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Rice Science, 2016, 23(1): 1−8

A Point Mutation in an F-Box Domain-Containing Protein Is Responsible for Brown Hull Phenotype in Rice

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Abstract: The accumulation of pigments affects the color of rice hulls while only limited information is known about its underlying mechanisms. In the present study, a rice *brown hull 6* (*bh6*) mutant was isolated from an ethane methyl sulfonate (EMS)-induced IR64 mutant bank. Brown pigments started to accumulate in *bh6* rice hulls after heading and reached a higher level in mature seeds. Some major agronomic traits including panicle length and 1000-grain weight in *bh6* were significantly lower than those in its corresponding wild type IR64, while other agronomic traits such as plant height, growth duration and seed-setting rate were largely similar between the two genotypes. The analysis of pigment content showed that the contents of total flavonoids and anthocyanin in *bh6* hulls were significantly higher than those in IR64 hulls. Our results showed that the brown hull phenotype in *bh6* was controlled by a single recessive gene which locates on the long arm of chromosome 9. Sequencing analysis detected a single base substitution (G/A) at position 1013 of the candidate gene (LOC_Os09g12150) encoding an F-box domain-containing protein (FBX310). Functional complementation experiment using the wild type allele can rescue the phenotype in *bh6*. Thus, we named this mutated gene as $OsFBX310^{bh6}$, an allele of *OsFBX310* functioning as an inhibitor of brown hull. The isolation of $OsFBX310^{bh6}$ and its wild type allele can provide useful experimental materials and will facilitate the studies on revealing the mechanisms of flavonoid metabolism in monocot plants.

Key words: *Oryza sativa* L.; brown hull mutant; *OsFBX310* gene; flavonoid metabolism

Pigment is an important character of plant taxonomy and plays an important role in plant development. In most plant species including rice (*Oryza sativa* L.), the coloration of flowers and fruits is achieved by the accumulation of flavonoid metabolites (Quattrocchio et al, 2006; Wang et al, 2014). The golden/brown hulls of rice are firstly described in 1917 and have been long used as a marker in rice breeding and genetic studies. However, the molecular basis of flavonoid metabolism and the formation of hull colors in rice have not been well understood.

Internal genetic factors are responsible for the coloration of rice hulls. The well known *C-A-P* control system is established in 1950s. This theory states that the formation of rice pigments is complementally controlled by three genes including *C* (chromogen), *A* (activator) and *P* (distributer) (Nagao, 1951; Takahashi, 1957; Reddy et al, 1995; Reddy, 1996). Subsequently, the *C* gene is mapped to the short arm of chromosome 6 (Kinoshita, 1984), and it encodes a protein belonging to the MYB transcription factor family (Saitoh et al, 2004). The genomic locations of the *A* and *P* genes have been determined but yet need to be isolated (Kinoshita, 1984). In addition, a small number of rice hull mutants have been reported (Iwata and Omura, 1977; Cui et al, 2007; Li et al, 2008; Li et al, 2012).

Received: 12 May 2015; **Accepted:** 6 July 2015

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Peer review under responsibility of China National Rice Research Institute

http://dx.doi.org/10.1016/j.rsci.2016.01.001

Compared with the normal straw color hulls, the abnormal rice hulls refer to golden, brown and black hulls. The *golden hull and internode-1* (*gh-1*) mutant was identified in early 1960s (Nagao and Takahashi, 1963) and is caused by the disruption of the *OsCHI* gene which encodes an enzyme involved in flavonoid pathway (Hong et al, 2012). The *golden hull and internode-2* (*gh-2*) mutant was identified in early 1970s (Iwata and Omura, 1971) and is controlled by the *GH2* gene which encodes a cinnamyl-alcohol dehydrogenase participating in the synthesis of monolignols to provide precursors for rice lignin biosynthesis pathway (Zhang et al, 2006). Interestingly, black hull is also a rare trait in common wild rice (Maekawa, 1984; Gu et al, 2005; Zhu et al, 2011). Recently, the *Rc* (brown pericarp and seed coat) gene encoding a bHLH protein which is a positive regulator involved in proanthocyanidin biosynthesis has been isolated (Furukawa et al, 2007). The inhibitor of brown furrow (IBF1) contains a putative Kelch domain of F-box proteins, and regulates pigment synthesis and deposition in rice hulls (Shao et al, 2012). Till now, the molecular mechanism of hull color formation in rice is largely unknown so that the identification and characterization of rice hull color mutants are necessary and will help to facilitate the studies on revealing the molecular basis of hull color formation.

