Expression analysis and nucleotide variation of *OsC1* gene associated with anthocyanin pigmentation in Thai rice cultivars

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ABSTRACT

OsC1 gene, encoding a R2R3-Myb transcription factor, regulates expression of structural genes in anthocyanins biosynthesis pathway in rice. It plays an important role in rice coloration. Expression of this gene in both leaf and seed tissues was examined in three rice cultivars covering white (Khao Dawk Mali 105; 105), red (Sukhothai Hom Dang; SKHD) and black (Kham; KN) rice. Expression of the *OsC1* gene was found in young leaves and seeds in all rice cultivars in this study. Higher level of gene expression was observed in seed tissues of Kham black rice at 25 days after flowering, associated with dark anthocyanin pigmentation on the pericarps. However, the expression patterns of this gene in leaves and seeds in white and red rice were stable and appeared at similar level, not corresponding with the phenotypic traits of red rice color at the late stage of development compared with the white one. Comparisons of nucleotide sequences in the exon 3 of the *OsC1* gene among those three cultivars revealed a 10-bp deletion in white and red rice, resulting in frameshift mutation in R3 region of Myb DNA binding domain, but not in the black. This deletion caused the functional loss in white (105) rice, leading to their anthocyanin pigment accumulation loss at all stages and tissue types. For red (SKHD) rice, although the expression of *OsC1* was detected at all stages, its similar 10-bp deletion in exon 3 of the *OsC1* gene indicated an alternative pathway for anthocyanin pigment to be accumulated in the pericarp tissues. The result from this study constitute a basis on how pigment would be developed in these three Thai rice cultivars and are applied to rice improvement by developing molecular markers for selection of rice anthocyanin pigmentation in future.

Keywords: *OsC1* gene, rice, anthocyanins, transcription factor

INTRODUCTION

Anthocyanins are water soluble pigments, classified as a major class of flavonoid. They contribute to the coloration of most flowers, fruits, and seeds, and are synthesized via the phenylpropanoid pathway (Koes *et al.*, 2005). To plants, different anthocyanin accumulations in their tissues and organs have diverse physiological functions, including hormone responses, ultra-violet radiation, and defenses to biotic and abiotic stresses (Reddy *et al.*,1994; Ithal and Reddy, 2004). To humans, they provide several biological activities such as antioxidants, prevention and reduction of risk of coronary heart disease and cancer, reduction of blood cholesterol and being antiviral, antimicrobial, and anti-inflammatory (Geekiyanage *et al.*, 2012). Antioxidant efficacy of anthocyanins is higher than that of vitamin C and E (Bagchi *et al.*, 1998 Cossins *et al.*, 1998; Shi *et al.*, 2003).

Rice is global staple food and the most important economic crop in the world. More than 40,000 varieties were reported under cultivated rice. Several of them have various important pigmented grain colors, with white, red, and black pericarp tissues due to anthocyanin pigment synthesis and accumulation (Reddy *et al.*, 1994). Anthocyanin synthesis in rice involves two major classes of genes, the structural genes and the regulatory ones. The first group encodes anthocyanin biosynthesis enzymes such as chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), dihydroflavonol 4-reductase (DFR), and anthocyanidin synthase (ANS) (Shih *et al.*, 2008). The other encodes transcription factors which are divided into two groups, Myc / basic helix-loop-helix (bHLH) and Myb type families.

In rice, Myc type bHLH transcription factors, *OSB1* and *OSB2* genes had been identified to be on the Pl^{w} locus on chromosome 4 (Hu *et al*, 1996; Sakamoto *et al.*, 2001; Inta *et al.*, 2013; Sakulsingharoj *et al.*, 2014). Dynamic response of the *OsB1* gene in black rice revealed a possible role in anthocyanin biosynthesis (Sakulsingharoj *et al.*, 2016). However, the regulatory genes of Myb type in most plant belong to R2R3 Myb transcription factors. Previously, the rice *OsC1* gene

