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SHORT REPORTS

Nucleotide variations of *9-cis-epoxycarotenoid dioxygenase 2* (*NCED2*) and pericarp coloration genes (*Rc* and *Rd*) from upland rice varieties

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Abstract

In this study, we analyzed the *Rc* and *Rd* genes that are responsible for the coloration of rice pericarps from six upland rice varieties. We also examined the association of pericarp coloration to the single nucleotide polymorphism in *9-cis-epoxycarotenoid dioxygenase 2* (*NCED2),* a key gene involved in abscisic acid (ABA) biosynthesis. Our findings demonstrated that all the upland rice varieties analyzed have a *Rd* gene which encodes a complete dihydroflavonol-4-reductase without early translational termination codon irrespective of their pericarp colors. However, the upland rice varieties with white pericarps were found to have a defective *Rc* gene with a 14-base deletion at exon 7 which could disrupt the function of a positive regulator of proanthocyanidin biosynthesis. In addition, the *NCED2* genes from the upland rice varieties with white pericarps in this study have a C-allele while the *NCED2* genes from Pandasan Red, Tomou and Taragang varieties that bear red pericarps were found to have a T-allele which was reported to be associated with a higher ABA level in upland rice. A better understanding of the gene sequences of upland rice varieties with red pericarp may provide important information for rice breeding programs.

Keywords Abscisic acid · Proanthocyanidin biosynthesis · Rice pericarp colors · Single nucleotide polymorphism

Introduction

The rice pericarps can be red, purple, brown, black and white in color. Purple and red pericarps are due to the accumulation of anthocyanins and proanthocyanidins, respectively (Reddy et al. 1995; Finocchiaro et al. 2007). Rice grains with red pericarp may be preferred in some regions of the world for their unique taste, texture, medicinal benefits, and cultural purpose. The red pigment is of interest for nutritional reasons because it has strong antioxidative properties that can potentially reduce cardiovascular disease (Ling et al. 2001).

The biosynthetic pathways of proanthocyanidins and anthocyanin shared two enzymes that are involved in the initial steps, i.e., flavanone-3-hydroxylase which catalyzes the flavonones to dihydroflavonols, and dihydroflavonol-4-reductase (DFR) which catalyzes the conversion of dihydroflavonols to leucoanthocyanidins. In the anthocyanin biosynthetic pathway, leucoanthocyanidins are converted to anthocyanidins by anthocyanidin synthase and subsequently to anthocyanins by anthocyanidin glucosyltransferase. However, anthocyanidin reductase can also change anthocynidins to epicatechins. Leucocyanidins, epicatechins and catechins (from leucocyanidins) are precursors of proanthocyanidins (Xie and Dixon 2005).

The red coloration in rice is primarily regulated by *Rc* and *Rd* genes (Furukawa et al. 2006). Rice varieties with both *Rc* and *Rd* genes have red grains while rice varieties with *Rc* but without *Rd* produce brown grains. Rice varieties

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with either *rc* and *rd,* or *rc* and *Rd* produce white rice grains (Furukawa et al. 2006). *Rc* which encodes a basic helix loop helix (bHLH) protein was mapped to chromosome 7 (Sweeney et al. 2006), corresponding to LOC_Os07g11020 in Nipponbare. *Rd* which encodes the DFR enzyme involved in both anthocyanin and proanthocyanidin biosynthesis was mapped to chromosome 1 (Furukawa et al. 2006), corresponding to LOC_Os01g44260 in Nipponbare. The *Rc* gene was reported to have eight exons separated by seven introns with a DNA binding motif bHLH at the seventh exon by Furukawa et al. (2006) and Gu et al. (2011). A 14-bp deletion at the seventh exon which causes a frame shift in the open reading frame and early termination of protein translation of this positive regulator of proanthocyanidin biosynthesis (Furukawa et al. 2006; Gu et al. 2011), was identified in a Nipponbare and other rice varieties that produce white grains. The truncated protein without a functional bHLH domain does not have any transacting regulatory activity. The rice *DFR* gene consists of three exons and two introns (Chen et al. 1998; Nakai et al. 1998). The occurrence of a stop codon at exon 1 (third codon) and 2 (55th codon) in the DFR of some rice varieties may render the gene non functional (Nakai et al. 1998). The *DFR* gene was reported to have alternative translation initiation predominantly in developing seeds (Furukawa et al. 2006).

