

Original Article

Complementary gene controlling the black hull phenotype and phenolics and flavonoids content in weedy rice (*Oryza sativa* f. *spontanea*) from northeastern Thailand

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Abstract

A complementary gene black seed hull 4 (*Bh4* and *Phr1*) is a hull color candidate gene that causes a black pigmentation in the ripened hulls of rice. The relationship between *Bh4* and *Phr1* in the black hull phenotype was elucidated previously. In this study, 82 ripened weed rice seed samples were collected to analyze the relationships between the phenotype and genotype of the two genes controlling seed hull color and to evaluate the phytochemical compounds in weedy rice grains. The results showed evidence which strongly supported the hypothesis of a complementary gene (*Bh4* and *Phr1*) controlling black seed hull in rice. The total phenolic content of brown weedy rice grains of black seed hull (2.58 mgGAE/ g) and straw seed hull (1.58 mg GAE/ g) with red pericarp was higher than weedy rice with black seed hull white pericarp (0.27 mg GAE/ g) and two traditional red rice varieties (Mun Pu, 1.51 mg GAE/ g), Niew Daeng, 1.24 mg GAE/ g). Total flavonoid content of the brown weedy rice grains revealed a similar trend with total phenolic content. This observation indicated that weedy rice is a rich source of phytochemicals that might have potential benefits for human health.

Keywords: weedy rice, seed hull color, phytochemical compounds

1. Introduction

Weedy rice (*Oryza sativa* f. *spontanea*) is found in cultivated rice fields worldwide. It has spread rapidly in areas where direct seeding or related technologies have been adopted and accompanied by less weed management (Yu, Bao, Shi, Dong, & Ge, 2005). It causes yield reduction and affects the quality of rice grain (Rathore, Singh, & Kumar, 2013). Weedy rice is classified as the same species as Asian cultivated rice (*O. sativa*), but it has morphological characteristics typical of wild rice species, e.g., seed dormancy and shattering, and cultivated rice, e.g., rapid growth, high selfing rate (Reagon *et al.*, 2010). The evolution and origins of weedy rice have been investigated in many locations both inside and outside the range of wild rice (*O. rufipogon*), which is the putative ancestor of Asian cultivated rice (Cao *et al.*,

2006; Londo & Schaal, 2007). Gene transfer from weedy rice to cultivated rice or from cultivated rice to weedy rice plays an important role in the evolution of both weedy rice and cultivated rice populations via its influences on genetic diversity and differentiation (Shivrain, Burgos, Gealy, Sale, & Smith, 2009; Xia, Wang, Xia, Zhao, & Lu, 2011).

Among rice genes, some loci have been used to study the genetic basis of evolution such as the seed shattering gene (*sh4*) in weedy rice populations (Zhu, Ellstrand, & Lu, 2012). The genes that control color expression in rice plants, such as the accumulation of anthocyanin in many plant tissues or organs in rice, leads to red, purple red, and purple phenotypes for protection from damage by biotic and abiotic stresses and for reproduction (Chin, Wu, Hour, Hong, & Lin, 2016). The hulls of cereal are considered to play a role in the protection of seeds from physical and oxidative damages (Ramarathnam, Osawa, Kawakishi, & Namiki, 1987). In the USA and Thai weedy rice populations, two main morphological groups, including straw-hull (SH) type and black-hull (BH) type, have been reported (Thurber *et al.*, 2010).

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The genetic mechanism involved in the transition from the black-colored seed hull of the ancestral wild rice to the straw-white seed hull of cultivated rice during grain ripening was reported by Zhu *et al.* (2011). They reported that the black hull of *Oryza rufipogon* was controlled by the *Bh4* gene, which was fine-mapped to an 8.8-kb region on rice chromosome 4. The *Bh4* gene encodes an amino acid transporter. A 22-bp deletion within exon 3 of the *bh4* variant disrupted the *Bh4* function, leading to a straw-white hull in cultivated rice. The black hull gene has been studied in terms of the parallel evolution of hull color variation in the domesticated and weedy rice system (Vigueira, Li, & Olsen, 2013). The results of that study showed that independent *Bh4* coding mutations have arisen in African and Asian rice that correlated with the straw hull phenotype which indicated that the *Bh4* gene is responsible for parallel trait evolution. The most important part of the study is that the weed forms can draw on both ancestral genes and crop genes as these genes respond to the selection pressures exerted by factors of modern agriculture.

