[Short Report]

Complementary Genes That Cause Black Ripening Hulls in F1 Plants of Crosses between Indica and Japonica Rice Cultivars

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Abstract: The F1 plants of crosses between indica and japonica rice cultivars often have black hulls during ripening, even though both of the parental cultivars have yellow hulls. Complementary genes are suggested to be necessary for the black hull phenotype, and one of them is predicted to be Phr1, which encodes polyphenol oxidase. On the other hand, Bh4, which encodes a tyrosine transporter, is known to cause the black hull phenotype in wild rice species, Oryza rufipogon. However, the relationship between Bh4 and Phr1 in the black hull phenotype has not been elucidated. In this study, a genotype analysis of the segregating populations from the cross between an indica cultivar, Habataki, and a japonica cultivar, Arroz da Terra, indicated that only those plants that had both functional genes, Bh4 and Phr1, showed the black hull phenotype, suggesting that a complementarity of Bh4 and Phr1 was necessary for the black hull phenotype.

Key words: Bh4, Black hull, Phr1, Polyphenol oxidase, Rice, Tyrosine transporter.

The hulls of cereals are considered to play a role in the protection of seeds from physical damages and also oxidative damages (Ramarathnam et al., 1986). Because the pigments in plants are considered to have important roles in antioxidant activity, defense against fungi and protection against UV radiation (Shirley, 1996; Huang et al., 2011), the pigments in hulls are suggested to protect the seeds. To identify the genes responsible for the pigmentation might assist the understanding of the role of the pigment. In this study, genes that caused a black pigmentation in the ripening hulls of rice (Oryza sativa L.) were analyzed.

The hulls of immature seeds are green, and the hulls of the mature seeds of cultivated rice are usually yellow. However, black hulls during the ripening stage sometimes occur in the F1 plants of crosses between the indica and japonica cultivars, even though both of the parental cultivars have yellow hulls (Nagao and Takahashi, 1954; Kuriyama and Kudo, 1967; Maekawa, 1984). It has been predicted that two or three complementary genes might be responsible for the black hull phenotype (Nagao and Takahashi, 1954; Kuriyama and Kudo, 1967; Maekawa, 1984). Maekawa (1984) reported that three complementary genes, termed Bh-a, Bh-b and Bh-c, controlled the black hull phenotype. Among them, Bh-c was suggested to correspond to Phr1, which encodes polyphenol oxidase (Kuriyama and Kudo, 1967; Maekawa, 1984; Yu et al., 2008). Phr1 causes the phenol reaction-positive phenotype of seeds, in which the hull color turns black after being soaked in a phenol solution (Yu et al., 2008). The frequency of the dominant allele of Phr1 was reported to be high in the indica and low in the japonica cultivars (Morishima and Oka, 1981; Yu et al., 2008). Polyphenol oxidase is known to oxidize phenols in plant tissues and to produce brown or black compounds (Piližota and Šubarić, 1998; Bittner, 2006); accordingly, the black hull pigmentation was suggested to be produced by the activity of polyphenol oxidase (Kuriyama and Kudo, 1967). Other complementary genes may be involved in the production of the phenol substrates (Kuriyama and Kudo, 1967); however, these genes have not yet been identified.

Recently, Bh4, that induces black hulls in the wild rice species, Oryza rufipogon, was identified using a cross between Oryza rufipogon W1943 and an indica cultivar Guangluai 4 (Zhu et al., 2011). Bh4 encodes a tyrosine transporter and expresses in hulls (Zhu et al., 2011). However, the relationship between Bh4 and Phr1 in the black hull phenotype has not been elucidated. In the present study, we analyzed the relationship between Bh4 and Phr1 in producing the black hull phenotype. We used an indica cultivar, Habataki, which has a functional Phr1, and a japonica cultivar, Arroz da Terra, which loses the Phr1 function.

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Materials and Methods

1. Polyphenol oxidase activities

The activities of polyphenol oxidase in rice seeds were analyzed using phenol (Yu et al., 2008) or tyrosine (Mahoney and Ramsay, 1992) as the substrates. Rice seeds were soaked in 2% phenol (Yu et al., 2008) or 0.01 M tyrosine in a 0.1 M Tris-HCl (pH 9.0) (Mahoney and Ramsay, 1992) at 30ºC for seven days. The seeds that had black pigmentation were determined to have a functional Phr1 gene (Fig. 1C and 1D), and a positive reaction to polyphenol oxidase in black hulls was determined by the darkening of the hull color.

2. Genotype analysis of Bh4 gene

The total DNA of the Habataki and Arroz da Terra cultivars was isolated from young leaves using the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). A 3.5-kb region of the Bh4 gene, including the entire coding sequence, was amplified using PCR primers 5'-GGTGCTAATGCATGGGTGGT-3' and 5'-CGTCGACATCAGGTGGTGTCGAC-3'. For the genotype analysis of the Bh4 gene, the region including the 22-bp deletion in the third exon was amplified by PCR with the primers: 5'-TCAAATATCCAGAATGCTAGTG-3', and 5'-CGGGAGGTTGAGCTACCTCCAG-3' (Fig. 2).

