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Genetic analysis and molecular mapping of *Rp,* a mutant gene encoding red pericarp in rice (*Oryza sativa* L.)

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Abstract: Coloured rice has pigments deposited in the grain pericarp; red rice is the most common type of coloured rice. Red rice is rich in essential nutrients and has been grown and consumed in China for a long time. In this study, we report the genetic characterisation and preliminary molecular mapping of a mutant gene encoding red pericarp in rice (*Oryza sativa* L.). To analyse the genetic basis of the red pericarp mutant, a reciprocal cross between GER-3 (red pericarp, indica cv.) and 898 (white pericarp, indica cv.) was made. The genetic analysis results confirmed that there was only one dominant gene, temporarily designated *Rp* (*Red pericarp*) controlling the segregation of the red pericarp in the F_2 population. For the molecular mapping of *Rp*, an F_2 population derived from an inter-subspecific cross between Gene Engineering Rice-3 (GER-3) and C418 (japonica cv., white pericarp) was constructed. The genotype of the pericarp colour of the F_2 individuals in the mapping population was validated by progeny testing of the $F_{2:3}$ families. Simple sequence repeat (SSR) markers and the bulked segregation analysis (BSA) method were used; *Rp* was mapped to the short arm of chromosome 7 between the SSR markers RM21182 and RM21268, with a genetic distance of 3.5 and 12.0 cM, respectively. In this paper, the potential origin of the red pericarp mutant gene *Rp* was also discussed.

Keywords: gene mapping; genetic characteristics analysis; coloured rice; red pericarp mutant

Most of the cultivated rice (*Oryza sativa*) consumed and grown worldwide has a white pericarp, but rice producing grains with red, purple, and brown pericarps is also common (Sweeney et al. 2006). Coloured rice has pigments deposited in the grain pericarp; red rice is the most common type of coloured rice. Among the wild ancestors of cultivated rice (*Oryza rufipogon*), a red pericarp is ubiquitous and, in some regions, is preferred for its ceremonial or medicinal value, texture, and taste (Sweeney et al. 2006; Finocchiaro et al. 2007; Qiu et al. 2010; Gunaratne et al. 2013; Mbanjo et al. 2020).

The red pigment in rice grains is due to the presence of proanthocyanidins (Oki et al. 2002). Proanthocyanidins provide nutritional value and are produced by a branch of the anthocyanin pathway and share many of the same biosynthetic genes (Ling et al. 2001; Winkel-Shirley 2001). Proanthocyanidins also serve as powerful antioxidants to reduce atherosclerotic plaque formation (Ling et al. 2001). Proanthocyanidins have been shown to have important deterrent effects on pathogens and predators (Bate-Smith 1973; Swain 1978; Scalbert 1991).

White rice was derived from the ancestral red rice by mutations in the *Rc* gene, which encodes an up-regulator of proanthocyanidin expression in the seed. Two mutations are responsible for the white trait of rice (Sweeney et al. 2006). The mutations were pre-

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dicted to be in exon 6 but were shown to be in exon 7 in the *Rc* locus for all the white rice studied (Sweeney et al. 2006; Furukawa et al. 2007). A single nucleotide polymorphism causes the premature termination of the Rc protein before the bHLH domain, and a 14-bp deletion results in a frame shift to a nonsense codon, finally resulting in a white pericarp (Sweeney et al. 2006; Furukawa et al. 2007).

Due to its health-promoting benefits, red rice has become increasingly popular. In recent decades, although several red rice varieties have been developed based on conventional breeding (Sharma et al. 2014; Zhang et al. 2015), most cultivated red rice varieties suffer from a low yield or other poor agronomic traits. Thus, the development of elite varieties would be of great significance for the production of red rice to meet the growing market.

In this paper, GER-3, an indica red pericarp mutant line possessed multiple desirable characteristics, and C418, a japonica white pericarp line was introduced for the genetic analysis and molecular mapping of *Rp* (Bao et al. 1994; Zhang & Yang 2006).

MATERIAL AND METHODS

Origins and genetic analysis of GER-3, a red pericarp mutant. By introducing the total genetic DNA of the maize donor, Yijingbai (maize cv., white pericarp), into the embryo of the rice receptor, Xiangzaoxian8 (indica cv., white pericarp), by means of embryo soaking, Gene Engineering Rice-3 (GER-3), a red pericarp mutant among the descendants of the trans-genetic line of maize-rice, was isolated in the maize-rice transgenic breeding programme (Bao et al. 1994).