Recently, the most important finding of genes correlated with black hull rice was reported by Fukuda *et al.* (2012). The complementary gene caused black hull is *Phr1* gene, which encodes polyphenol oxidase (Yu *et al.*, 2008). They found that only the plants that had both functional *Bh4* and *Phr1* genes could produce black hull which resulted from an analysis of the segregated populations in crosses between *indica* and *japonica* rice.

Evolution of hull color in weedy rice populations was reported by (Vigueira *et al.*, 2013). They found that a test for selection indicated that the straw hull of weedy rice deviated from neutrality at this gene. This meant that there was possible selection on the hull color gene (*Bh4*) during both rice domestication and de-domestication. The black hull seeds of weedy rice fall off easily from the panicle of the rice plant at the maturation stage. Therefore, the black hull color can protect them from being targeted by birds and, as such, the allele frequency of black hull color would increase in the next generation. Recent studies show that birds around the rice field play an important role in the selection of straw-white seed hull in rice (Zhu *et al.*, 2011).

Rice strains with red pericarp show important attributes that are the bioactive phytochemicals present in whole grains more than white pericarp (Finocchiaro *et al.*, 2007; Zhou, Chen, Zhang, & Blanchard, 2014). These phytochemical compounds can reduce many chronic diseases such as cardiovascular and heart diseases, diabetes, and obesity (Choi, Kim, & Friedman, 2012). Studies of the benefit attributes of weedy rice in natural populations are now addressing questions that although weedy rice is an unwanted plant in paddy fields, whole grains with red pericarp of these unwanted plants are still carry phytochemical compounds that are beneficial for humans. In this study, weedy rice was sampled from paddy fields of Hom Mali rice in the northeastern Thailand to further determine the phytochemical compounds (flavonoids and phenolic acid) in whole grains and determine the relationship among the two genes controlling black hull phenotype. The study aimed to yield data on the level of bioactive compounds in weedy rice grains and the genetic background of weedy rice in the paddy fields to determine valuable implications for the evolution of weedy rice populations.

2. Materials and Methods

2.1 Weedy rice samples

Weedy rice samples (82 weedy rice plants) were taken between March and October of 2015 and 2016 from Thung Kula Ronghai area in the northeastern region of Thailand, including 33 accessions from Roi Et Province and 49 accessions from Mahasarakham Province. Each plant was collected and the seed hull color was recorded. In addition, flag leaf samples of the 82 weedy rice samples were also individually collected for DNA extraction. These samples were classified further for pericarp color of the seed following hull removal.

2.2 Evaluation of seed hull and pericarp color

To evaluate seed hull and pericarp color, a collection of weedy rice seed samples was classified based on hull color (black hull (BH) or straw hull (SH) seeds) and red or white pericarp color.

2.3 Determination of phytochemical compounds

Three phenotypes of weedy rice samples are used to determine the phytochemical compounds which included black hull seed with white pericarp (BH-W), black hull seed with red pericarp (BH-R), and straw hull with red pericarp (SH-R). In addition, two traditional rice varieties with red pericarp (Niew Daeng and Mun Pu) were used as materials for analysis as well. The total phenolic compounds were determined and expressed in terms of milligrams of gallic acid equivalents per gram of aqueous extract using the Folin-Ciocalteu assay (Zhou *et al.*, 2014). Briefly, the brown rice was ground using a pestle and mortar and rice flour was mixed with pure hexane (1:20 g/v) in a glass container with a stopper. A 0.1 mL aliquot of the extract was mixed with 0.25 mL of Folin-Ciocalteu reagent and held for 3 min. Then 3.0 mL of distilled, deionized water was added to the mixture and incubated at room temperature for 2 min in the dark. After that, 1.0 mL of 15% sodium carbonate (w/v) was added and left for 1 min and then made to a total volume of 10.1 mL with distilled and deionized water and allowed to stand at room temperature for 90 min in the dark. The absorbance of the developed color was measured by spectrophotometry at 760 nm. The total content of phenolic compounds in each sample extract was then determined by a standard curve prepared with gallic acid. The total flavonoid content of rice samples was determined by using a colorimetric method (Bakar, Mohamed, Rahmat, & Fry, 2009). The total flavonoid content was expressed as mg rutin equivalents per gram of dried sample (mg RE/g dry weight). Briefly, 0.5 mL of aliquot of the extract was mixed with 2.25 mL of distilled, deionized water in a test tube and then 0.15 mL of 5% NaNO₂ solution (w/v) was added and allowed to stand at room temperature for 6 min. Then 0.3 mL of 10% AlCl₃ (w/v) was added to the mixture, and after 6 min, 2 mL of 1 M NaOH was added. The mixture was mixed well by vortex. The absorbance was measured immediately at 510 nm using a spectrophotometer.

