotide sequence of *OSB1* gene from Khum was compared to the sequences of *OSB1* genes from black rice varieties reported in GenBank which were *OSB1* gene of *japonica* rice Taichung 65-Plw (AB021080), *Ra* genes (likely identical to *OSB1*) of *japonica* purple rice Chuanheinuo (EU096986) and *indica* purple rice Yunanheixiannuo (EU095985). All sequences of *OSB1* genes from black rice showed almost complete identity (99-100 %) (Figure 3). The *OSB1* gene cloned from white rice Sasanishiki contained the ORF of 1,725 bp. When the nucleotide sequence of *OSB1* gene from Sasanishiki was compared to the sequence of *OSB1* gene of *Oryza japonica* rice group Nipponbare reported in GenBank (NM_001060067), it showed almost complete identity of 99 % (Figure 3).

When the full-length coding sequences of *OSB1* gene from black rice (Khum, Taichung 65-Plw, Chuanheinuo, and Yunanheixiannuo) and white rice (Sasanishiki and Nipponbare) were compared, three differences could be identified in white rice. The first change was at nucleotide position 191 within the 2nd exon which was a base substitution from C (black rice) to T (white rice), resulting in an amino acid substitution at position 64 (T64M) (Figure 4 and 5a) located within the conserved N-terminal interacting domain of basic helix-loop-helix (bHLH) MYC transcription factor (Goff *et al*., 1992). The T residue at position 64 was strictly conserved in rice *Ra1*, T65-Plw *OSB1* and T65-Plw OSB2, maize *B-Peru* and *Lc*, and *Arabidopsis TT8* (Shih *et al*., 2008). The second difference was 2-bp (GT) addition at position 1,633 in white rice located within the 7th exon that gave rise to a frameshift mutation starting at amino acid 545 at C-terminus, resulting in a premature termination of protein sequence containing 574 amino acid residues (Figure 4 and 5b). On the other hand, the *OSB1* gene in black rice had ORF of 1,767 bp which was translated to the protein sequence of 588 amino acid residues (Figure 4). The third change was at position 1,722 which was a 1-bp deletion of nucleotide G located on the 8th exon (Figure 5b). The 2-bp addition and 1-bp deletion in white rice resulted in the occurrence of a premature stop codon, leading to the truncation of 14 amino acids at C-terminus of OSB1 protein.