Because it possessed multiple desirable characteristics, GER-3 was released widely for rice field production.

To analyse the genetic basis of the red pericarp mutant, a reciprocal cross between GER-3 (red pericarp, indica cv.) and 898 (white pericarp, indica cv.) was made. The plant materials of GER-3, 898 and the reciprocal F_1 and F_2 were field planted at the experiment station.

Mapping population development. To mapping the *Rp*, an inter-subspecific cross between GER-3, the red pericarp mutant (indica cv., red pericarp) and C418 (japonica cv., white pericarp) was made, and an F_2 population derived from this cross was constructed. The F_2 individuals derived from this inter-subspecific cross were selfed to obtain the $F_{2:3}$ families. Genotypes of the F_2 individuals can be deduced using the $F_{2:3}$ population.

Field planting and trait recording. The plant materials were planted in the field during the rice-growing seasons at the experimental stations in Hefei (31°N, 117°E), Sanya (18°N, 109°E), and Tianjin (39°N, 117°E), China. The planting spacing was 13.3 cm between the plants in a row and 16.7 cm between the rows with 11 plants per row. The field management followed the local agricultural practices. The irrigation of the field was maintained to avoid drought stress (Yang & Tu 2003). The pericarp colour of these materials was recorded after harvest in autumn.

DNA extraction and PCR analysis. The genomic DNA was extracted from the fresh leaves of individuals in the F_2 mapping population using the modified cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle 1987; Ragers & Bendich 1998). Nearly 2000 SSR markers selected from 12 rice chromosomes were used to mapping the *Rp* (http://www.gramene. org; International Rice Genome Sequencing Project 2005). The polymerase chain reactions (PCRs) consisted of 2.5 mL of a 10× reaction buffer with $(NH4)_2SO_4$, 100 mM, 2.0 mL $MgCl_2$ (25 mM), 1.0 mL dNTPs (10.0 mM), 1.0-unit Taq DNA polymerase (Takara Bio (Dalian) Inc., Japan), 100 ng template DNA and 1.5 mL of primer (10 mM), in a final volume of 25.0 mL with distilled water, and then covered with a drop of mineral oil. The PCR amplification was performed with the T100TMPCR (Bio-Rad Laboratories, Hercules, USA). The amplifications were performed using the following profile: 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 5 min. The amplification products were analysed on 4% agarose gels stained with ethidium bromide and photographed using the Gel Doc 2000 system (Bio-Rad Laboratories, Hercules, USA). The amplification products were further analysed on 6% polyacrylamide gels stained with 0.1% silver nitrate, when necessary.

BSA and mapping of the red pericarp mutant gene *Rp*. The bulked segregant analysis (BSA) method (Michelmore et al. 1991) was used to identify the polymorphic molecular markers linked to the mutant gene *Rp* in this study. The genomic DNA from 30 red pericarp individuals and 30 white pericarp individuals in the F_2 segregated progenies was pooled to create the red pericarp and white pericarp DNA bulks, respectively. The parental DNA and the two bulks were used for the BSA. The markers

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were examined for polymorphism between GER-3 and C418. The polymorphic markers from the two parents were screened against the two DNA bulks, and the polymorphic markers between the two DNA bulks were screened against the entire F_2 segregated population. Polymorphic markers were used for the linkage analysis with the pericarp colour genotype. The pericarp colour of the F_2 individuals in the population was validated by progeny testing of $F_{2:3}$.

χ^2 test of the goodness of the fit was applied to analyse the segregation ratio in the population (Gai 2000). To determine the linkage relationship between the *Rp* locus and the molecular markers, the genotype of the pericarp colour was combined with the DNA marker data for the linkage analysis. The linkage analysis was conducted using the Mapmaker/Exp 3.0 program at a logarithm of the odds (LOD) threshold of 3. 0 to construct a local genetic map for the *Rp* genomic region. The map distance between the marker and the red pericarp colour gene was estimated by the Kosambi mapping function (Lincoln et al. 1992).