2.4 Genomic DNA extraction and Bh 4 and Phr1 genotyping

Flag leaves of each individual weedy rice sample were used as materials for DNA extraction. Genomic DNA was extracted by 1% CTAB with a modification of the method by Doyle and Doyle (1987). For the genotype analysis of *Bh4* gene, the region including the 22-bp deletion in the third exon was amplified by polymerase chain reaction (PCR) with the primers: 5'-TCAAATATCAA CCAGATGCTAGTG-3', and 5'-CGGGAGGTTGAGC GTCAC-3'. For the genotype analysis of *Phr1* gene, the region including the 18-bp deletion in the third exon was amplified by PCR with the primers: 5'-CTGCGCTT CACGTACCAGG-3' and 5'-CGTCGAACTT GACGTA CGTGG-3'. The PCR reactions were carried out in a volume of 20 μ L containing 1x buffer, 1 mM each of dATP, dCTP, dGTP and dTTP, 2 mM MgCl₂, 10 mM of SSR primer, 50 ng of genomic DNA, and 1 unit of *Taq* polymerase (Promega, Madison, WI, USA). The PCRs were performed using the following cycle: 94 °C for 4 min followed by 36 cycles of 40 s at 94 °C, 30 s at 55 °C, 40 s at 72 °C, and 10 min at 72 °C for the final extension. The PCR products were separated on 3% agarose gel electrophoresis. Migration distance of each allele was determined by comparison with a known molecular-weight standard (100 bp ladder, New England Biolabs, Beverly, MA, USA) after performing electrophoresis. The results from gel electrophoresis of both genes were recorded as (+) for functional allele (non-deletion) and (-) for loss of the function as reported by Fukuda *et al.* (2012).

3. Results and Discussion

Mature seed hull colors of weedy rice samples were classified into two groups: straw (SH) and black seed hull (BH). Each group exhibited both the white or red pericarp (Figure 1). From this sample collection, weedy rice plants with straw seed hull were dominant (74.4%), whereas the mature seeds with black hull was 25.6%. Evolution of hull color in weedy rice populations was reported by (Vigueira *et al.*, 2013). They found that the test for selection indicated that the straw hull of weedy rice deviated from neutrality at this gene. This meant possible selection on the hull color gene (*Bh4*) during both rice domestication and de-domestication. It was observed that straw and black hull seeds of weedy rice in northeast Thailand fall off easily from the panicle of the rice plant at the maturation stage. During the maturing stage, it is possible that the black hull seeds are noticed easily and are targeted by birds or rats in the natural habitats. Thus, the allele frequency of straw hull seeds would increase in the next generation. This observation was supported by previous studies that indicated that birds around the rice field play an important role in the selection of straw seed hull in rice (Zhu *et al.*, 2011).

Most varieties of rice have white grains, but some varieties have brown, red or black grains. Red grains are characterized by red pericarp due to the deposition and oxidative polymerization of proanthocyanidins (Furukawa *et al.*, 2006). Red pericarp was associated with coding sequence of the *Rc* gene located in chromosome 7 (Sweeney, Thomson, Pfeil, & McCouch, 2006). *Rc* was the dominant red allele that differed from the recessive white allele by a 14-bp deletion



Figure 1. Examples of weedy rice seeds with straw and black hull used in this study.

within exon 6. This 14-bp sequence that is present in rice plants with white pericarp induces a frame shift in the sequence which results in two premature stop codons before the end of exon 6.