encoding Myb factors for anthocyanin synthesis had been identified (Martin and Paz-Ares, 1997). It was a homolog of the *C1* gene in maize and provided C1/Pl proteins for Myb DNA binding domains regulating downstream gene expression in the flavonoid pathway (Chandler *et al.*, 1989). Several studies on rice coloration showed that the *OsC1* gene was located on the short arm of chromosome 6 where 3 exons and 2 introns were designated (Saitoh *et al*, 2004). The 10-bp deletion in the R3 domain of exon 3 caused frameshift mutation, leading to truncated transcription factor of OsC1 which, in turn, halting of anthocyanin pigmentation eventually (Saitoh *et al.*, 2004; Chin *et al.*, 2016; Choudhury *et al.*, 2014). Although *OsC1* gene make-up was unravelled, there was still rare information concerning the *OsC1* gene in Thai color rice.

The present study was carried out to investigate the relationship between the expression of *OsC1* regulatory gene and the anthocyanin pigmentation in three cultivars of Thai rice in different tissues. In addition, nucleotide sequences of *OsC1* in exon 3 among those cultivars were compared and investigated for the basis on the allelic variation linking to their phenotypic attributes.

MATERIALS AND METHODS

Plant materials and RNA extraction

Three Thai rice cultivars Khao Dawk Mali 105 (kindly provided by Maejo University), Sukhothai Hom Dang (kindly provided by Khao Thammachad, Co, Ltd, Sukhothai, Thailand), and Kham (collected from Nong-Tao-Kham village, Sansai district, Chiang Mai, Thailand) were employed in this study (Figure 1). Phenotypic traits were recorded from rice in the vegetative and reproductive stages. The seedlings were collected two weeks after germination. The rice plants were grown in greenhouse and developing seeds were collected at 15 and 25 days after flowering. At maturity, the seeds were harvested. The seeds were dehulled and pericarp colors were observed at different developmental stages. RNA was extracted from leaves and seeds at corresponding stages using the TRIzol reagent (ThermoFisher Scientific, USA) and treated with DNaseI (New England Biolabs, USA) before use.

