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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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, eighty-two ripened weed rice seed samples were collected for analysis of the relationships between phenotype and genotype of the two genes controlling seed hull color and for evaluation phytochemical compounds in weedy rice grains. The results showed evidences which are strongly supported the hypothesis of a complementary gene (Bh4 and Phr1) controlling black seed hull in rice that proposed by Fukuda et al. (2012). 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 showed higher than that of weedy rice with black seed hull white pericarp (0.27 mg GAE/ g) and two traditional red rice (Mun Pu, 1.51 mg GAE/ g), Niew Daeng, 1.24 mg GAE/ g). Total flavonoid content of brown weedy rice grains revealed similar trend with total phenolic content. This observation indicates that weedy rice is a rich sources of phytochemicals that might be a potential benefits on human health.

Key words: weedy rice, seed hull color, phytochemical compounds
32 **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*), the putative ancestor of the Asian cultivated rice (Cao et al., 2006; Londo & Schaal, 2007). Gene transfer from weedy rice to cultivated rice and/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 (Xia, Wang, Xia, Zhao, & Lu, 2011; Shivrain, Burgos, Gealy, Sale, & Smith, 2009).

Among rice genes, some loci have been used for studying the genetic basis of evolution such as the seed shattering gene (*sh4*) in weedy rice populations (Zhu, Ellstrand, & Lu, 2012). There are genes controlling color of morphology in rice plant, such as anthocyanin accumulates in many plant tissues or organs in rice, leading 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 physical and oxidative damages.
(Ramarathnam, Osawa, Kawakishi, & Namiki, 1987). In the US 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).

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 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). In the current study the results show that independent *Bh4* coding mutations have arisen in African and Asian rice that correlated with the straw hull phenotype, this indicated that the *Bh4* gene is responsible for parallel trait evolution. The most important part of this 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 of 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 can produce black hull, resulting from the analysis of the segregating 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 test for selection indicates the straw hull of weedy rice deviates from neutrality at this gene. This means that there is 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 maturation stage and a black hull color can protect them from being targeted by birds, 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 shows important attributes that is 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, however whole grains with red pericarp of these unwanted plants are still carrying phytochemical compounds that are benefit for humans. In this study, weedy rice was sampled from paddy fields of Hom Mali rice in the northeastern Thailand to further determine of phytochemical compounds (flavonoids and phenolic acid) in whole grains, and to determine the relationship among the two gene controlling black hull phenotype. The results would yield data of level amount of bioactive compounds in weedy rice grains and the genetic background of weedy rice in the paddy fields, which are valuable implications for the evolution of weedy rice populations.
Materials and methods

Weedy rice samples

Weedy rice samples (82 weedy rice plants) had been collected from March to October 2015-2016 from Thung Kula Ronghai area in the northeastern region of Thailand, including 33 accessions from Roi Et and 49 accessions from Mahasarakham provinces. Each plant was collected and recorded seed hull color, in addition flag leaf samples of the eighty-two weedy rice samples were also individually collected for DNA extraction. These samples were classified further for pericarp color of hull removed seed.

Evaluation of seed hull and pericarp color

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

Determination of phytochemical compounds

There are three phenotypes of weedy rice samples used for determination of phytochemical compounds, including black hull seed with white pericarp (BH-W), black hull seed with red pericarp (BH-R), 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 by 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, deionised 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 deionised water and allowed to stand at room temperature for 90 min in the dark.

The absorbance of the developed color was measured by a spectrophotometer 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 colormetric 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, deionised water in a test tube, and then 0.15 mL of 5% NaNO$_2$ solution (w/v) was added and allowed to stand at room temperature for 6 min. Then 0.3 mL of a 10% AlCl$_3$ (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.