In the previous study, we had isolated a *brown hull 6* (*bh6*) mutant from an IR64 mutant bank (Wu et al, 2005). This study presents the results of phenotypic characterization, genetic analysis, map-based isolation and validation of the mutation. The mutation happened in a single recessive gene, $OsFBX310^{bh6}$, locating on the long arm of chromosome 9. It encodes an F-box domain containing protein (FBX310). A single base substitution (G/A) was detected in $OsFBX310^{bh6}$. Functional complementation using the wild type allele can rescue the phenotype of $OsFBX310^{bh6}$ mutation. Thus, our results indicate that function loss of *OsFBX310* is responsible for the brown hull phenotype in *bh6*.

MATERIALS AND METHODS

Rice materials

The *brown hull 6* (*bh6*) mutant was obtained from an ethane methyl sulfonate (EMS)-induced IR64 mutant bank (Wu et al, 2005). This mutant has been selfed for more than nine generations and the target trait has been stably expressed in both greenhouse and field conditions in Fuyang, Hangzhou, Zhejiang Province and Lingshui, Hainan Province, China. This mutant was crossed with the wild type IR64 and the variety CPSLO17 with normal hull, respectively, so as to construct F_2 populations for genetic analysis and genomic mapping.

Methods

Phenotypic observation

In the summer of 2012, the mutant *bh6* and the wild type IR64 were grown in the paddy fields of the China National Rice Research Institute (CNRRI) in Fuyang, Hangzhou, Zhejiang Province, China. The experiment was performed under a completely randomized block design with three replications. The plant height, growth duration, panicle length, numbers of productive panicles per plant and filled grains per panicle, seed-setting rate and 1000-grain weight of mutant and wild type rice were recorded. The data from the three replications were used for statistical analysis.

Measurement of anthocyanin content

The content of anthocyanin was detected according to the method of Mirecki and Teramura (1984) with minor modification. In brief, mature rice hulls were ground in liquid nitrogen and anthocyanin was extracted with HCl/methanol (1:99) solution at 4 °C for 24 h. The extract was precipitated by centrifugation at 12 000 × *g* for 30 min. The supernatant was used for detecting absorbance values at 530 and 657 nm with a Lambda-25 spectrophotometer (PerkinElmer, USA). The anthocyanin content was calculated by the absorbance (*A*) difference between A_{530} and A_{657}. The data from three measurements were used for statistical analysis.

Measurement of total flavonoids

Total flavonoids were detected according to the method of Shao et al (2012) with minor modification. Briefly, 1.5 g of mature rice hulls were ground in liquid nitrogen and incubated in 80% methanol at 25 °C for 24 h. The extract was precipitated by centrifugation at 12 000 × *g* for 30 min. The supernatant was recovered by adding 10% $AlCl_3$ solution to a final concentration of 1%. Absorbance values were detected at the wavelength of 420 nm. The data from three measurements were used for statistical analysis.

Genetic analysis

The *bh6* mutant as the female parent was crossed with

the male parents IR64 and CPSLO17 in Lingshui, Hainan Province, China in 2010. F_1 plants from these two crosses were grown in the paddy fields at CNRRI and were selfed to produce F_2 seeds in the same year. The presence or absence of brown hull was recorded in individual F_2 plant from the crosses *bh6*/IR64 and *bh6*/CPSLO17 in the paddy fields at CNRRI in 2011. Three segregating F_3 lines from the cross *bh6*/IR64 were grown at CNRRI in 2012 for phenotyping.

DNA extraction and PCR amplification

The DNA of parents and F_2 individuals with brown hulls were extracted following the mini-preparation method (Lu and Zheng, 1992). PCR amplification was performed according to Shi et al (2009), and the PCR products were separated and visualized on 6% non-denaturing polyacrylamide gels using silver staining.