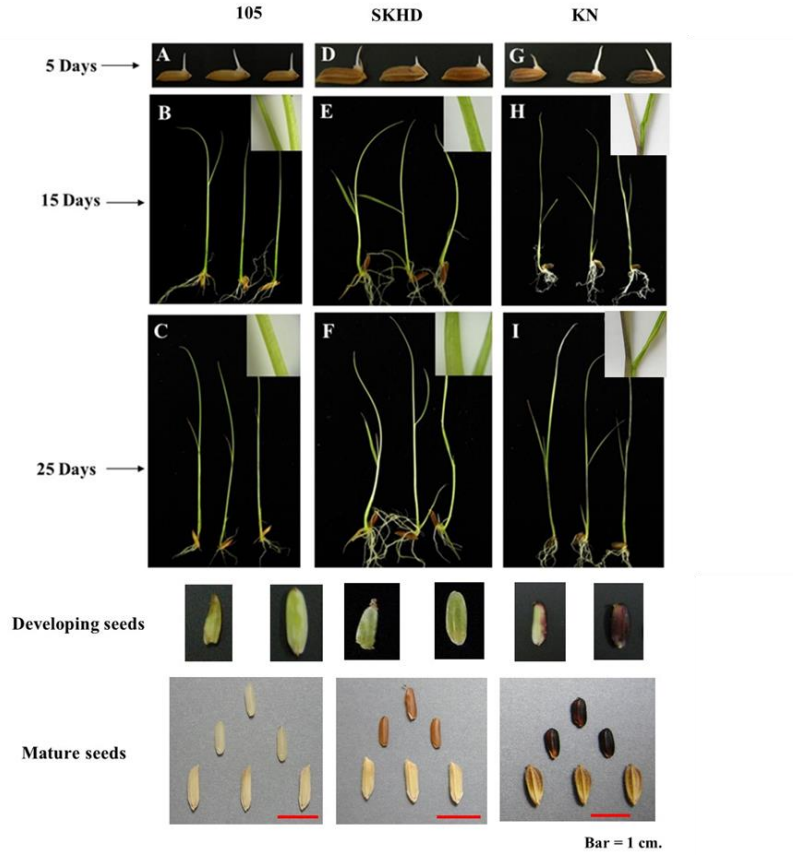

Figure 1 Phenotypic traits of seedlings and seeds of three rice cultivars. (A-C) Khao Dawk Mali 105 seedlings at the age of 5, 15 and 25 days, respectively. (D-F) Sukhothai Hom Dang seedlings at the age of 5, 15 and 25 days, respectively. (G-I) Kham seedlings at the age of 5, 15 and 25 days, respectively. Developing seeds at 15 days (left) and 25 days (right) after flowering. Mature seed, (lower row) and pericarp colors of dehulled seeds (upper row).

Investigation of *OsC1* gene expression at the early stage of development

RNA from seedlings 2-week after germination and 5-day-old developing seeds were investigated for their *OsC1* gene expressions. Two micrograms of total RNA of each sample were reverse transcribed using oligo (dT) primers in 20 uL reaction by High-Capacity cDNA Reverse Transcription Kit according to manufacturer's instructions (Applied Biosystems, USA). Gene-specific primers were designed based on nucleotide sequence of the *OsC1* gene (Y15219.1), OC1_F: 5´-CGGGTTCTTCTTCCACGAC-3´ and OC1_R: 5´-CCCGCAACTGCACTTAAAAT-3´ to provide the expected length of about 360 bp products. The *OsActin* gene was used as an internal control and its primer set was OsActinF 5´-TGATGCGCCCAGGGCTGTCT-3´and OsActinR 5´-CGATTGGCCTTGGGGTTGAG-3´. Amplification of target cDNA was performed with MyTaq Red Mix (Bioline, USA). Both PCR profiles were 95 ºC for 3 min, 35 cycles at 95 ºC for 30 sec, 55 ºC for 30 sec, and 72 ºC for 30 sec, and 5 min at 72 ºC for final extension. Aliquots of PCR products were analysed on a 1.8 % (w/v) agarose gel by electrophoresis.

Examination of *OsC1* gene expression in different tissues by semi-quantitative RT-PCR

RNA from leaf and seed tissues at the age of 15 and 25 days was extracted (Figure 1). The DNase I-treated RNA samples were reverse transcribed using oligo (dT) primers by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). For the template, 2 ug of total RNA was employed in a 20 uL Reverse Transcription (RT) reaction, according to manufacturer's instructions. Gene-specific primers were OC1_F and OC1_R, generating the expected RT-PCR products of about 360 bp. The primers of *OsActin* gene (OsActinF and OsActinR) were also used as an internal control. Amplification of target cDNA was performed with My Taq Red Mix (Bioline, USA). The PCR profile was 95 ºC for 3 min, 28 cycles at 95 ºC for 30 sec, 55 ºC for 30 sec, and 72 ºC for 30 sec, and 5 min at 72 ºC for final extension. Aliquots of PCR products were analysed on a 1.8 % (w/v) agarose gel by electrophoresis. The expression levels of *OsC1* gene were determined by the program GelAnalyzer 2010 (http://www.GelAnalyzer.com). The relative expression levels were based on the expression ratios of a target *OsC1* gene to a reference *OsActin* gene.

Measurement of anthocyanin contents

Total anthocyanin contents of 2-week-old seedlings extracts from Khao Dawk Mali 105 (105), Sukhothai Hom Dang (SKHD) and Kham (KN) were quantified. Fifty milligrams of fresh tissues were homogenized with 1 ml of 1 % HCl in 80 % methanol (V/V) as modified from Chin *et al*. (2016). The supernatant was collected after centrifugation at 12,000 rpm for 5 min and absorbances at 530 nm and 675 nm were measured using a spectrophotometer (Meterech SP-830 plus, Taiwan). The anthocyanin contents were determined as follow: (A530-A675)/gram fresh weight. Three replicates were analyzed for each sample. Statistical analysis was performed using R-3.4.0 program (http://www.r-project.org).