3. Genotype analysis of Phr1 gene

A 3.0-kb region including Phr1 gene of the Habataki and Arroz da Terra cultivars was amplified using PCR primers 5'-GGATGAGGGATATGTGTG-3' and 5'-CCGTTACTGCGGTTGTG-3'. The region including the third exon was sequenced using the following primers: 5'-CTGCGCTTCACGTACCAGG-3' and 5'-CGTCGAACTTGACGTTG-3' as described above. 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'-CTGCGCTTCCAGTACCAGG-3' and 5'-CGTCGAACTTGACGTTG-3' (Fig. 3).

4. Analysis of the segregating populations

The presence of the functional Bh4 and Phr1 genes was
analyzed using 80 Habataki / Arroz da Terra F₂ plants, 104 Habataki // Habataki / Arroz da Terra BC₁F₁ plants and 91 Habataki // Habataki / Arroz da Terra BC₁F₄ plants. BC₁F₄ plants were developed by single seed descent method from the BC₁F₁ plants. DNA was extracted from the fresh leaves according to Thomson and Henry (1995). The genotypes of the \textit{Bh₄} and \textit{Phr₁} genes were determined by PCR as described above (Fig. 2 and Fig. 3).

### Result

1. **Mature hull colors and polyphenol oxidase activities**

   The parental cultivars, Habataki and Arroz da Terra, had yellow hulls (Fig. 1B), whereas the mature seeds of the F₁ plants that resulted from the cross between Habataki and Arroz da Terra had black hulls (Fig. 1A and 1B). However, the hulls of the empty seeds of the F₁ plants were yellow (Fig. 1A and 1B).

   The Habataki cultivar demonstrated polyphenol oxidase activity and a black pigmentation was produced when using either phenol or tyrosine as the substrates (Fig. 1C and 1D), suggesting that Habataki had a functional \textit{Phr₁} gene. In contrast, Arroz da Terra did not demonstrate polyphenol oxidase activity (Fig. 1C and 1D). Both the normal seeds and empty seeds of the F₁ plants exhibited polyphenol oxidase activities (Fig. 1C and 1D).

2. **Genotype analysis of \textit{Bh₄} and \textit{Phr₁}**

   The sequences of the coding regions of \textit{Bh₄} in Habataki and Arroz da Terra were compared with those of \textit{Oryza rufipogon} W1943, which had a black hull and a functional \textit{Bh₄} gene (GenBank accession number FQ377546; Zhu et al., 2011). The sequences of the exon regions of \textit{Bh₄} in Arroz da Terra were identical to those of W1943. The Habataki cultivar had a 22-bp deletion in the third exon (Fig. 2), which induced a frame shift resulting in a premature stop codon and the loss of the \textit{Bh₄} function (Zhu et al., 2011). Habataki had a single point mutation in the third exon in addition to the 22-bp deletion (Fig. 2).

   Most of the \textit{japonica} cultivars have been reported to have 18-bp deletion in the third exon of \textit{Phr₁}, which induced the loss of the \textit{Phr₁} function (Yu et al., 2008). The sequences of the third exon of \textit{Phr₁} in Habataki and Arroz da Terra were compared with the \textit{indica} cultivar, MH63, which had a functional \textit{Phr₁} gene (GenBank accession number DQ532375; Yu et al., 2008). The sequencing analysis and PCR analysis revealed that Arroz da Terra had the 18-bp deletion in the third exon of \textit{Phr₁}, though Habataki had the complete \textit{Phr₁} (Fig. 3).

### Table 1. The genotype of \textit{Bh₄}, \textit{Phr₁} and hull colors in the segregating populations.

| Population                                      | Genotype | Number of Plants |
|-------------------------------------------------|----------|------------------|
|                                                 | \textit{Bh₄} | \textit{Phr₁} | Black hull | Yellow hull | Total |
| Habataki // Arroz da Terra F₂                    | ++       | ++              | 5 | 0 | 5 |
|                                                 | ++       | −−              | 7 | 0 | 7 |
|                                                 | ++       | −−              | 0 | 16 | 16 |
|                                                 | −−       | ++              | 7 | 0 | 7 |
|                                                 | −−       | −−              | 0 | 6 | 6 |
|                                                 | −−       | −−              | 0 | 6 | 6 |
|                                                 | ++       | −−              | 0 | 13 | 13 |
|                                                 | −−       | ++              | 20 | 0 | 20 |
| Habataki // Habataki // Habataki // Arroz da Terra BC₁F₁ | ++       | −−              | 0 | 32 | 32 |
| Habataki // Habataki // Arroz da Terra BC₁F₄     | −−       | ++              | 0 | 14 | 14 |
|                                                 | ++       | ++              | 11 | 0 | 11 |
|                                                 | ++       | −−              | 3 | 0 | 3 |
|                                                 | ++       | −−              | 0 | 3 | 3 |
| Habataki // Habataki // Arroz da Terra BC₁F₄     | −−       | ++              | 0 | 3 | 3 |
|                                                 | ++       | −−              | 0 | 1 | 1 |
|                                                 | −−       | ++              | 0 | 5 | 5 |
|                                                 | −−       | −−              | 0 | 2 | 2 |
|                                                 | −−       | −−              | 0 | 15 | 15 |
|                                                 | ++       | −−              | 17 | 0 | 17 |
| Habataki // Habataki // Arroz da Terra BC₁F₄     | −−       | ++              | 41 | 0 | 41 |
|                                                 | −−       | −−              | 0 | 32 | 32 |
|                                                 | −−       | −−              | 0 | 14 | 14 |
|                                                 | ++       | ++              | 3 | 0 | 3 |
|                                                 | −−       | ++              | 0 | 3 | 3 |
|                                                 | −−       | −−              | 0 | 15 | 15 |
|                                                 | ++       | −−              | 17 | 0 | 17 |
|                                                 | −−       | ++              | 41 | 0 | 41 |
|                                                 | −−       | −−              | 0 | 32 | 32 |
|                                                 | −−       | −−              | 0 | 14 | 14 |

