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Characterization and molecular mapping of a dwarf mutant in wheat

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ABSTRACT. A spontaneous dwarf mutant of wheat was found in an F_5 generation line derived from a cross between Huamai No. 9 and Een No. 1 in 1998; it was named Huaai 01. We characterized the genetic pattern of Huaai 01 and mapped the gene controlling the dwarf trait. This dwarf mutant was found insensitive to exogenous gibberellic acid treatment, based on the length of the first leaf and the coleoptile at the seedling stage, suggesting that it plays a crucial role in the gibberellin response pathway. Genetic analysis revealed that a single gene that is partially recessive controls the dwarf phenotype in Huaai 01. We named the dwarfing gene *Rht-B2*. Simple sequence repeats (SSR) were examined as identifying markers linked to the *Rht-B2* gene in an F_2 population. We screened 904 pairs of primers and identified 5 SSR markers linked to the *Rht-B2* gene. Two markers, barc1096 and xgwm495, were located on the flanking region of the *Rht-B2* gene at genetic distances of 2.9 and 3.3 cM, respectively. Based on published SSR linkage data for wheat, the *Rht-B2* gene was mapped to the long arm of chromosome 4B. This identification and characterization of the *Rht-B2* dwarfing gene will facilitate its utilization in wheat breeding.

Key words: Wheat; Genetic analysis; Gibberellic acid; Dwarf mutant; SSR analysis

INTRODUCTION

Dwarfing genes have played an important role in the Green Revolution to improve lodging resistance. The application of dwarfing genes has resulted in increased yields, higher fertility, early maturity, and high tillering capacity (Khush, 2001; Hedden, 2003). In wheat, the reduced height (*Rht*) alleles, *Rht-B1b* and *Rht-D1b*, have been found in more than 70% of modern wheat cultivars grown worldwide (Evans, 1998). Twenty-one genes with major effects on plant height in wheat have been reported and assigned as *Rht* (McIntosh et al., 2008). However, only 4 or 5 dwarfing genes of these mutations have been widely used (Börner et al., 1993; Ahmad and Sorrells, 2002). Among these major dwarfing genes, the semi-dwarfing alleles *Rht-B1b* (formerly *Rht1*) and *Rht-D1b* (formerly *Rht2*) are widely used in breeding programs (Gale and Youssefian, 1985; Börner et al., 1996).

Gibberellin is an essential endogenous regulator of plant growth. The important dwarfing genes that have been used in agriculture are mutants in the gibberellin biosynthesis or response pathways. Mutations of genes in the biosynthesis pathway cause gibberellic acid (GA) deficiency and dwarf phenotypes, and exogenous GA application can restore wild-type phenotype in these mutants (Phillips, 1998). Dwarf mutants in the GA response pathway display a similar phenotype as GA biosynthesis mutants, although they fail to respond to exogenous GA treatment (Sun, 2000). The dwarfing genes *Rht-B1* and *Rht-D1* are mutants in the GA response pathway, and most modern commercial wheat cultivars contain one of these *Rht* mutant alleles (Silverstone and Sun, 2000).

In 1998, we found a dwarf individual in line 50063 of the F_5 generation of a cross between Huamai No. 9 and Een No. 1 at the Huazhong Agricultural University, Wuhan, China. Since Huamai No. 9 (95 cm) and Een No. 1 (100 cm) were tall cultivars, and no dwarf individual appeared in their F_2, F_3, and F_4 generations, the dwarf trait of this individual was most likely a mutation. The dwarf individual was named "Huaai 01". The plant height of Huaai 01 was about 45 cm, and no segregation was found over 3-year experiments for plant height. The pedigree of the 2 parents did not contain any dwarfing genes; the plant height of Huaai 01 was much shorter than that of most dwarf germplasms found worldwide.

To efficiently use this dwarf germplasm in plant breeding programs, we characterized the inheritance of this dwarfing gene, and mapped this gene using molecular markers in common wheat. The effect of exogenous GA_3 on seedling growth was also determined.

MATERIAL AND METHODS

Plant materials

The dwarf mutant (Huaai 01) was a spontaneous variation obtained from the progeny of Huamai No. 9 and Een No. 1. The tall cultivar Een No. 1 was crossed with Huaai 01 to generate a mapping population. To map the dwarfing gene in Huaai 01, 333 F_2 individuals were genotyped. For genetic analysis, Huaai 01 was also crossed with 2 other tall varieties, Huamai No. 9 and Huamai 2152, to determine the inheritance of genes controlling the dwarf trait in Huaai 01.