PCR analysis and comparison of *OsC1* gene sequences

Genomic DNA was extracted from young rice seedlings using modified CTAB method (Hwang and Kim, 2000). The extracted DNA was subjected to PCR using primers specific to a partial exon 3 region of *OsC1* gene (OC1ex3_F: 5´-GATCGATCGTGTATAT ATGTTGTCAGGT-3´ and OC1ex3_R: 5´-GTTGCTG TGTCGGTGTCGGCG-3´) (Saitoh *et al.*, 2004). The PCR amplification was carried out in a 20 uL reaction using Phusion Flash High-Fidelity PCR Master Mix (ThermoFisher Scientific, USA) with temperature profile as following, 98 ºC for 30 sec, 35 cycles at 98 ºC for 10 sec, 55 ºC for 15 sec, and 72 ºC for 45 sec, and 5 min at 72 ºC for final extension. Aliquots of PCR products were analysed on a 1.8 % (w/v) agarose gel by electrophoresis. The amplified fragments of about 250 bp were cut, purified and subjected to sequencing by the 1st BASE (Malaysia). The nucleotide and deduced amino acid sequences of exon 3 region of rice cultivars were analyzed and compared with *OsC1* gene available in GenBank via the ClustalX 1.83 and GeneDoc 2.7 programs.

RESULTS AND DISCUSSION

Phenotypic traits of anthocyanin pigmentation in rice tissues at different growth stages

Three Thai rice cultivars, differing in mature pericarp colors, white (Khao Dawk Mali 105; 105), red (Sukhothai Hom Dang; SKHD) and black (Kham; KN), provided no drastic phenotypic variants in tissues related to anthocyanin pigmentation except for the seeds (Figure 1). After germination, the 105 and SKHD had green seedlings and remained green to whole stages, including at 15 and 25 days while the black rice KN accumulated low amount of anthocyanins on leaf sheath at 15 days of age and gained increasing anthocyanin accumulation at the 25-day-old stage (Figure 1). Similarly, the developing seeds of 105 and

SKHD were green at 15 and 25 days after flowering while the KN appeared to accumulate small amount of anthocyanins at the first 15 days followed by the rapid increasing at 25 days (Figure 1). When the seeds reached the maturity stage, their pericarps turned white, red, and black, referring to different rice cultivars used in this experiment.

Expression of the *OsC1* gene in leaves and seeds

The spatial distribution of the *OsC1* gene expression in leaf and seed tissues at the early stage of development of white, red, and black rice cultivars revealed a similar pattern of expression among leaves and seeds. The expression was rather stable in all three rice varieties as compared with that of the internal *OsActin* gene (Figure 2). In several cultivars of rice, the *C* locus of the *OsC1* gene on chromosome 6 was responsible for anthocyanin accumulation in apiculus (Saitoh *et al.*, 2004; Choudhury *et al*., 2014) and leaf sheath (Chin *et al.*, 2016). Previous semi-quantitative RT-PCR analysis revealed that the *OsC1* expression was light-inducible in both colored and non-colored rice, including seedlings of *japonica* Taichung 65 (Shin *et al.*, 2008). In this study, the *OsC1* gene expression was found in young leaves and seeds of all three Thai rice cultivars, regardless of their colors. These provided a clue on *OsC1* regulation in Thai rice in general.

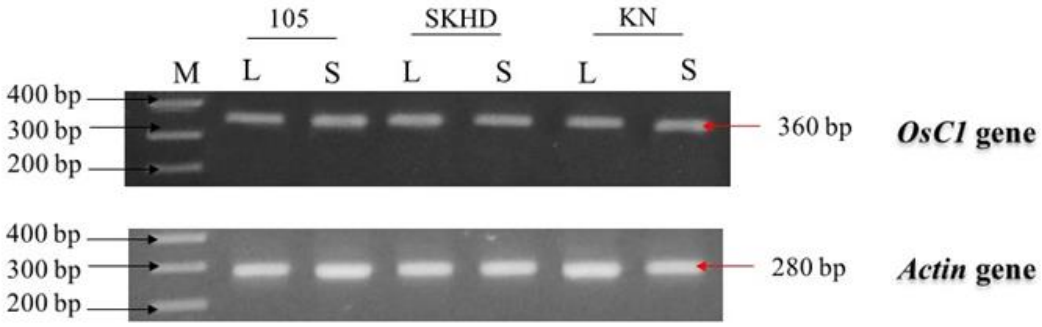

Figure 2 Expression analysis of the *OsC1* gene in rice by RT-PCR technique. The cDNA from young leaves (L) and developing seeds (S) were used as templates. Expression of the *OsActin* gene was used as an internal control. Khao Dawk Mali 105 (105), Sukhothai Hom Dang (SKHD) and Kham (KN).