The pigments in rice pericarps have deterrent effects on pathogens or predators in nature (Shirley 1998). The genes involved in the biosynthesis of anthocyanin and proanthocyanidins can be induced by stresses through transcription factors in the basic domain/leucine zipper family and Myb family (Ithal and Reddy 2004; Hartmann et al. 2005). In addition, al. (2011) reported that *Rc* not only regulated *Rd* gene but also promoted biosynthesis and accumulation of abscisic acid (ABA) in early developing seeds from a pair of perfect rice isogenic lines. ABA is a phytohormone related to environmental stresses including water deficit (Finkelstein 2013). ABA is also able to promote or inhibit the biosynthesis of anthocyanin in fruits by crosstalking with other phytohormones including jasmonic acid, gibberellin, auxin and cytokinin (Jaakola 2013). The antioxidant properties of anthocyanins and proanthocyanidins may enable the plants to cope better and survive under environmental stresses. The production of xanthoxin through oxidative cleavage of 9-cisepoxyxanthophylls by 9-*cis*-epoxycarotenoid dioxygenase (NCED) (Finkelstein 2013) is an irreversible reaction and rate-limiting step in ABA biosynthesis. Li et al. (2019) reported that NCED could affect the ABA level in *Lycium* fruits, which regulates three transcription factors that can improve the production of anthocyanin by upregulating the gene expression of genes related to anthocyanin biosynthesis. ABA-mediated anthocyanin biosynthesis pathways have also been reported in other plants (Xie et al. 2012; An et al. 2018).

The aims of this study were to analyze the *Rc*, *Rd* and *NCED* genes from six upland rice varieties and to examine the association between coloration of rice pericarp and the single nucleotide polymorphism (SNP) in *NCED*. A better understanding of the gene sequences of upland rice varieties with red pericarp will provide important information for rice breeding programs.

Materials and methods

Plant materials and DNA extraction

Rice seeds of selected Malaysian rice varieties were obtained from Rice Genebank, Malaysian Agricultural Research and Development Institute (MARDI), Kepala Batas, Malaysia; and Kebun Bahagia Bersama, Sungai Buloh, Malaysia (Supplementary Table S1). Rice husk was removed from 20 seeds randomly selected from each rice variety to record the colour of the rice pericarps.

Seeds were germinated on filter paper after sterilization using 40% (v/v) Clorox®-Bleach (The Clorox Company, USA). Fourteen day-old rice seedlings were transplanted into pails containing 15 kg of a mix of top soil and sand (70:30) in a perforated pail. Total genomic DNA was extracted from the leaves of 2 month-old rice using a modified protocol described by Doyle and Doyle (1987); Doyle and Dickson (1987); and Cullings (1992).

Cloning of selected gene regions

Primers were designed based on the *Rc*, *Rd* and *NCED2* genes of a rice reference sequence from *japonica* Nipponbare using Primer3 Ver. 0.4.0 (bioinfo.ut.ee/primer3-0.4.0/) (Table 1). PCR was performed in a total volume of 50 µL containing 1 × KAPA HiFi Fidelity buffer, approximately 500 ng DNA template, 0.3 mM dNTPs, 0.3 µM forward and reverse primers, and 0.2 U KAPA HiFi DNA polymerase

Table 1 List of primers used in this experiment

Primer	Sequence (5′ → 3′)
NCED-forward primer	TTCGTCTCGCAGTTTACAGG
NCED-reverse primer	ACTGGCACTTGGCGTCTTAG
Rc-forward primer	AAGCCTACCCTCTCACAGCA
Rc-reverse primer	CGGTTCCTTAGCTGCTTCAC
Rd-forward primer	CCATCACCAAGTGCAAGGTA
Rd-reverse primer	TCTCTTGCTTTGCTGCTTCA
Rd-internal forward primer	TGGGTTAGGAACAACGATCC
Rd-internal reverse primer	GGGCTCTCGAAGAGGAAGAT

(KAPA Biosystems, Switzerland). The PCR was conducted using the following parameters: 95 °C for 7 min, followed by 30 cycles of denaturation at 98 °C for 30 s, annealing for 45 s at the optimum annealing temperature (Table 1) and extension at 72 °C for 1 kb per minute. The PCR products were purified using MEGAquick-spin™ Total Fragment DNA Purification Kit (Intron Biotechnology, Korea) according to the manufacturer's instructions. The PCR products were either sequenced directly or cloned into TA cloning vector before sequencing.