Genomic DNA extraction and Bh 4 and Phr1 genotyping

Flag leaves of each individual of weedy rice samples were used as materials for DNA extraction. Genomic DNA was extracted by 1% CTAB with a modification of the method of Doyle & Doyle (1987). For the genotype analysis of Bh4 gene, the region including the 22-bp deletion in the third exon was amplified by 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’-CTCGCGCTT
CACGTACCAGGG-3’ and 5’-CGTCGAACTTGACGTA CGTGGN3’. The PCR reactions were carried out in a volume of 20 µL 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, Wisconsin, USA). The polymerase chain reactions were performed using the following cycle: 94 °C for 4 min and followed by 36 cycles of 40 s at 94 °C, 30 s at 55 °C and 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 comparing with a known molecular-weight standard (100 bp ladder, New England BioLabs, New England, USA) after performing electrophoresis. The results from gel electrophoresis of both gene were recorded that (+), for functional allele (non-deletion) and (−), for loss of the function as reported by Fukuda et al. (2012).

Results and discussion

Mature seed hull colors of weedy rice samples were classified into two groups, straw (SH) or black seed hull (BH). Each group exhibited both white or red pericarp (Fig. 1). From this collection sample, weedy rice plants with straw seed hull were dominant (74.4%), whereas the mature seeds with black hull is of 25.6%. Evolution of hull color in weedy rice populations was reported by (Vigueira et al., 2013). They found that test for selection indicates the straw hull of weedy rice deviates from neutrality at this gene. This means that there is possible selection on the hull color gene (Bh4) during both rice domestication and de-domestication. In terms of weedy rice in
northeast Thailand, from the observation straw and black hull seeds of weedy rice fall
off easily from the panicle of the rice plant at maturation stage. During maturing stage
this is possible that black hull color seed were noticed easily and being targeted by
birds or rat in natural habitats, thus the allele frequency of straw hull seed 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 were 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 \textit{Rc} gene, located in chromosome 7
(Sweeney, Thomson, Pfeil, & McCouch, 2006). \textit{Rc} was the dominant red allele
differed from the recessive white allele (rc) by a 14-bp deletion within exon 6. This 14-bp
sequence was present in rice plant with white pericarp induces a frame shift in the
sequence, resulting in two premature stop codons before the end of exon 6.

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 \textit{Bh4} and had lost the function of \textit{Bh4}
(Zhu et al., 2011), in addition a functional \textit{Bh4} gene was insufficient to produce black
pigmentation in hulls and that the complementary function of \textit{Phr1} gene was
necessary.
Current studies report that there is a relationship between the two loci (\textit{Bh4} and \textit{Phr1}) that causes a black pigmentation in the ripening hulls of rice (Fukuda et al., 2012). They found that only rice plants that had both functional alleles of \textit{Bh4} and \textit{Phr1} genes could produce black hull at ripening time.

For \textit{Bh4} gene, the genotype of weedy rice was determined by using PCR assay. It was found that there are three genotypes (\textit{Bh4+/Bh4+}, \textit{Bh4+/Bh4-,Bh4-/Bh4-}) among the 82 weedy rice accessions. All 82 accessions showed association between phenotype of seed hull (straw or black) with \textit{Bh} gene genotype is that \textit{Bh4 +/Bh4+}, black seed hull,\textit{Bh4 -/Bh4-}, straw seed hull. Interestingly, two weedy rice accessions, i.e. SPRE24 and SPRE26 exhibited heterozygous genotype (\textit{Bh4+/Bh4-}) with black seed hull (Fig. 2a). Furthermore analysis for \textit{Phr1} gene was performed on the 82 weedy rice accessions, the two sample of weedy rice showed genotypes (\textit{Phr1+/Phr1+}) as shown in Figure 2b. This means that two weedy rice accessions, SPRE24 and SPRE26 carried a functional allele (\textit{Bh4+/Bh4-}) for \textit{Bh4} and two functional gene (\textit{Phr1+/Phr1+}) for \textit{Phr1} gene. In addition, 80 weedy rice accessions showed genotype (\textit{Phr1-/Phr1-}) or (\textit{Phr1+/Phr1+}) for straw seed hull and black seed hull, respectively. These evidences are strongly supported the hypothesis of a complementary gene (\textit{Bh4} and \textit{Phr1}) controlling black seed hull in rice that is “a functional \textit{Bh4} gene was insufficient to produce black pigmentation in hulls and that the complementary function of \textit{Phr1} was necessary” that proposed by Fukuda et al.(2012). This experiment suggests that weedy rice accessions from northeast Thailand showed a relationship between functional allele and/or non-functional allele of the two loci, \textit{Bh4} and \textit{Phr1} and seed hull color.