Genetic mapping

Equal amount of leaf blades from each of 10 wild type plants and 10 mutant plants were collected for DNA extraction, and a wild type DNA pool and a mutant DNA pool were established, respectively. These two DNA pools and 72 randomly selected F_2 individuals with brown hulls were used to rapidly locate the mutation site. A total of 1 014 simple sequence repeat (SSR) markers evenly covering 12 chromosomes were applied in polymorphism survey on the two parents *bh6* and CPSLO17. The polymorphic markers were then used for screening the above two DNA pools, and genotyping 72 randomly-selected F_2 individuals for confirming the linkage between markers and the mutation. Subsequently, 877 F_2 individuals were used for fine mapping. SSR markers were obtained from the Gramene database (www.gramene.org) (Table 1), while insertion/deletion (InDel) markers were designed using the Primer 5.0 software and DNAStar 8.0 software after comparing the sequences between the japonica variety Nipponbare and the indica variety 9311 in public databases, RGP (http://rgp.dna.affrc.go.jp/E/toppage.html), Gramene (http://gramene.org/genome_browser/index.html) and the Gene Research Center of the Chinese Academy of Sciences (http://rice.genomics.org.cn/rice/index2.jsp). Primers were synthesized by Sangon Biotech Co. Ltd (Shanghai, China).

Cloning of mutated sequence and complementation analysis

Open reading frames were predicted by the rice genome automated annotation system (http://rice.plantbiology.msu.edu/). The candidate gene was amplified with the following primers (BH6F: 5′-TATGACCATGATTACGAATTCTTTAGACATACAATGAGATGACCTC-3′ and BH6R: 5′-ACGACGGCCAGTGCCAAGCTTCCCCTATTATAAGTAGTCACTTTAG-3′). A 9 218 bp wild type genomic DNA fragment containing a 5 018 bp upstream sequence, a 1 185 bp coding sequence, and a 3 015 bp downstream sequence was amplified and inserted into the binary vector pCAMBIA1300 to generate a construct (p1300-9.3) for complementation test. The complementation vector p1300-9.3 was introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation and then transformed into the mature embryo-induced calli of *bh6* according to the *A. tumefaciens*-mediated method (Zhu et al, 2001).

RESULTS

Phenotypic performance of *bh6* mutant

Brown pigments started to accumulate in hulls of *bh6* mutant after heading and peaked in mature seeds under field conditions in the summer of 2012 in Fuyang, Hangzhou, Zhejiang Province, China. By contrast, the hull color of the wild type IR64 remained straw-yellow (Fig. 1). Additionally, the mutant plants grew more slowly than the wild type, and the heading date of *bh6* was about one week later than that of IR64. Some other agronomic traits of the mutant, including panicle length and 1000-grain weight, were also significantly lower than those of the wild type, while the number of productive panicles per plant, plant height, seed-setting rate and number of filled grains per panicle were similar between these two genotypes

Table 1. Molecular markers used for gene mapping.

Marker	Forward primer (5′→3′)	Reverse primer (5′→3′)
RM23804	GAGCCGACTCAATCCAATCCTCTCC	ATCGTCTTCCAAGCAAGTGCAAGC
RM23893	GGCGGCTTAAGAGTGTTTGTAGG	AGAGGATTGCTTTGCTGATGAGG
RM23904	CTCACCGGAGCACCACTAACC	GAGAGCAAGACTGTGAAGTGTGAACC
RM7038	GATTAGAGCTTTGGTGGTTCTTGG	ACTTGTGGTCGGTCTGGTAGTCC
InDel_6	GAATCCGGTTCCGAGACTAAA	TCAATCTTGCAGCAAACAAGGC
InDel_10	ACCTGATCGATGTCTAATTTGCAC	CGTTTACGAAAGGCAGAGGACA

Fig. 1. Phenotypes of the mutant *bh6* and its wild type IR64.

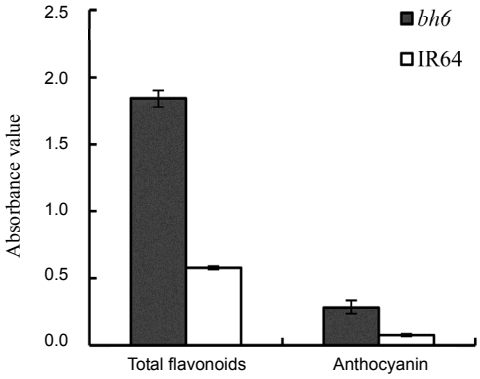

Fig. 2. Total contents of flavonoid and anthocyanin in rice hulls.