Chuanheinu
T65-Plw
Yunanheixi
pKNlB1
Nipponbare
pSasalB1

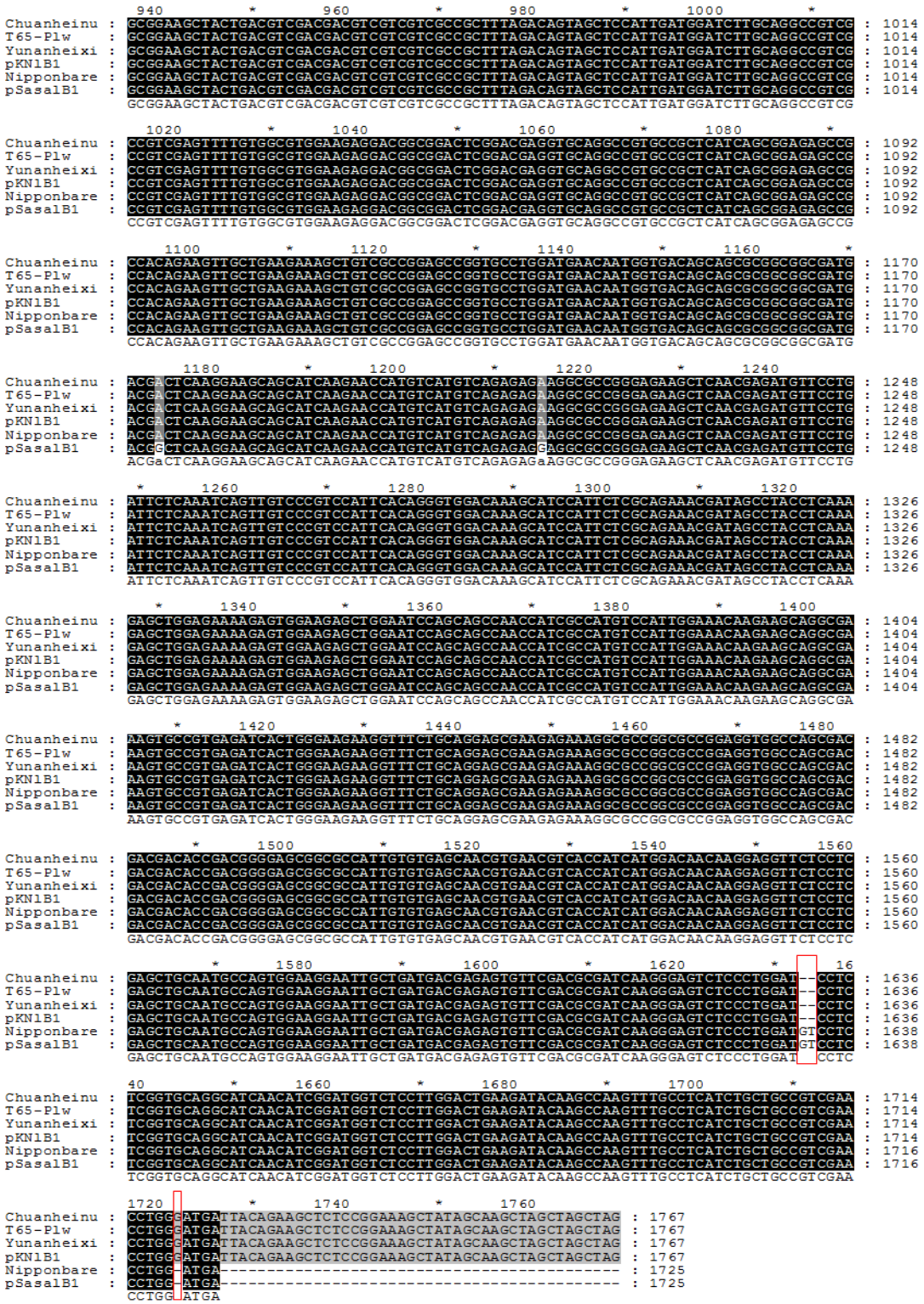


Figure 3 Alignment of the full-length coding sequences of *OsB1* genes cloned from leaves of white rice Sasanishiki (pSasalB1) and black rice Khum (pKNlB1) and *OsB1* genes from GenBank which were *OSB1* gene of *japonica* purple rice Taichung 65-Plw (T65-Plw) (AB021079), *Ra* genes of *japonica* purple rice Chuanheinuo (EU096986) and *indica* purple rice Yunanheixiannuo (EU095985), and *OSB1* gene of *Oryza japonica* rice group Nipponbare (white rice) (NM_001060067). The red boxes show the base substitution at nucleotide position 191, the 2-bp (GT) addition at position 1,633 and the 1-bp (G) deletion at position 1,722.