Figure 1. Phenotypes of red pericarp mutants
Top row, left to right: seeds from 898, [898 × GER-3]/F_1, [GER-3 × 898]/F_1 and GER-3; bottom row, left to right: seeds from [898 × GER-3]/F_2, and [GER-3 × 898]/F_2

RESULTS AND DISCUSSION

Genetic analysis of *Rp* gene. The genetic results revealed that the pericarp colour of the reciprocal F_1 was red for all the red individuals, exhibiting the same red pericarp as that of the GER-3 parent, which was significantly different from that of the white pericarp parent 898 (Figure 1). No segregation of the pericarp colour within one plant (panicle) was founded, because the tissue of the pericarp grain is completely identical to the mother plant, and it is the rest of the cover tissue of the pistil that has not undergone the process of fertilisation.

In the [GER-3 × 898]/F_2 population, there was segregation in the pericarp colour (Figure 1). Of the 1034 individuals, 759 individuals had a red pericarp and 275 individuals had a white pericarp; the segregation ratio of the red pericarp individuals to the white pericarp individuals was 2.76 : 1, which was a good fit to a 3 : 1 ratio (χ^2_c = 1.3204 < 3.84, P > 0.05, Table 1; Gai 2000).

A similar segregation ratio of the red pericarp to white pericarp individuals was obtained in the reciprocal F_2 population between GER-3 and 898 in another trial. In the [GER-3 × 898]/F_2 population, of the 766 individuals, 537 individuals had a red pericarp and 229 individuals had a white pericarp. In the [898 × GER-3]/F_2 population, of the 1 625 individuals, 971 individuals had a red pericarp and 294 individuals had a white pericarp. Combining the data for the reciprocal F_2 population, the segregation ratio of the red pericarp individuals to the white pericarp individuals was 2.867 : 1, which was also a good fit to a 3 : 1 ratio (χ^2_c = 0.5713 < 3.84, P > 0.05, Table 1; Gai 2000). The result indicated that only a single dominant gene is responsible for the red pericarp of GER-3 and that the red pericarp expression of the mutant gene *Rp* is not affected by the cytoplasm.

Mapping population development. To mapping the red pericarp mutant gene *Rp*, an inter-subspecific cross was carried out between the mutant GER-3 (indica rice cultivar, red pericarp) and C418 (japonica

Table 1. The results of the χ^2 test of the F_2 population for the genetic analysis of the *Rp*

F_2 population	Year	Total	Red	White	Red/white ratio	χ^2_c	P
[GER-3 × 898]/F_2	2002	1 034	759	275	2.76 : 1	1.3204	> 0.05
[GER-3 × 898]/F_2	2003–2005	766	537	229	2.867 : 1	0.5713	> 0.05
[898 × GER-3]/F_2	2003–2005	1 625	971	294			

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Table 2. The results of the χ^2 test of the F_2 population for the *Rp* mapping

Population	Total	Red	White	Red/white ratio	χ^2_c	*P*
[GER-3 × C418]/F_2	864	626	238	2.288 : 1	0.9698	> 0.05
[C418 × GER-3]/ F_2	679	514	165			

rice line, white pericarp), and an F_2 mapping population was developed. In the F_2 mapping population and the $F_{2:3}$ progenies derived from F_2 individuals, segregation in the pericarp colour occurred.

In the [GER-3 × C418]/F_2 population, of the 864 individuals, 626 had a red pericarp and 238 had a white pericarp, and in the [C418 × GER-3]/F_2 population, of the 679 individuals, 514 had a red pericarp and 165 had a white pericarp. Combining the data, of the 1 543 individuals in the F_2 segregation population, 1 140 had a red pericarp and 403 had a white pericarp. The segregation ratio of the red pericarp individuals to the white pericarp individuals was 2.8288 : 1, which was an excellent fit with the segregation ratio of 3 : 1 ($\chi^2_c = 0.9698 < 3.84$, $P > 0.05$, Table 2; Gai 2000).