(Table 2).

Total flavonoids and anthocyanin contents

Total flavonoids and anthocyanin contents were detected in the seeds of *bh6* and IR64. The contents of total flavonoids in *bh6* and IR64 were 1.86 ± 0.06 and 0.60 ± 0.04, respectively. The contents of anthocyanin in *bh6* and IR64 were 0.30 ± 0.02 and 0.09 ± 0.01, respectively. These results showed that both the contents of total flavonoids and anthocyanin in *bh6* were significantly higher than those in IR64 (Fig. 2).

Genetic analysis of *bh6*

The mutant *bh6* was used as a female parent and crossed to the male parents IR64 and CPSLO17 with normal hull color to construct two populations. All F_1 plants from these two crosses showed normal hull color, indicating that the brown hull trait was controlled by recessive gene(s). The number of recessive gene(s) was determined based on the segregation of F_2 individuals derived from the crosses *bh6*/IR64 and *bh6*/CPSLO17. In F_2 plants, all individuals were similar to their parents, and no intermediate types were observed from these two crosses. In the population containing 2 107 plants derived from *bh6*/CPSLO17, 1 600 plants had normal hull color and 507 plants had brown hull, matching the 3:1 Mendelian segregation ($\chi^2 = 0.962 < \chi^2_{0.05} = 3.84$). In the population containing 684 plants derived from *bh6*/IR64, 507 plants had normal hull and 177 plants showed brown hull, again matching the predicted 3:1 Mendelian ratio ($\chi^2 = 0.236 < \chi^2_{0.05} = 3.84$) (Table 3). These results indicate that the mutated trait is controlled by a single recessive gene. In addition, the ratios of wild type to mutant type in the three F_3 segregation lines derived from *bh6*/IR64 were 3:1 (Table 4), suggesting that the mutation is indeed governed by a single recessive gene.

Genomic mapping of *bh6*

Bulk segregant analysis was used to rapidly locate the mutation on chromosome, and 72 randomly selected F_2 individuals with brown hulls were used for further confirmation. Four polymorphic SSR markers (RM23804, RM23893, RM23904 and RM7038) from chromosome 9 between parents and between two pools were identified (Table 1). Our results indicate

Table 2. Performance of agronomic traits between wild type IR64 and mutant *bh6*.

Material	Plant height (cm)	Panicle length (cm)	No. of productive panicles per plant	No. of filled grains per panicle	Seed-setting rate (%)	1000-grain weight (g)
IR64	120.0 ± 1.5	27.4 ± 0.3	15 ± 3	89 ± 12	71.6 ± 1.3	24.3 ± 0.1
bh6	119.4 ± 1.4	26.1 ± 0.4*	16 ± 2	81 ± 16	70.7 ± 1.6	22.5 ± 0.1**

* and ** represent significant difference at the 0.05 and 0.01 levels, respectively.

Table 3. Genetic analysis of brown hull mutant *bh6*.

Cross combination	F_1	F_2			$P_{(3:1)}$
		Total No. of plants	No. of wild type plants	No. of mutant type plants	
bh6/CPSLO17	Normal	2 107	1 600	507	0.32
bh6/IR64	Normal	684	507	177	0.60

Table 4. Genetic analysis of F_3 segregation lines derived from *bh6*/ IR64.

F_3 line	Total No. of plants	No. of wild type plants	No. of mutant type plants	$P_{(3:1)}$
Line 1	276	216	60	0.21
Line 2	212	163	49	0.53
Line 3	132	107	25	0.11

that the mutation may locate on chromosome 9. After genotyping 72 randomly-selected F_2 individuals using these four markers, the numbers of crossing-over plants were 10, 3, 12 and 30, respectively (Fig. 3-A). Therefore, these four markers were linked to the target gene on chromosome 9. To further delimit the gene location, additional polymorphic SSR and InDel markers around the chromosomal region were identified and then used for further genotyping 877 F_2 mutant type individuals. The subsequent results showed that the numbers of crossing-over plants when using the four markers RM23893, InDel_6, InDel_10 and RM23904 were 3, 1, 2 and 12, respectively. It indicates that the mutation may locate at the interval between InDel_6 and InDel_10, covering approximately 106 kb region (Fig. 3-B).