Expression levels of the *OsC1* gene in tissues at different growth stages

To examine whether expression levels of *OsC1* genes from different tissues at various growth stages was correlated with the phenotypes of anthocyanin accumulations, the cDNA was prepared from RNA samples extracted from seedlings and developing seeds at 15 and 25 days of development and subjected to semi-quantitative RT-PCR. As shown in Figure 3, in leaves and seeds, the expression patterns of *OsC1* transcripts in white and red rice were stable and appeared at similar level. However, expression of *OsC1* gene in KN, black rice, was slightly different especially at late development. The relative expression levels of *OsC1* gene in seed tissues of KN at 15 and 25 days of development showed 3.36 and 5.0 folds, respectively, relative to those of white 105 rice (Figure 3D). Higher level of gene expression was observed in KN seed tissues at 25 days after flowering, associated with dark anthocyanin pigmentation on the pericarps (Figure 1) and consistent with high anthocyanin contents in seeds at 25 days of age of black rice in previous reports (Jiamyangyuen *et al.*, 2017; Rahman *et al.*, 2015).

In leaf tissues, the relative expression of *OsC1* gene in KN at 15 days after germination showed slightly increased level of 1.13 fold relative to that of white 105 rice (Figure 3B). The *OsC1* expression was associated with higher athocyanin contents of 15.01 per gram fresh weight of KN seedlings at 15 days of age (Figure 4). Although the phenotypic traits of KN seedlings at 15 days were slightly different from those at 25 days (Figure 1), the anthocyanin contents of seedlings at 15 and 25 days of 15.01 and 10.37 per gram fresh weight, respectively, (Figure 4) were correlated with the relative levels of *OsC1* gene expression (Figure 3B). The result in KN was in consistent with that of anthocyanin enhancement in other rice cultivars, having a dark purple leaf sheath as previously investigated by real-time RT-PCR (Chin *et al.*, 2016).