For TA cloning, 100 ng of purified PCR product was added to 1X PCR buffer, 0.2 mM dATP (New England Biolabs, USA) and 0.2 U *Taq* DNA polymerase (Genedirex, USA) in a total volume of 100 µL and incubated at 72 °C for 1 h. The A-tailed PCR product was then purified using MEGAquick-spin™ Total Fragment DNA Purification Kit (Intron Biotechnology, Korea) according to the manufacturer's instructions. The purified A-tailed product was cloned into TA cloning vector (Yeastern Biotech, Taiwan) and transformed into *Escherichia coli* DH5α competent cells prepared using rubidium chloride (Green and Rogers 2013). The transformed clones were verified by colony PCR with M13 primer pairs and restriction enzyme digestion prior to sequencing.

Sequence analysis of *Rc*, *Rd* and *NCED2* genes

The sequences were analyzed using BioEdit Sequence Alignment Editor, version 7.2.1 (Hall 1999). Multiple sequence alignment was conducted with ClustalW algorithm (Thompson et al. 1994) with reference sequences from different subpopulations; *japonica* Nipponbare, *indica* IR64 and *aus* Kasalath retrieved from Rice SNP-Seek Database (http://www.snp-seek.irri.org).

Mining of single nucleotide polymorphism (SNP) in *NCED2* gene

The single nucleotide polymorphism (SNP) at position 14,233,796 in the *NCED2* gene (LOC_Os12g24800) which was reported to be associated with a higher production of ABA (Lyu et al. 2013; Alexandrov et al. 2015) was analyzed in 3024 rice varieties available in the Rice SNP-Seek Database (http://www.snp-seek.irri.org). The frequencies of C and T alleles at the mentioned position were calculated in 184 upland (Supplementary Table S2), 506 *japonica* and 1154 *indica* rice varieties with different pericarp colors (brown, light brown, speckled brown, red, purple, variable purple or a mixture colors) available at the Rice SNP-Seek Database (http://www.snp-seek.irri.org).

Results and discussion

Rd and *Rc* genes from upland rice varieties

The pericarps of Malaysian upland rice varieties analyzed in this study were found to be in red and white colors (Fig. 1). Among the upland rice varieties analyzed in this study, Pandasan was found to have both white and red grains, Tomou and Taragang bear red grains while the other rice varieties bear white grains.

Since the loss of red pigment in rice could be due to the SNP in *Rd* gene which causes an early termination of DFR protein which is involved in the red pigment biosynthesis, or due to a deletion in *Rc* gene encoding the BHLH transcription factor which regulates the red pigment biosynthesis, both *Rc* and *Rd* genes were analyzed in this study. Nipponbare which has white pericarp was found to have a SNP (A-nucleotide) that causes early translational termination of DFR whereas IR64, Kasalath and all the upland rice varieties analyzed in this study were found to have C-nucleotide at the corresponding SNP position, irrespective of the pericarp colors (Fig. 2). The *Rc* gene of Bokilong, Gonsulak, Kuku Balam and Pandasan White with white pericarps demonstrated a 14-base deletion (ACGCGAAAAGTCGG) at exon 7, causing a frame shift in protein translation leading to an early truncation of bHLH transcription factor. With a defective *rc* gene