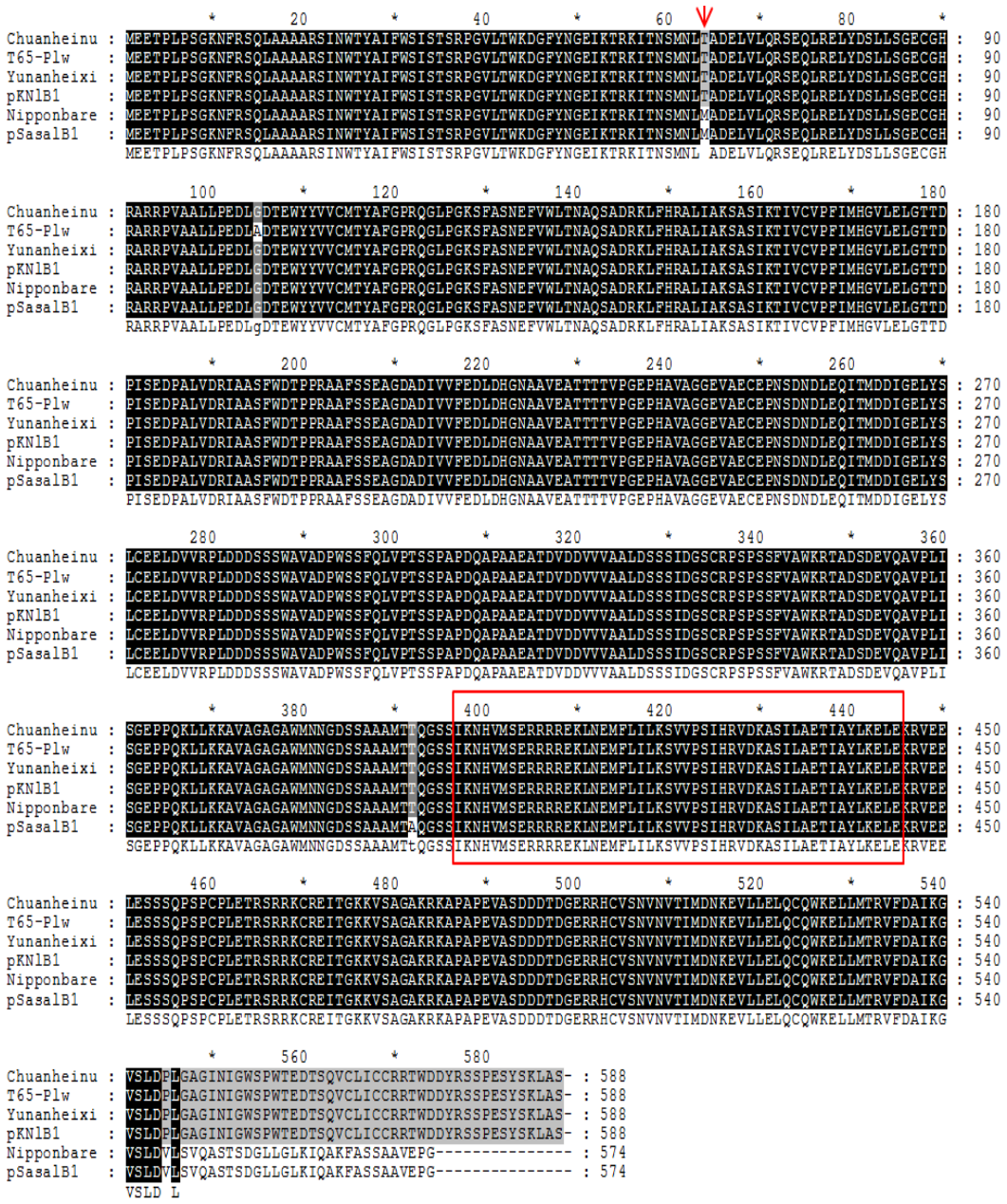


Figure 4 Alignment of deduced amino acid sequences from ORFs of *OsB1* genes cloned from leaves of white rice Sasanishiki (pSasalB1) and black rice Khum (pKNlB1) and amino acid sequences of *OsB1* genes from GenBank which were *OSB1* gene of *japonica* purple rice Taichung 65-Plw (T65-Plw) (BAB64301), *Ra* genes of *japonica* purple rice Chuanheinuo (ABW89745) and *indica* purple rice Yunanheixiannuo (ABW89744), and *OSB1* gene of *Oryza japonica* rice group Nipponbare (white rice) (NP_001053532). The basic helix-loop-helix (bHLH) domains are in the red box at 397-445 amino acid position. The T64M mutation was indicated by the red arrow at amino acid position 64.