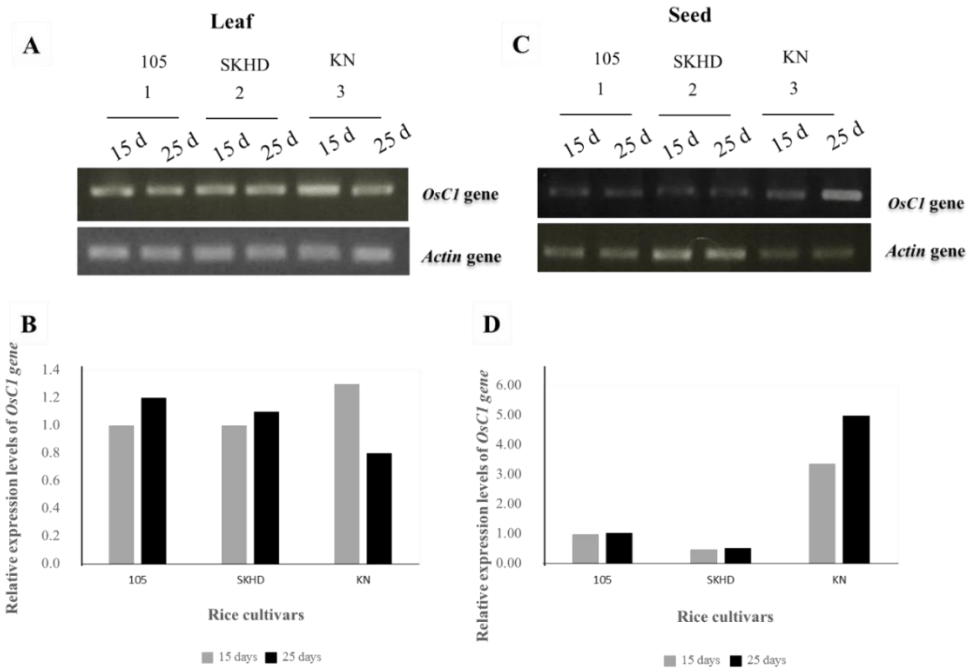

Figure 3 Semi-quantitative RT-PCR expression analysis of the *OsC1* gene in rice leaves (A) and seeds (C) at different ages of 15 and 25 days. *OsActin* expression was used as an internal control. The white, red and black rice were Khao Dawk Mali 105 (105), Sukhothai Hom Dang (SKHD) and Kham (KN), respectively. The relative expression levels of *OsC1* gene (the expression ratios of *OsC1* gene to *OsActin* gene) in leaves (B) and seeds (D) at different ages of 15 and 25 days. The values represented the expression levels of each sample relative to that of white rice (105).

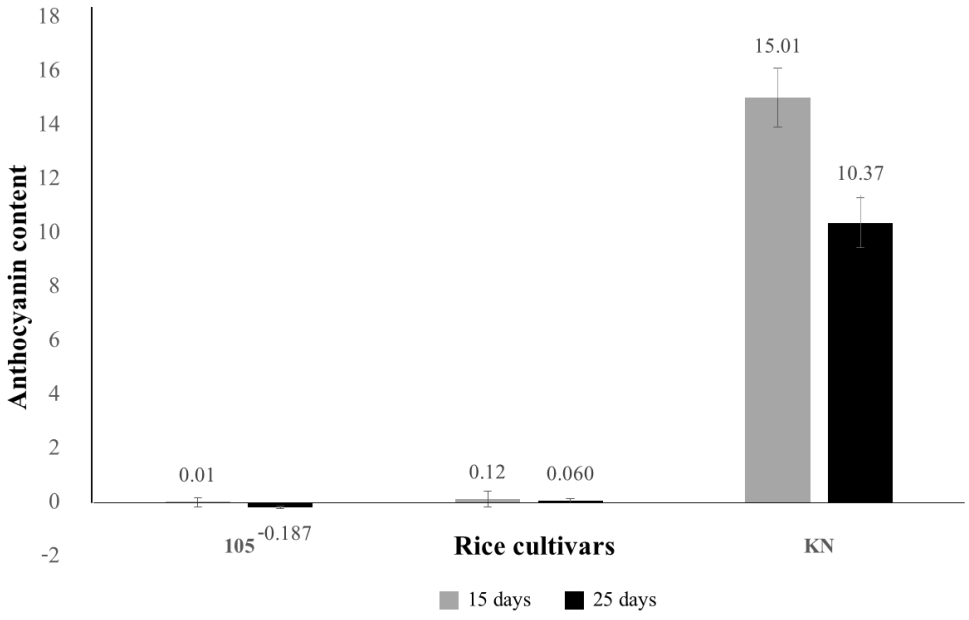

Figure 4 Anthocyanin contents of seedling extracts from Khao Dawk Mali 105 (105), Sukhothai Hom Dang (SKHD) and Kham (KN). Anthocyanin contents were calculated as follow: [(A_{530}-A_{675})/gram fresh weight]. Bar represented standard deviation. The anthocyanin content in KN black rice at 15 and 25 days showed significant difference ($p < 0.05$) using paired t-test.

As described earlier, expression of *OsC1* gene could be induced by seveal factors including light, stress, and plant growth regulator treatments. Semi-quantitative RT-PCR analysis in seedlings of *japonica* rice confirmed that *OsC1* gene expression was induced by light exposure in both pigmented and nonpigmented ones (Shih *et al.*, 2008). In *indica* rice, it was found that *OsC1* transcripts levels analyzed by northern blot were increased in rosponsive to dehydration stress, high salt, and abscisic acid (ABA) treatments (Ithal and Reddy, 2004). The *OsC1* which is Myb factor was thought to be a potential transcription activator, especially for *OsDFR* and *OsANS*, the late structural genes in the anthocyanin biosynthesis pathway. In this study, expression level of *OsC1* was enhanced in seed tissues at late stage of development, suggesting a possible major role of regulatory *OsC1* gene that might regulate the structural genes spatially and temporally in rice.