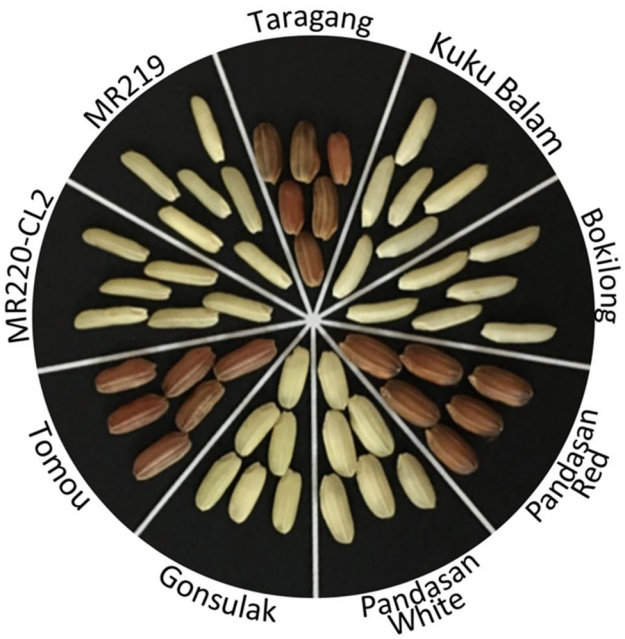

Fig. 1 The coloration of rice pericarp from Malaysian rice varieties. Tomou and Taragang have red pericarps while Pandasan has either red or white pericarp. MR220-CL2, MR219, Gonsulak, Bokilong and Kuku Balam have white pericarps

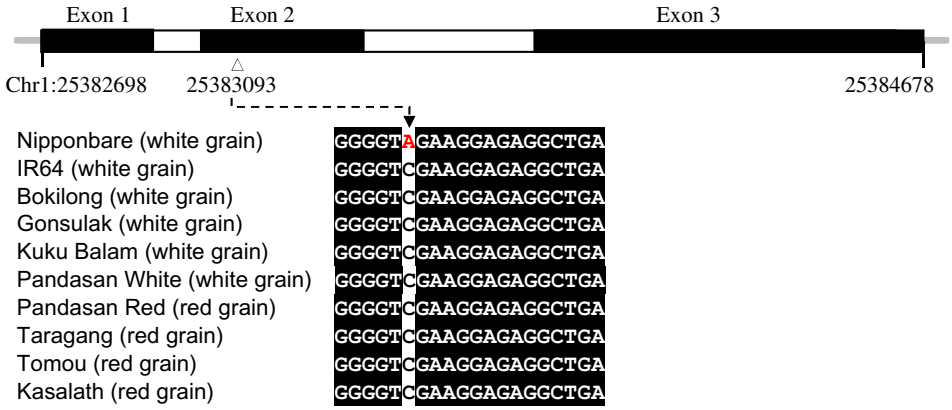

Fig. 2 The gene structure of *Rd* gene (LOC_Os01g44260) and the SNP associated with the grain color of rice. The upper panel shows the schematic representation of *Rd* gene whereby the exons and introns are shown by black and white boxes, respectively. The open triangle indicates the location of single nucleotide substitution from A to C in exon 2 at position 25,383,093 of chromosome 1 that causes an early translational termination of dihydroflavonol-4-reductase

hence a non functional BHLH, these rice varieties could not produce anthocyanin even though the DFR in these rice varieties could be fully functional. Only the Pandasan variety germinated from red grains (Pandasan Red), Tomou and Taragang varieties that bear red pericarps have a non-defective *Rc* gene as in the Kasalath variety which also produces red pericarp (Fig. 3). In summary, the white grain phenotype in Nipponbare and the upland rice varieties analyzed in this study (Bokilong, Gonsulak, Kuku Balam, and Pandasan White) was caused by different pericarp coloration genes, i.e., mutation of *Rd* gene in Nipponbare and a 14-bp deletion in *Rc* gene of the upland rice varieties.