Isolation of candidate gene

Based on the gene annotation in the Gramene database, 12 open reading frames and an F-box domain containing protein (LOC_Os09g12150) were predicted in the 106 kb region on rice chromosome 9. Based on the previous report (Shao et al, 2012), the F-box domain containing protein (LOC_Os09g12150) should be the candidate gene which is thus termed as *OsFBX310* here afterwards. The open reading frames of *OsFBX310* contained a 1 185 bp coding sequence, encoding a predicted protein with 395 amino acid residues. Sequence analysis between the wild type IR64 and the mutant *bh6* detected a single base substitution (G/A) at position 1013 in the mutated allele of $OsFBX310^{bh6}$ (Fig. 3-C). It further indicates that $OsFBX310^{bh6}$ is responsible for the brown hull phenotype in *bh6* mutant.

Genetic complementation

To further clarify whether the above candidate gene *OsFBX310* was indeed responsible for the brown hull phenotype, a genetic complementation test was performed by transforming the wild type genomic clone of *OsFBX310* encompassing upstream sequence, coding region and downstream sequence to the mutant (Fig. 4-A). A total of 14 independent transgenic lines were obtained, and all of them exhibited normal straw-yellow color in hulls, similar to the wild type (Fig. 4-B). In addition, sequencing analysis of transformants revealed that the wild type *OsFBX310* sequence has been integrated into the *bh6* genome (Fig. 4-C). Therefore, it is concluded that the mutation in *OsFBX310* encoding an F-box domain containing protein is responsible for the brown hull phenotype in *bh6* rice plants.

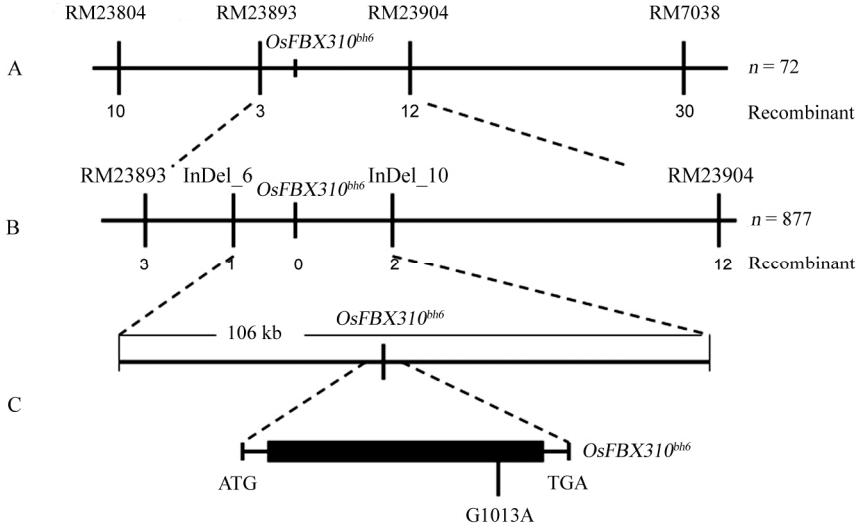

Fig. 3. Fine mapping of the mutation on chromosome 9.

A, *bh6* is linked with SSR markers RM23804, RM23893, RM23904 and RM7038; B, *bh6* is located in a 106 kb region between InDel_6 and InDel_10; C, Single base substitution at position 1013 (G1013A).

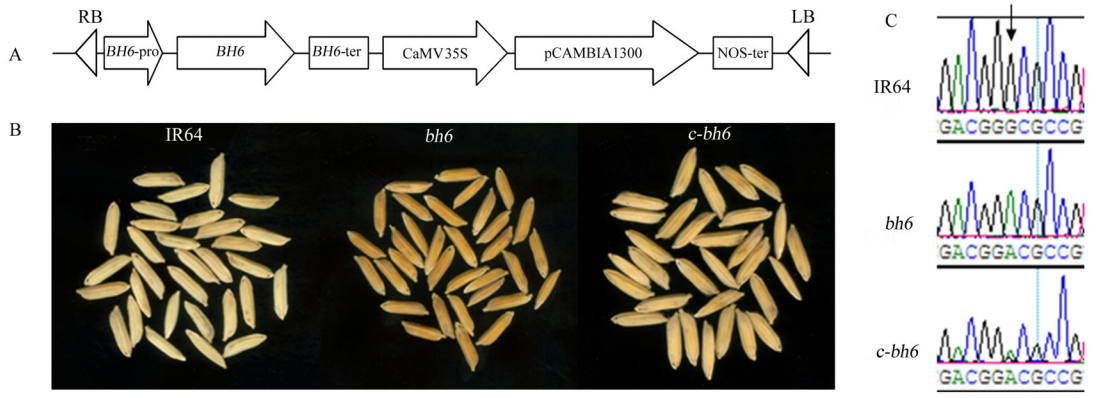

Fig. 4. Functional complementation of the mutation.