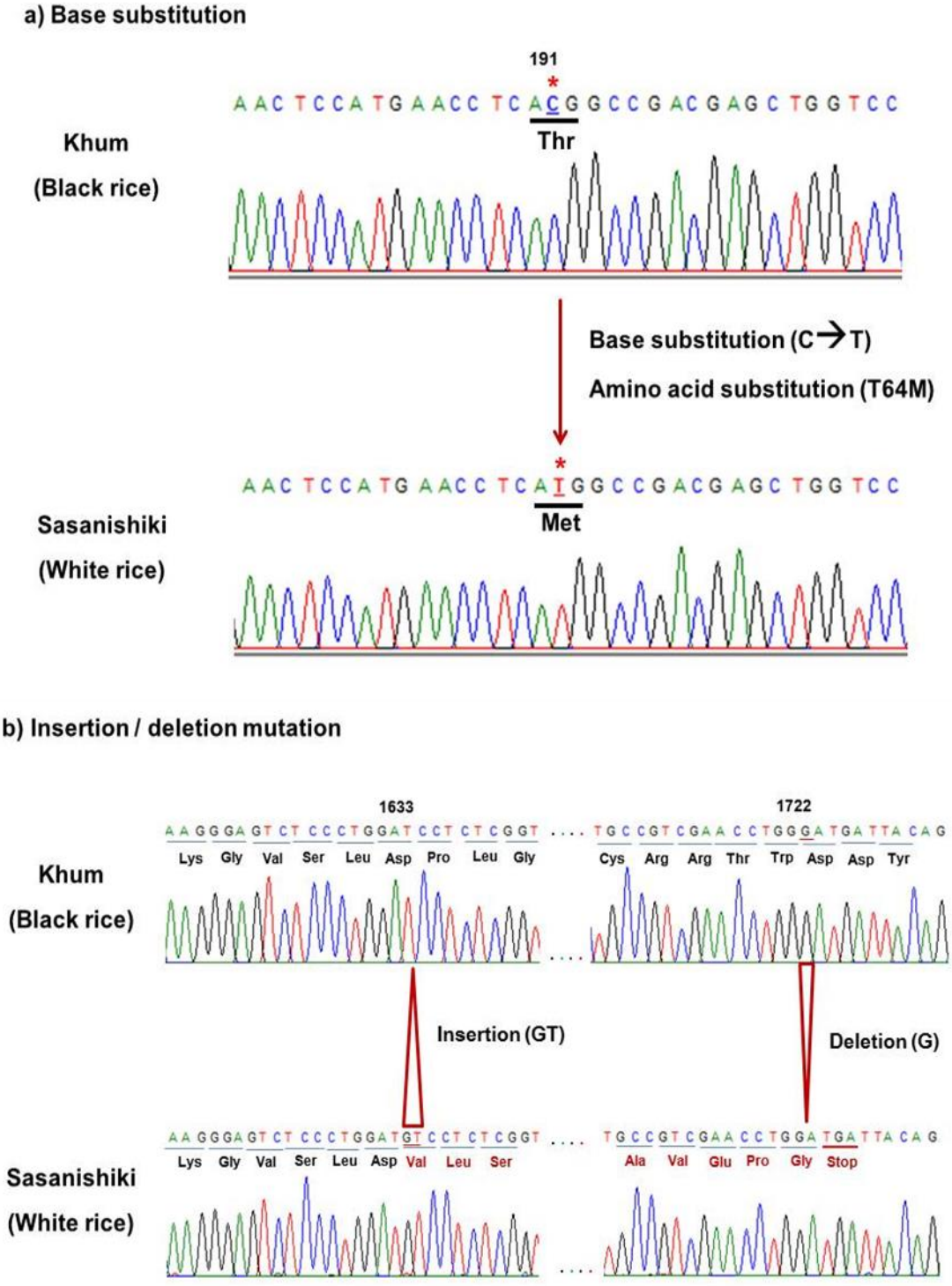


Figure 5 Sequencing chromatograms of *OSB1* gene from black rice Khum and white rice Sasanishiki. a) Base substitution was found at position 191 (indicated by asterisks) from C (Khum) to T (Sasanishiki), leading to amino acid substitution at position 64 from threonine (T) to methionine (M) (T64M). b) Insertion / deletion mutation was detected in white rice. The 2-bp (GT) insertion at position 1633 and the 1-bp (G) deletion at position 1722 were present in Sasanishiki, causing a frameshift mutation at C-terminus and a premature stop codon. The chromatograms displayed the forward sequences on the coding DNA strand.

N-terminal region required for the functional interaction of *R/B* gene family

In rice, at the *Pl* locus on chromosome 4, two members of *R/B* gene family were identified, *OSB1* and *OSB2*, isolated from T65-Plw with purple leaves and pericarp, which are homologous to the maize *B-Peru* gene (Sakamoto *et al*., 2001). Hu *et al*. (1996) identified rice *Ra1* gene isolated from rice with purple color in leaves but not pericarp, which appeared to be allelic to *OSB1* gene and was homologous to maize *Lc* gene. We previously cloned the full-length coding sequences of *OSB2* gene from leaves of Thai rice Khum which has black (dark purple) leaves and pericarp (Inta *et al*., 2013). The *OSB1* gene cloned from this study and *OSB2* gene of *indica* black rice Khum were compared to the members of *R/B* genes reported in GenBank which were rice sequences including, *Ra1* (AAC49219), Nipponbare *OSB1* (NM_001060067), T65-Plw *OSB1* (BAB64301) and T65-Plw *OSB2* (BAB64302), maize sequences including *B-Peru* (CAA40544) and *Lc* (AAA33504), and *Arabidopsis* sequence, *TT8* (AEE82802). It was found that amino acid position at T-64 residue in the N-terminal region was strictly conserved in rice *Ra1*, *OSB1* and *OSB2*, maize *B-Peru* and *Lc*, and *Arabidopsis TT8* (Figure 6a, b). The amino acid substitution (T64M) in white rice Nipponbare and Sasanishiki (in this study) corresponded to the inability of anthocyanin accumulation.