NCED2 genes from upland rice varieties

The gene structure of *NCED2* gene (LOC_Os12g24800) and SNP associated with drought tolerance of upland rice varieties reported by Lyu et al. (2013) is shown in Fig. 4. A single nucleotide substitution from C to T at position 14,233,796 at chromosome 12 causes the change of valine to isoleucine in the protein. *NCED2* with the substitution of isoleucine at the corresponding amino acid position was reported to be dominant in many upland rice varieties and these rice varieties exhibited a higher level of ABA and were more tolerant to drought (Lyu et al. 2013). The *NCED2* gene from Bokilong, Gonsulak, Kuku Balam, and Pandasan White belong to the C-type while Pandasan Red, Tomou and Taragang varieties have the T-type *NCED2*

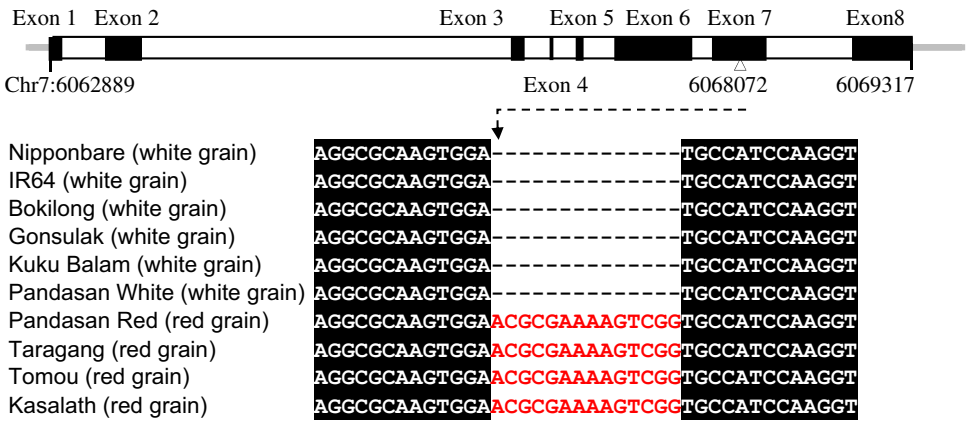

Fig. 3 The gene structure of *Rc* gene (LOC_Os07g11020) and the changes in nucleotide sequence associated with the grain color of rice. The upper panel shows the schematic representation of *Rc* gene whereby the exons and introns are shown by black and white boxes, respectively. The open triangle indicates the location of 14-base deletion (ACGCGAAAAGTCGG) at exon 7 in rice varieties with white grains which causes a frame shift in protein translation leading the truncation of bHLH transcription factor

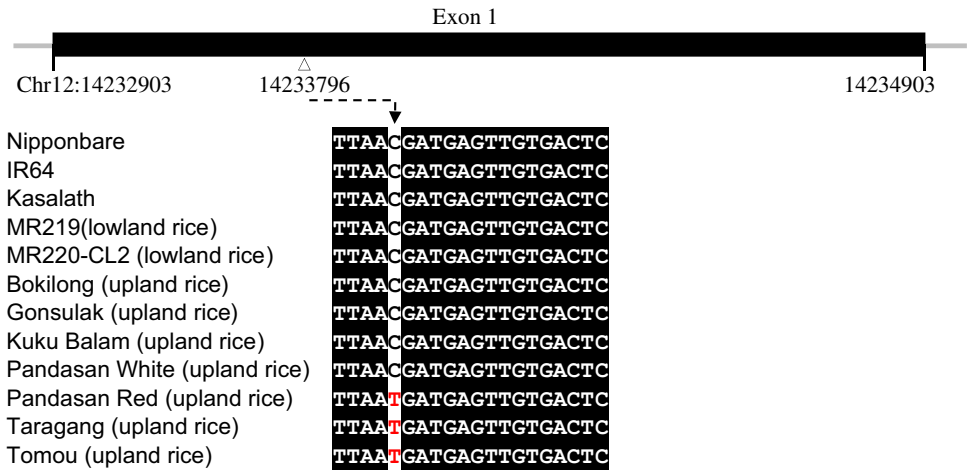

Fig. 4 The gene structure of 9-cis-epoxycarotenoid dioxygenase 1 (*NCED)* gene (LOC_Os12g24800) and SNP associated with drought tolerance of upland rice varieties. The upper panel shows the schematic representation of *NCED* gene whereby the exon is shown by a black box. The open triangle indicates the location of single nucleotide substitution from C to T at position 14,233,796 at chromosome 12 that causes the change of valine to isoleucine in the protein

(Fig. 4). Coincidently, all three rice varieties with the T-type *NCED2* were found to bear red pericarps (Fig. 2). Since the T-allele in *NCED2* gene was also found to be associated with a higher ABA level in upland rice (Lyu et al. 2013), Pandasan Red, Tomou and Taragang which have the T-allele in their *NCED2* genes may have a higher level of ABA. Nevertheless, Kasalath which bears red pericarp was found to have a C-type *NCED2* (Fig. 4).