Although the above results did imply *OsC1* role in anthocyanin accumulation in black rice, similar level of *OsC1* expressions between the 105 and SKHD was not in corresponding with the phenotypic traits of red color rice at late stage of development compared with the white one. Thus, for red rice, care should be taken to identify further factor (s) that timely control anthocyanin biosynthesis and accumulation. Likewise, for the 105, more investigations on the *OsC1* gene makeup and its role for the loss control of anthocyanin accumualtion would be needed.

Comparisons of nucleotide and amino acid sequences of *OsC1* genes

For further investigation on the allelic variation linking with their anthocynanin biosynthesis and accumulation, nucleotide sequence and the deduced amino acid prediction were determined. Nucleotide sequence analysis of exon 3 of *OsC1* gene showed 10-bp deletion at position 796-805 in white and red rice, 105 and SKHD, respectively (Figure 5), but not of the black (KN) and Purpleputtu, a reference purple rice from GenBank (BAD04024.1). The white (105) and red (SKHD) rice had 10-bp deletion in the R3 Myb domain which was a core domain in exon 3 of *OsC1* gene. This deletion caused a frameshift mutation, leading to a premature stop codon (Figure 6).

This finding agreed with the report of Saitoh *et al*., (2004), at same location and deletion type for the colorless apiculus lines. In addition, most colorless apiculus rice varieties (Choudhury *et al.*, 2014) and green rice varieties (Chin *et al.*, 2016) were reported to have similar deletion, suggesting a key role of *OsC1* gene in genetic makeup of anthocyanin accumulations. The result here was consistent with the previous ones, suggesting a loss-of function mutation by the *OsC1* alleles in 105, SKHD, affected directly to R3 Myb domain, leading to no anthocyanin biosynthesis. Although SKHD had mutated *OsC1* gene, the accumulation of red pigment still appears in their

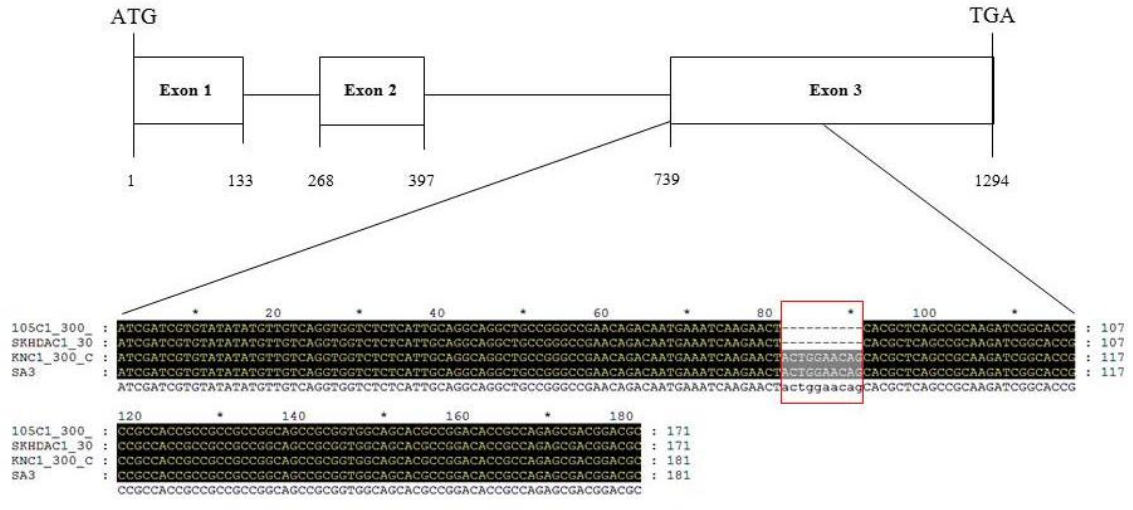

Figure 5 Comparison of nucleotide sequences of exon 3 of *OsC1* gene among different rice cultivars. Exons and introns were indicated by open boxes and thin lines, respectively. The numbers 1-1294 indicated the positions of full-length genomic sequences of *OsC1* gene. The red box showed the 10-bp deletion in exon 3 in white (105C1_300) and red (SKHDC1_300) rice, comparing with that in black (KNC1_300) rice cultivar. SA3 was the *OsC1* gene from purple rice Purpleputtu (AB111869.1) showing sequence of exon 3.