\textit{Total Phenolic and flavonoid content in weedy rice}
The concentration of total phenolic compounds and total flavonoid content of weedy rice and traditional color rice were measured and listed in Table 1. The total phenolic content of brown weedy rice of black seed hull (2.58 mg GAE/g) and straw seed hull (1.58 mg GAE/g) with red pericarp showed higher than that of weedy rice with black seed hull white pericarp (0.27 mg GAE/g) and two traditional red rice (Mun Pu, 1.51 mg GAE/g), Niew Daeng, 1.24 mg GAE/g). Total flavonoid content of brown weedy rice grains revealed similar trend with total phenolic content. Phenolic compounds are played important role in their various potential biological activities, such as ferulic acid found in rice grains (Zhou, Robards, Helliwell, & Blanchard, 2004; Zhou et al., 2014; Prathepha, Siriamornphun, & Sakdakham, 2017). Ferulic acid is known for its antioxidant activities, which result in inhibition of inflammation and tumor initiation and can serve as a food preservative (Adom & Liu, 2002). In this study weedy rice with different phenotypes based on seed hull color and pericarp color that revealed different total phenolic compounds, this would be supported the idea proposed by Zhou et al. (2014) is that the concentration and distribution of phenolic compounds in rice are phenotype-specific parameters. Nowadays, there is no evidence demonstrating the association between hull color and phytochemical contents in rice grains. In oat, there is a report by Kovacova & Malinova (2007) that evaluation of ferulic and coumaric acids in selected oat genotypes. Interestingly, future investigation on the evaluation of phytochemical contents in selected weedy rice genotypes would be considered for seeking a new functional food components.

Conclusion

This paper presented a complementary gene controlling seed hull color in weedy rice occurring in natural habitats. The results are strongly supported the previous
In addition, weedy rice is considered as unwanted plant in paddy fields, but some characteristics of weedy rice such as pericap color of rice grain showed a significant amounts of bioactive compounds, such as phenolic and flavonoid contents. This observation indicate that weedy rice is a rich sources of phytochemicals that might be a potential benefits on human health.

**Acknowledgements**

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References


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

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<th>Rice sample</th>
<th>Total phenolic content (mg GAE/g)**</th>
<th>Total flavonoid content (µg RE/g)</th>
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<tr>
<td><strong>Weedy rice</strong></td>
<td></td>
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<tr>
<td>BH-W</td>
<td>0.27±0.01</td>
<td>12.36±0.39</td>
</tr>
<tr>
<td>BH-R</td>
<td>2.58±0.06</td>
<td>25.45±1.55</td>
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<tr>
<td>SH-R</td>
<td>1.58±0.07</td>
<td>20.81±0.23</td>
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<td><strong>Traditional rice landrace</strong></td>
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<tr>
<td>Mun Pu</td>
<td>1.51±0.03</td>
<td>22.48±1.17</td>
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<tr>
<td>Niew Daeng</td>
<td>1.24±0.02</td>
<td>19.91±0.16</td>
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*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.
Figure 1. Example of weedy rice seeds with straw and black hull used in this study.

Figure 2. Sample of agarose gel electrophoresis showed DNA banding performed by using PCR technique and primer pairs (forward: 5’-TCAAATATCAA CCAGATGCTAGTG-3’, and reverse: 5’-CGGGAGGTGTAGC GTCAC-3’ of the BH4 gene (A) and
with a primer pairs of the Phr1 gene (forward: 5'-CTGCGCTT CACGTACCAGG-3' and reverse: 5'-CGTCGAACTTGACGTACGTGG-3') (B). For 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.