Since ABA level and the expression of *NCED* (which is involved in ABA biosynthesis) were associated with pigment production (Li et al. 2019; Karppinen et al. 2018; Jia et al. 2011), we also examined whether the red pigmentation was correlated to the T-allele in *NCED2* by analyzing the frequencies of C- and T- alleles in the *NCED2* gene of 1967 rice varieties with different pericarp colors available at the Rice SNP-Seek Database (http://www.snp-seek.irri.org), i.e., 1547 white, 2 brown, 14 light brown, 9 speckled brown, 371 red, 18 purple, 1 variable purple, and 5 mixture rice varieties (Supplementary Table S2) (Mansueto et al. 2017). The percentage of T-allele in *NCED2* gene was 13.10%, 19.01% and 10.82% for the upland, *japonica* and *indica* rice varieties with pigmented grains, respectively (Table 2). The C-allele was found to be the major allele for all three rice groups with pigmented grains (i.e., 86.90%, 80.99% and 89.18% for the upland, *japonica* and *indica* varieties, respectively). C-allele was predominant in the *NCED* gene from rice varieties with white grains, i.e., 84.29%, 86.23% and 92.20% for the upland, *japonica* and *indica*, respectively. Our findings did not support the association of the T-type *NCED2* with pericarp colour. Since the rice genome has five *NCED* homologs (*NCED1-5*; Huang et al. 2018; Hwang et al. 2018), a higher activity of other NCEDs may also increase the ABA level and improve anthocyanin/proanthocyanidin production.

Table 2 Allele percentage at rice *NCED2* at position14233796 in chromosome 12

Pericarp color[a]	Upland[b]		*Japonica*[b]		*Indica*[b]	
	C-allele (%)	T-allele (%)	C-allele (%)	T-allele (%)	C-allele (%)	T-allele (%)
White	84.29	15.71	86.23	13.77	92.20	7.80
Pigmented	86.90	13.10	80.99	19.01	89.18	10.82
Brown	N/A	N/A	100	0	100	0
Light brown	100.00	0	N/A	N/A	90.00	10.00
Speckled brown	N/A	N/A	100	0	100	0
Red	84.29	15.71	79.31	20.69	88.96	11.31
Purple	100	0	88.89	11.11	94.44	5.56
Variable purple	100	0	N/A	N/A	100	0
Mixture	100	0	75.00	25.00	90.00	10.00

[a]Pericarp color was categorized following the Rice Standard Evaluation, IRRI (https://snp-seek.irri.org/phenotype_dict.pdf) based on the Methuen Handbook of Colours (Kornerup and Wanscher 1967). Please refer to the footnote of Supplementary Table S2 for more information

[b]The number of upland, *japonica* and *indica* varieties analyzed were 184, 506 and 1154; respectively

Conclusions

We conclude that all six upland rice varieties analyzed in this study have a *Rd* gene encoding a complete DFR (without an early translational termination codon). However, the upland rice varieties with white pericarps were found to have a 14-base deletion at exon 7 in *Rc* gene which could cause a frame shift in protein translation, leading to early truncation of bHLH transcription factor which regulates the red pigment biosynthesis. The upland rice varieties with white pericarps in this study have C-type *NCED2* gene while Pandasan Red, Tomou and Taragang varieties that bear red pericarps were found to have T-type *NCED2,* which could possibly accumulate a higher level of ABA that upregulates the expression of genes in the anthocyanin/proanthocyanidin biosynthetic pathway. However, not all rice varieties with pigmented grains in the Rice SNP-Seek Database have T-type *NCED2*. A better understanding of the gene sequences of upland rice varieties with red pericarp may provide important information for rice-breeding programs.

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Author contributions CLH and CYT designed the experiments; MAH, NAMK, AS, NM. NIMR conducted the experiments and analyzed the data; CLH wrote the article with the contributions of all authors; all authors approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declared no conflict of interest.

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