The genotype of \textit{Bh₄} without (\textit{Bh₄}+) or with 22-bp deletion (\textit{Bh₄}−), and the genotype of \textit{Phr₁} without (\textit{Phr₁}+) or with 18-bp deletion (\textit{Phr₁}−) were analyzed. \textit{Bh₄}−\textit{Phr₁}− plants in \textit{F₂} and \textit{Bh₄}+\textit{Phr₁}+ plants in \textit{BC₁F₄} were not found in this experiment.
the black hull phenotype, the presence of the functional 
\textit{Bh4} and \textit{Phr1} genes in \textit{F}_2, BC_F, and BC_F plants from 
crosses between Habataki and Arroz da Terra was analyzed. The 
genotype of \textit{Bh4}, \textit{Phr1} and mature hull colors are 
provided in Table 1. Only the plants that had both functional \textit{Bh4} (\textit{Bh4} +) and \textit{Phr1} (\textit{Phr1} +) produced black 
hulls (Table 1).

**Discussion**

The analysis of the segregating populations in crosses 
between Habataki and Arroz da Terra indicated that only 
the plants that had both functional \textit{Bh4} and \textit{Phr1} genes 
produced black hulls (Table 1), suggesting that a 
complementarity of \textit{Bh4} and \textit{Phr1} were necessary for the 
black hull phenotype. \textit{Bh4} was reported to control the 
black hull phenotype of ancestral wild rice species, \textit{Oryza 
rufipogon} (Zhu et al., 2011). Most of the cultivated rice 
lines, which had yellow 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 this study, Arroz da 
Terra, a \textit{japonica} rice cultivar, was found to have a complete 
\textit{Bh4} gene (Fig. 2), suggesting the retention of the 
\textit{Bh4} function. However, the hulls of Arroz da Terra were yellow 
(Fig. 1B). This indicated that merely possessing a 
functional \textit{Bh4} gene was insufficient to produce black 
pigmentation in hulls and that the complementary function of \textit{Phr1} was necessary.

In higher plants, the brown or black pigmentation of 
tissues is caused mainly by polyphenol oxidase (Piližota and 
Šubarić, 1998; Bittner, 2006). Phenols are oxidized by 
polyphenol oxidases, and the resulting quinonic compounds 
associate with amino acids or proteins, producing brown 
pigmentation (Piližota and Šubarić, 1998; Bittner, 2006). Furthermore, \textit{Bh4} was predicted to encode a tyrosine 
transporter, and the concentrations of tyrosine in hulls 
increased in the transformed rice plants with functional \textit{Bh4} 
gene (Fig. 1A and 1B). Because the hulls of the empty seeds had polyphenol oxidase activity (Fig. 1C and 1D), the yellow hulls of the empty seeds might be 
caused by the lack of the substrates for polyphenol oxidase. 
The expression of \textit{Bh4} in the hulls might be induced by 
the development of the seeds. Another possibility is that 
the substrates are produced in the developing embryo or 
endosperm and transported to the hull.

Several functions for black hulls are predicted. The 
black seeds have the same color as the soil and, thus, may 
be unnoticed by birds (Furuhata et al., 2009). Indeed, the 
seeds of ancestral wild rice species that have black hulls, 
shatter easily to the ground at maturation, and the black 
seeds of ancestral wild rice species that have black hulls, 
shatter easily to the ground at maturation, and the black 
hull pigmentation is considered to protect the seeds from 
the ground (Zhu et al., 2011). Dark pigmentation of melanin is also known to screen UV 
radiation and act as an antioxidant (Huang et al., 2011); therefore, the black 
hull pigmentation might protect the seeds from UV 
radiation and oxidative damage. Further analysis of the 
black hull phenotype might reveal the function of the hulls 
in the protection of the seeds from the environment.

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* In Japanese with English summary.