3.1 Genotype variation and hull color among weedy rice accessions

The japonica cultivated rice lines, which had yellow (straw) hulls, were found to have a 22-bp deletion within the third exon of *Bh4* and had lost the function of *Bh4* (Zhu *et al.*, 2011). In addition, a functional *Bh4* gene was insufficient to produce black pigmentation in the hulls and that the complementary function of the *Phr1* gene was necessary.

Current studies have reported a relationship between the two loci (*Bh4* and *Phr1*) that causes black pigmentation in the ripening hulls of rice (Fukuda *et al.*, 2012). They found that only rice plants that had both functional alleles of *Bh4* and *Phr1* genes could produce black hulls at the ripening time.

For the *Bh4* gene, the genotype of weedy rice was determined using PCR assay. Three genotypes (*Bh4+*/*Bh4+*, *Bh4+*/*Bh4-*, *Bh4-*/*Bh4-*) were found among 82 weedy rice accessions. All 82 accessions showed an association between the phenotype of seed hull (straw or black) with the *Bh* gene genotype (i.e. *Bh4+*/*Bh4+* was black seed hull and *Bh4-*/*Bh4-* was straw seed hull). Interestingly, two weedy rice accessions (i.e. SPRE24 and SPRE26) exhibited heterozygous genotype (*Bh4+*/*Bh4-*) with black seed hull (Figure 2a). Furthermore, an analysis for the *Phr1* gene was performed on the 82 weedy rice accessions. Two samples of weedy rice showed genotypes (*Phr1+*/*Phr1+*) (Figure 2b). This meant that the two weedy rice accessions, SPRE24 and SPRE26, carried a functional allele (*Bh4+*/*Bh4-*) for *Bh4* and two functional genes (*Phr1+*/*Phr1+*) for the *Phr1* gene. In addition, 80 weedy rice accessions showed the genotypes *Phr1-*/*Phr1-* and *Phr1+*/*Phr1+* for straw seed hull and black seed hull, respectively. The evidence strongly supports the hypothesis of complementary genes (*Bh4* and *Phr1*) controlling black seed hull in rice. In other words, "a functional *Bh4* gene was insufficient to produce black pigmentation in hulls and that the complementary function of *Phr1* was necessary", which was proposed by Fukuda *et al.* (2012). This experiment suggests that weedy rice accessions from northeast Thailand showed a relationship between the functional allele or a non-functional allele or both of the two loci, *Bh4* and *Phr1* and seed hull color.