In the [C418 × GER-3]/$F_{2:3}$ progeny, the populations could be categorised into three groups according to the pericarp colour. Group 1, derived from the homozygous red pericarp F_2 individuals, exhibited red pericarp non-segregation; 81 $F_{2:3}$ progenies were categorised in this group. Group 2, derived from the heterozygote red pericarp F_2 individuals, showed continued segregation and resulted in both red pericarp and white pericarp individuals; 141 $F_{2:3}$ progenies were categorised in this group. Group 3, derived from the homozygous white pericarp F_2 individuals, exhibited white pericarp non-segregation; 77 $F_{2:3}$ progeny were categorised in this group. The ratio of group 1 to group 2 progeny was 0.57447. The ratio of the segregating red pericarp $F_{2:3}$ progenies to the non-segregating red pericarp $F_{2:3}$ progeny showed a good fit to 2 : 1 ($\chi^2_c = 0.8565 < 3.84$, $P > 0.05$, Figure 2; Gai 2000). The results confirmed that there was only one dominant gene, temporarily designated *Rp* (*Red pericarp*) controlling the segregation of the red pericarp in the F_2 mapping population. Because the genetic background of the individuals in the segregation population derived from the inter-subspecific cross is very different, the segregation population is especially suitable for the primary mapping of *Rp.*

Preliminary mapping of the red pericarp mutant gene *Rp.* A total of 119 F_2 individuals in the mapping population were selected for the preliminary mapping of the red pericarp mutant gene *Rp*. Nearly 2 000 SSR markers selected from 12 rice chromosomes were tested (http://www.gramene.org). Initially, the markers were examined for polymorphism between GER-3 and C418. Then, the polymorphic markers were screened against the white DNA bulks, and finally, the polymorphic markers between the white DNA bulks were screened against the entire F_2 mapping population. Polymorphic markers were used for co-segregation analysis with the pericarp colour genotype.

By using SSR markers and the BSA approach, the red pericarp mutant gene *Rp* was finally mapped to the short arm of chromosome 7 at the interval between two SSR markers RM21182 and RM21268, with a genetic distance of 3.5 and 12.0 cM (Figure 3), respectively.

Potential sources of the red pericarp mutant gene *Rp.* The study provides important information for the better understanding of the genetics and molecular biology of the red pericarp and for the better management of the features associated with red rice. Lee et al. (2009) identified a genetic difference between the cultivar Perla and its natural red rice mutant Perla Rosso in the *Rc* gene. Lost by the original 14-bp deletion that gave rise to white rice,

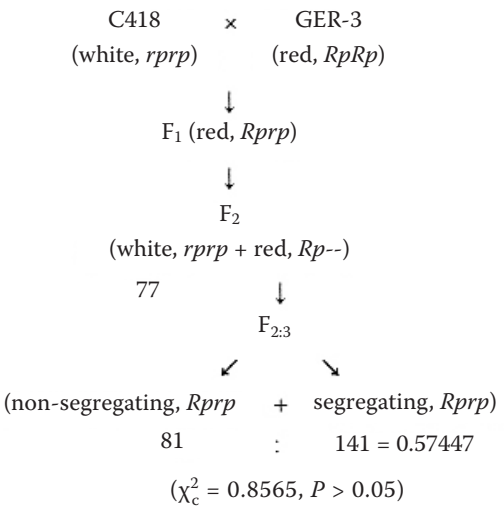

Figure 2. Results of the χ^2 test of the progeny testing of the red pericarp lines of the $F_{2:3}$ families

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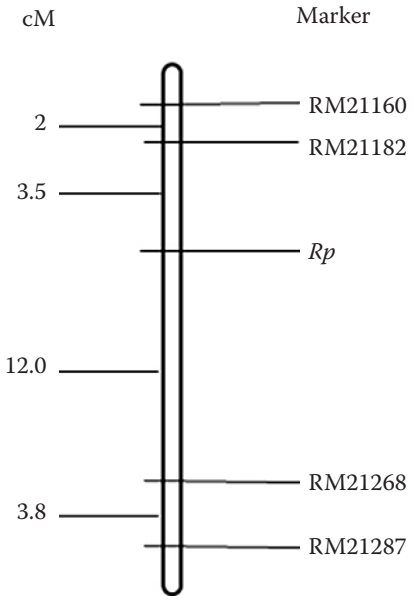

Figure 3. Preliminary mapping of the *Rp* gene on the short arm of chromosome 7

the deletion of a G base restores the reading frame for the *Rc* gene. A new allele that arose by natural mutation within the *Rc* pseudogene of the cultivar Wells has been identified by Brooks et al. (2008); the mutation restored the reading frame of the gene and reverted the bran layer pigmentation to red (wild-type). Both restorative mutations of the red pericarp gene occurred independently, which might suggest that such events may not be rare.