A, Complementation construct p1300-9.3; B, Phenotype of IR64, *bh6* and complementary transgenic line (*c-bh6*); C, Sequencing analysis of complementary transgenic lines. The arrow refers to the mutation site.

DISCUSSION

In plants, flavonoids provide blue, red and purple pigments for flowers, fruits and leaves while usually only provide brown pigments for the coloration of seeds (Shirely, 1996). Besides coloration, flavonoids have many other important physiological functions such as dormancy and viability (Lepiniec et al, 2006). Flavonoids are the secondary metabolites and consist of several major categories such as anthocyanins (red to purple pigments), flavonols (colorless to pale yellow pigments), and proanthocyanidins known as condensed tannins (colorless pigments but brown after oxidation). The amount and distribution of flavonoids vary among different plant species, organs and developmental stages, and depend on growth conditions (Debeaujon et al, 2001). In plants, brown color usually results from phenolic compounds which are oxidated into highly reactive state and spontaneously polymerize to form brown products (Pourecl et al, 2005).

In the present study, we have isolated *OsFBX310* and confirmed that a point mutation is responsible for the phenotype in the mutant *bh6*. The contents of anthocyanins and total flavonoids in *bh6* were much higher than those in the wild type IR64, suggesting that the *OsFBX310* in rice has a similar role with the *I* (Inhibitor) allele in soybeans. In soybeans, the seed coat color is determined by the classically defined *I* locus. The dominant *I* allele inhibits seed coat pigmentation, and it has been suggested that there is a correlation between the inhibition by *I* allele and the silence of *chalcone synthase* (*CHS*) gene in the seed coat (Senda et al, 2002). In rice, the *gold hull and internode 1* (*gh-1*) phenotype is caused by the disruption of the *OsCHI* gene which encodes an enzyme involved in flavonoid pathway, while the *gh-2* phenotype is controlled by the *GH2* gene which encodes a cinnamyl-alcohol dehydrogenase engaging in monolignols synthesis to provide precursors for rice lignin biosynthesis (Zhang et al, 2006; Hong et al, 2012). In the present study, the elevated accumulation of anthocyanins may be the direct cause of brown hulls in *bh6*. Han et al (2006) reported that anthocyanidin firstly pigmentizes at the part of caryopsus away from embryo, then gradually extends to the part near the embryo, and makes the whole pericarp pigmentized. Similarly, *bh6* exhibited an obvious accumulation of slight-brown pigment in the furrows of glumes after pollination, and hull color gradually became darker and finally turned into typical brown during the desiccation of seeds.

In *Oryza sativa*, flavonoid pathway has been characterized mainly by mutants with altered seed colors, especially brown. To date, about eight genes related to seed coat color have been identified in rice, including the genes encoding flavonoid biosynthesis related enzymes (*OsCHS*, *OsCHI* and *Cfi*), transcription factor MYB (*C* gene), WKRY domain proteins (*P1* and *Ra*) and MYC proteins (*Rb* and *OSB1*). Based on the previous report, $OsFBX310^{bh6}$ is an allele of *IBF1* functioning as a negative regulator in flavonoid biosynthesis. The *bh6* mutant shows a single base substitution (G-A) while the *ibf1* allele contains three nucleotides (109G, 110C and 409G) deletion. In addition, all these changes do not result in significant phenotypic difference between *bh6* and *ibf1*. The isolation of *OsFBX310* and its recessive allele in the present study can facilitate the further studies on flavonoid metabolism in the monocot rice.

ACKNOWLEDGEMENT

This research was supported by the National High Technology Research and Development Program of China (Grant Nos. 2012AA101102 and 2011AA10A101).

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(Managing Editor: LI Guan)