Our results corresponded to the report of Shih *et al*. (2008) that found the same substitution identified in several white rice varieties. We also found the conserved T-64 residues in both *OSB1* and *OSB2* genes of Thai black rice Khum. Goff *et al*. (1992) reported that the N-terminal region of bHLH MYC protein was responsible for interaction between two classes of regulatory proteins, the *R/B* family and *C1/Pl* family, to activate anthocyanin biosynthesis pathway. Such interaction did not require the bHLH motif.

Although the conserved region of bHLH which is a DNA-binding motif of transcription factors was conserved in all different plants analyzed (Figure 6c), the T64M mutation at N-terminus in white rice might cause the impaired function of *OSB1* gene.

The 2-bp addition in *OSB1* gene correlated with white rice pericarp phenotype

Ra1 gene was isolated from cDNA library prepared from rice Purple 522 which has purple phenotype in leaves but not in seeds (Hu *et al*., 1996). *OSB1* gene was identified from leaf cDNA library of purple T65-Plw line displaying purple leaves and black seeds (Sakamoto *et al*., 2001). Sequence comparison revealed *Ra1* gene had a 2-bp addition which resulted in a frameshift and changed amino acid sequence (Shih *et al*., 2008). In addition, the *OSB1* gene in black rice was active in anthocyanin accumulation, but the inactive *OSB1* gene with a 2-bp addition in white and red rice caused the inability to produce anthocyanin (Lim and Ha, 2013). The *Ra* genes, identical to *OSB1* gene, of two black rice lines Yunanheixiannuo and Chuanheinuo which had green leaves but purple pericarp, were sequenced and compared with those of white rice lines. This analysis revealed the 2-bp (GT) insertion/deletion within the 7th exon corresponded to the white/purple color difference. White pericarp rice might be caused by the GT addition within 7th exon of *Ra* gene (Wang and Shu, 2007).

These corresponded to our results that the *OSB1* gene in *indica* Thai black rice Khum might be functional for the activation of anthocyanin biosynthesis while the inactive *OSB1* gene containing 2-bp insertion in white rice Sasanishiki lost anthocyanin pigmentation. Moreover, we found the active *OSB1* gene in black rice Khum displaying black color phenotype in leaves and seeds and also detected *OSB1* expression in both tissues. This suggested that the *OSB1* gene might play an important role as one of the main regulators of anthocyanin biosynthesis in rice seed. The 2-bp difference in the 7th exon of *OSB1* gene in white and black rice could be used to develop marker which could discriminate rice seeds with various pericarp color phenotypes. The application may be used for identification of rice pericarp color before seed setting in rice breeding programs.

CONCLUSIONS

Anthocyanin biosynthesis in rice is regulated by the regulatory genes including *OSB1* gene. The expression of *OSB1* gene was detected in all white and colored rice and also in leaves and seeds. The *OSB1* full-length coding sequences were cloned from leaves of *indica* Thai black rice Khum and *japonica* white rice Sasanishiki. Sequence comparison of the *OSB1* gene form black and white rice revealed the differences found in white rice which were T64M mutation at N-terminus of amino acid sequence, 2-bp insertion and 1-bp deletion in the 7th and 8th exons, respectively. The changes at the conserved N-terminal interacting domain might affect the function *OSB1* gene in white rice. The InDel mutation was present in white rice lines, affecting the C-terminus of regulatory protein. Although this affected C-terminal region was not located in bHLH domain, it caused frameshift

mutation and alteration of amino acid sequences downstream from 2-bp addition site in white rice. Our results, which showed the functional *OSB1* gene expressing in both black leaf and seed phenotype of Thai *indica* rice Khum, provide supporting evidence that the *OSB1* gene might play an important role in regulation of anthocyanin pigmentation in rice seeds. The cloned *OSB1* genes will be further studied for their functions to gain more insight of anthocyanin biosynthesis in rice, and especially of molecular mechanism of pericarp pigmentation. The nucleotide differences of the *OSB1* genes between white and colored rice could also be applied for developing molecular markers for seed color selection during early plant development stages, which will be useful for rice improvement.