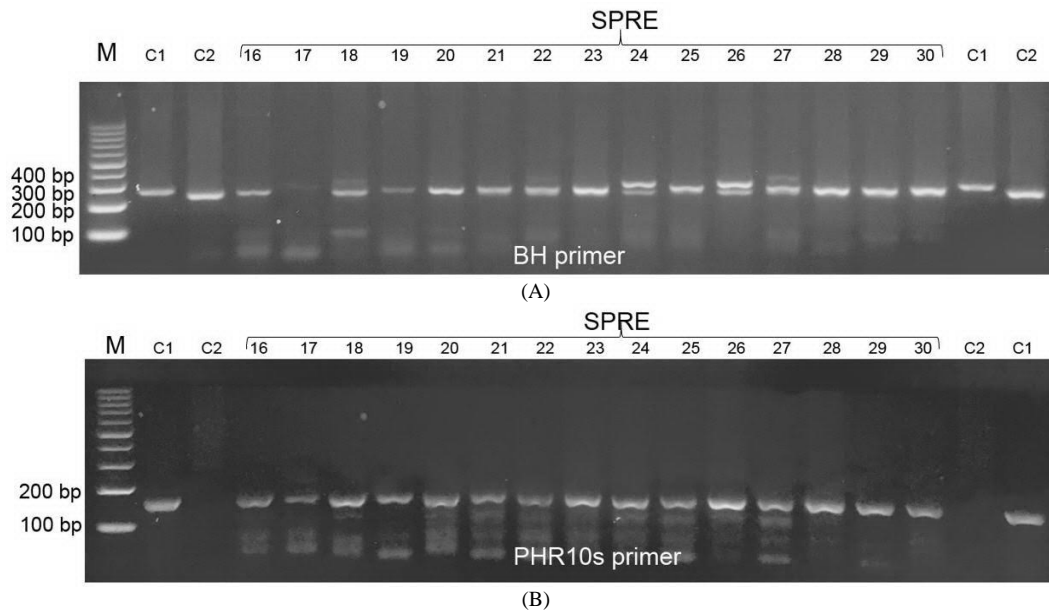


Figure 2. Samples of agarose gel electrophoresis that show DNA banding using PCR and primer pairs (forward: 5'-TCAAATATCAA CCAGATGCTAGTG-3', and reverse: 5'-CGGGAGGTTGAGC GTCAC-3') of the *BH4* gene (A) and with primer pairs of the *Phr1* gene (forward: 5'-CTGCGCTT CACGTACCAGG-3' and reverse: 5'-CGTCGAACTTGACGTACGTGG-3') (B). For the BH4 gel (A), M=100 bp DNA Ladder. C1=control (black hull seed), C2=control (straw hull seed). Weedy rice DNA samples with straw hull seeds indicated in sample code: SPRE16-21, 23, 25,28,29,30. Sample code: SPRE22, 24, 26, 27 showed black hull seeds. For Phr1 gel (B), C1=control (straw hull seed, (Phr1-/Phr1-) genotype), C2=without DNA (negative control). All DNA samples showed (Phr1+/Phr1+) genotype.

3.2 Total phenolic and flavonoid content in weedy rice

The concentrations of total phenolic and total flavonoid contents of weedy rice and traditional color rice were measured (Table 1). The total phenolic contents of brown weedy rice of black seed hull (2.58 mgGAE/g) and straw seed hull (1.58 mg GAE/ g) with red pericarp were higher than weedy rice with black seed hull white pericarp (0.27 mg GAE/ g) and two traditional red rice varieties (Mun Pu, 1.51 mg GAE/g) and Niew Daeng, 1.24 mg GAE/g). The total flavonoid contents of brown weedy rice grains revealed a similar trend as the total phenolic contents. Phenolic compounds play important roles in their various potential biological activities, such as ferulic acid found in rice grains (Prathepha, Siriamornphun, & Sakdakham, 2017; Zhou, Robards, Helliwell, & Blanchard, 2004; Zhou *et al.*, 2014;). Ferulic acid is known for its antioxidant activities which results in inhibition of inflammation and tumor initiation and can serve as a food preservative (Adom & Liu, 2002). This study of weedy rice with different phenotypes based on seed hull color and pericarp color revealed different total phenolic compounds. This supports the idea proposed by Zhou *et al.* (2014) that the concentration and distribution of phenolic compounds in rice have phenotype-specific parameters. Until now, no evidence has demonstrated an association between hull color and phytochemical contents in rice grains. In oat grains, a report by Kovacova & Malinova (2007) evaluated ferulic and coumaric acids in selected oat genotypes. Interestingly, future investigations on the evaluation of phytochemical contents in selected weedy rice genotypes should be considered to find new functional food components.

Table 1. Total phenolic and total flavonoid contents in brown weedy rice grain and two traditional rice landrace (Mun Pu and Niew Daeng).

Rice sample	Total phenolic content (mg GAE/g)**	Total flavonoid content (µg RE/g)
Weedy rice*		
BH-W	0.27±0.01	12.36±0.39
BH-R	2.58±0.06	25.45±1.55
SH-R	1.58±0.07	20.81±0.23
Traditional rice landrace		
Mun Pu	1.51±0.03	22.48±1.17
Niew Daeng	1.24±0.02	19.91±0.16

*BH-R: black hull with red pericarp; BH-W: black hull with white pericarp; SH-R: straw hull with red pericarp.

**All data are expressed as the means±standard deviation (S.D.) of three replicate measurements.

4. Conclusions

Complementary genes control seed hull color in weedy rice occurring in natural habitats. The results strongly support previous reports. In addition, weedy rice is considered as an unwanted plant in paddy fields, but some characteristics of weedy rice, such as pericarp color of rice grain, showed significant amounts of bioactive compounds such as phenolic

and flavonoid contents. This observation indicates that weedy rice is a rich source of phytochemicals that might have potential benefits for human health.

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