The red pericarp mutant gene *Rp* represents a novel germplasm resource for rice specialty breeding. In the process of exploring the potential sources of the red pericarp mutant gene *Rp*, we first considered an outcross as leading to the red pericarp in GER-3. However, there was no red pericarp rice cultivars planted in the experiment field (Bao et al. 1994). In the maize-rice transgenic breeding programme, the colour of the pericarp of Yijingbai, the maize donor, is white; thus, the assumption that the red pericarp mutant gene *Rp* originated from the maize donor was also rejected.

The red pericarp mutant gene *Rp* was mapped to the region between the SSR markers RM21182 and RM21268 and is located in the same vicinity of the *Rc* gene (*Os07g0211500*) on the short arm of chromosome 7 (Sweeney et al. 2006). In addition, the chalcone synthase gene (*Os07g0214900*) in the *Rp* region may function for the anthocyanin biosynthesis (Tsutsui et al. 2012). Referring to the two restorative mutations of the red pericarp gene reported by Brooks et al. (2008) and Lee et al. (2009), it was deduced that the potential origin of *Rp* may be a gene mutation associated with the anthocyanin in the tissue culture or transgenic process in the maize-rice transgenic breeding programme. Of course, the above hypothesis needs to be further studied and confirmed.

Studies on red rice. Extensive research has been previously conducted the inheritance and mechanism of the underlying red colouration in the rice pericarp. Using classical genetic analysis, two loci, *Rc* (brown pericarp and seed coat) and *Rd* (red pericarp and seed coat), have been identified. *Rc* and *Rd* are inherited monogenically, and they were mapped on chromosome 7 and on chromosome 1, respectively (Nagao & Takahashi 1947; Dong et al. 2008). The red pericarp in rice is controlled by the complementary effect of two major genes; the *Rd* gene increases the pigment content in the pericarp and the *Rc* gene is responsible for the accumulation of the pigments (Nagao et al. 1957). Studies have shown that: (1) *Rd* alone has no phenotype; (2) in the absence of *Rd*, *Rc* produces brown seeds; and (3) when *Rc* and *Rd* are both present, these loci produce a red seed colour (Kato & Ishikawa 1921). Furukawa et al. (2007) reported that *Rc* and *Rd* were involved in the proanthocyanidin synthesis in the red pericarp of rice (Dong et al. 2008). The *Rd* gene encodes a dihydroflavonol-4-reductase (DFR) protein (Furukawa et al. 2007), and the *Rc* gene encodes a transacting regulatory factor with a basic helix-loop-helix (bHLH) motif (Sweeney et al. 2006). Zhu et al. (2019) developed a CRISPR/Cas9-mediated method to functionally restore the recessive *Rc* allele through reverting the 14-bp frame-shift deletion to in-frame mutations in which the deletions were in multiples of three bases, and successfully converted three elite white pericarp rice varieties into red ones (Zhu et al. 2019). The red rice discussed in this paper, is not the same as purple rice. The phenotype of purple rice is controlled by other *Pb* (synonym *Prp-b*) and *Pp* (synonym *Prp-a*) genes (Rahman et al. 2013).

CONCLUSION

In the maize-rice transgenic breeding programme Gene Engineering Rice-3 (GER-3), a red pericarp mutant line that possessed multiple desirable characteristics was isolated and used for the genetic analysis and molecular mapping of the red pericarp mutant gene in this study.

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The genetic analysis results revealed that the red pericarp of the red pericarp mutant was controlled by a single dominant gene, temporarily designated red pericarp (*Rp*), and that the red pericarp expression of the mutant gene *Rp* is not affected by the cytoplasm.

An F_2 population derived from an inter-subspecific cross between GER-3 (indica cv., red pericarp) and C418 (japonica cv., white pericarp) was constructed for the molecular mapping of *Rp*. There was only one dominant gene controlling the segregation of the red pericarp in the F_2 mapping population, it confirmed that the F_2 segregation population is especially suitable for the primary mapping of *Rp.*

Referring to the preliminary mapping result of *Rp*, and the restorative mutations of the reported red pericarp gene, it was deduced that the potential origin of *Rp* may be a gene mutation in the tissue culture or transgenic process in the maize-rice transgenic breeding programme.

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