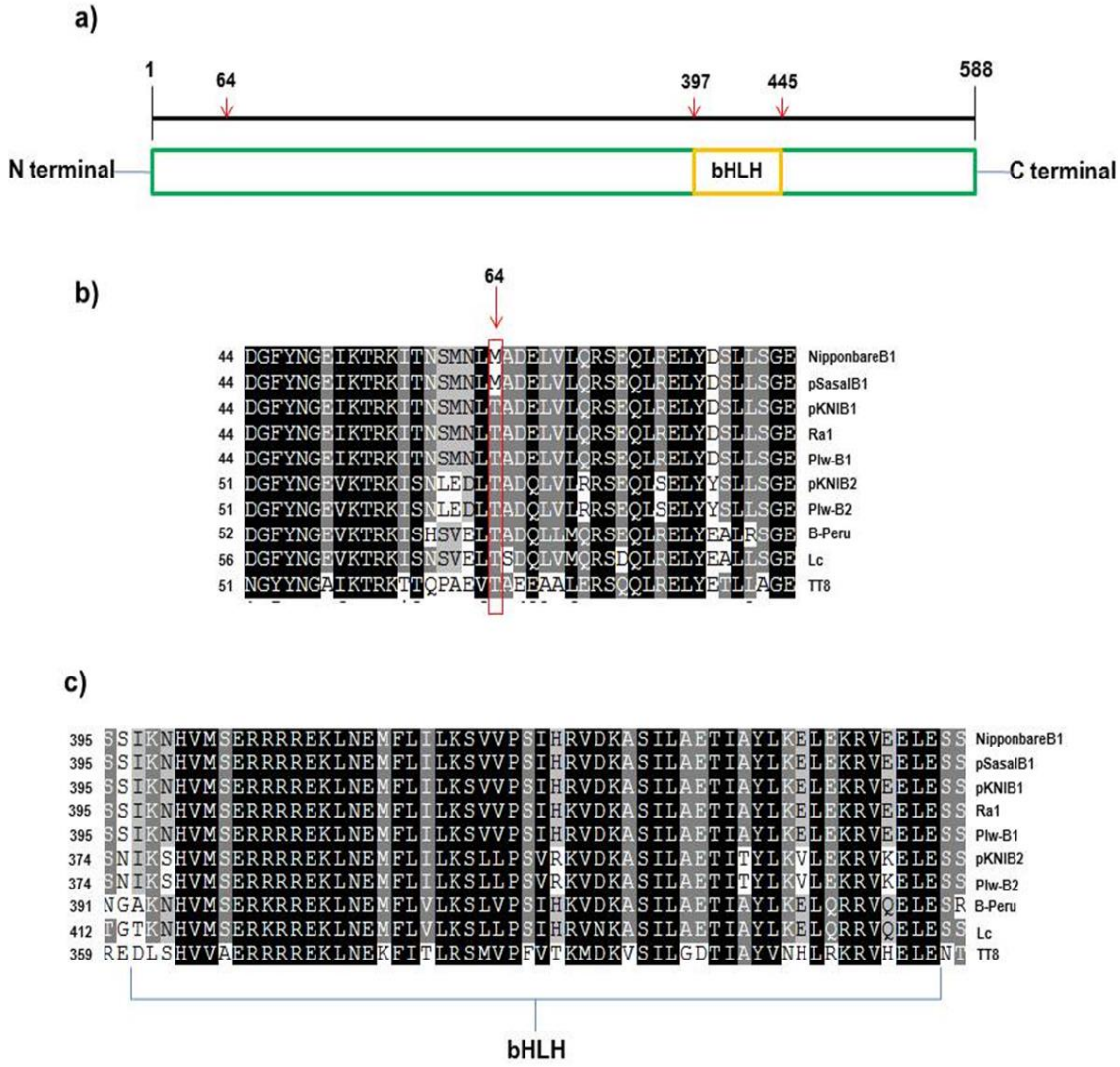


Figure 6 Comparison of different plant anthocyanin-related bHLH regulatory proteins. a) Schematic diagram of amino acid sequences of bHLH regulatory proteins which had the number based on the 588-residue *OSB1* amino acid sequence of purple rice T65-Plw. b) Alignment of a selected region at the N-terminal interacting domains of bHLH transcription factors from various plants. The amino acid position at 64 was indicated by the red arrow and the red box. c) Alignment of bHLH motif at position 397-445 based on T65-Plw OSB1 protein which was highly conserved among the regulatory proteins of rice, maize and *Arabidopsis*. The amino acid sequences from GenBank were rice Nipponbare *OSB1* (NP_001053532), T65-Plw *OSB1* (BAB64301), T65-Plw *OSB2* (BAB64302) and *Ra1* (AAC49219), maize *B-Peru* (CAA40544) and *Lc* (AAA335014), and *Arabidopsis TT8* (AEE82802). pSasalB1 and pKNlB1 were the *OSB1* genes cloned from leaves of white rice Sasanishiki and black rice Khum, respectively. pKNlB2 was the *OSB2* gene cloned from leaves of black rice Khum.

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