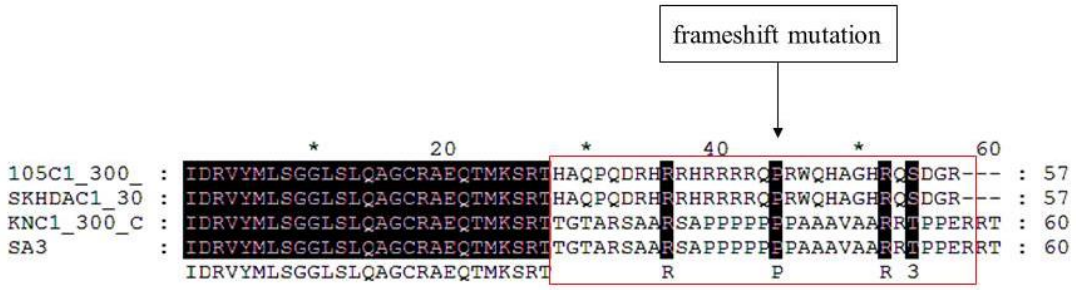

Figure 6 Alignment of deduced amino acid sequences of exon 3 of *OsC1* gene from different rice cultivars. The red box showed the frameshift mutation caused by the 10-bp deletion in exon 3 of white (105C1) and red (SKHDC1) rice, comparing with that in black (KNC1) rice cultivar. Purpleputtu was the amino acid sequences of exon 3 of *OsC1* gene from purple rice Purpleputtu (BAD04024.1).

pericarps, suggesting an alternative pathway for this control. For red color rice, proanthocyanidins, a branch of anthocyanin biosynthesis pathway has been proposed. It shares common structural genes encoding biosynthesis enzymes comparably. This proanthocyanidin biosynthesis is controlled by other regulators containing basic helix-loop-helix transcription factor (Furukawa *et al.,* 2007; Sweeny *et al.,* 2006),

Although the results in the present study suggested that the *OsC1* gene plays a key role in anthocyanin pigmentation in Thai rice, mutations in other genes linked with no anthocyanin pigmentation were also confirmed (Sakamoto *et al*., 2001; Inta *et al.,* 2013; Sakulsingharoj *et al.,* 2016). Therefore, further studies on other genetic makeups and their controls especially on regulatory genes or on structural genes in anthocyanin biosynthesis need to be carried out.

In conclusion, expression of regulatory *OsC1* gene was found in young leaf and seed tissues of all three Thai *indica* rice independent of the colors. However, only in the leave and seeds of black rice, Kham, had an enhanced expression of the *OsC1* gene, which in turn appeared to associate with the phenotypic traits of anthocyanin accumulation directly with *OsC1* regulator. Thus for Kham cultivar, the result suggested that *OsC1* was a key role for anthocyanin accumulation. The presence of a 10-bp deletion in exon 3 of *OsC1* gene that caused functional loss in white rice led to their loss of anthocyanin pigment accumulation at all stages and tissue types. For red (SKHD) rice, although the expression of the *OsC1* gene was detected at all stages, its similar 10-bp deletion in exon 3 of *OsC1* gene indicated an alternative pathway for anthocynanin pigment to be accumulated in the pericarp tissues. Further conclusive demonstrations on *OsC1* expression and nucleotide variations with different alleles at the *C* locus linked to phenotypes of anthocyanin accumulation among various colored rice varieties need to be investigated. These will not only constitute a basis on how pigment would be developed but also beneficial for understanding genetic diversity in rice toward a goal for marker development for rice breeding in the future.

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