Field trial and trait measurements

A field trial was conducted at the Experimental Station of Huazhong Agricultural Uni-

versity, Wuhan, China. The F_2 population, F_1 plants and parents, of 3 different cross combinations (Huaai 01 x Huamai No. 9, Huaai 01 x Een No. 1, and Huaai 01 x Huamai 2152), were planted in the field in 2008-2009. Young leaves from F_2 plants of the Huaai 01 x Een No. 1 combination were collected for simple sequence repeat (SSR) analysis. Each F_2 plant was self-pollinated to obtain F_3 seeds, and each F_3 family was grown in a row (20 plants/row; 2 m), the segregation of plant heights were used to confirm the genotype of the corresponding F_2 plants in 2009-2010. At maturity, 15 plants of each F_3 family were measured for plant height from the ground to the top of the main inflorescence (without awn). Length of the spike, uppermost, the first, second, third, and fourth internodes (numbered from the top to the bottom of plant stem) of these individuals were also measured.

To test the allelic relationship of Huaai 01 with other dwarfing genes in wheat, the F_1 of Huaai 01 x Een No. 1 was crossed with 3 dwarf varieties, Chuannongmai No. 1, Emai No. 11, and Yangmai 158, which have the *Rht-B1b* gene. The seeds were planted in the field as described above.

GA_3 seedling growth test

Huaai 01, Een No. 1, and Chinese Spring seeds were planted on filter paper moistened with distilled water and kept at 4°C. Two days later, seedlings with synchronized germination were transferred to plastic pots (8 cm in diameter). The pots were grown in a standardized nutrition solution supplemented with 20, 50, or 100 ppm GA_3 in a growth chamber in the dark at 20°C. Ten seedlings per treatment were compared with the same number of seedlings grown under standard conditions with distilled water. After reaching the 3-leaf stage, the first leaf length and the coleoptile length were measured for 10 individuals of each treatment. The data were analyzed and compared using the SPSS software (http://www.spss.com).

DNA extraction and molecular marker analysis

Total genomic DNA was extracted from fresh leaves using the cetyltrimethylammonium bromide (CTAB) method (Stein et al., 2001). DNA concentration was measured using a Beckman spectrophotometer (Beckman, Fullerton, USA) and adjusted to a final concentration of 20 ng/μL in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Bulked segregant analysis (Michelmore et al., 1991) combined with the SSR markers were used to identify molecular markers linked to the dwarf gene. Equal amounts of genomic DNA from 10 extremely short plants and 10 extremely tall plants from the F_2 mapping population were pooled to construct the dwarf bulk and tall bulk, respectively.

SSR markers were synthesized based on sequence information from IPK Gatersleben (prefix: gwm and gdm; Röder et al., 1998; Pestsova et al., 2000), the Wheat Microsatellite Consortium (prefix: wmc; Gupta et al., 2002), the John Innes Centre (prefix: psp; Bryan et al., 1997), DuPont (prefix: dupw; Eujayl et al., 2002), R. Ward (prefix: barc; Song et al., 2005), INRA Clermont-Ferrand (prefix: cfd, cfa, and gpw; Guyomarc'h et al., 2002; Sourdille et al., 2003, 2005). PCR was performed in a volume of 10 μL in a Bio-Rad thermocycler. The reaction mixture contained 40 ng template DNA, 1X PCR buffer, 2 mM Mg^{2+}, 0.2 mM each dNTP, 0.2 μM each forward and reverse primer, and 0.5 U *Taq* DNA polymerase (TakaRa Bio, Dalian, China). The amplification reactions were carried out using the following profile: one

cycle of 94°C for 3 min, followed by 35 cycles at 94°C for 1 min, 1 min between 50 to 62°C (depending on the individual primer set), and 1 min at 72°C, and ending with an elongation step of 10 min at 72°C. The PCR product was separated on a 6% denaturing polyacrylamide gel and visualized using silver staining (Xu et al., 2002).

A total of 904 SSRs randomly arranged on the whole wheat chromosome were screened for polymorphism between the parental lines. The polymorphic SSR markers were further used to analyze the 2 DNA bulks. The polymorphic markers between 2 DNA bulks were used to analyze the 333 individuals from the F_2 population. The linkage maps were constructed using the MAPMAKER software (Lander et al., 1987), and the genetic distance (cM) was derived using the Kosambi function (Liu and Meng, 2003).

RESULTS

GA response in seedling stage

A seedling growth test was performed to test for sensitivity to GA. Huaai 01 showed no significant response to all levels of GA_3 tested for both the first leaf length ($P = 0.178 > 0.05$) (Figure 1A) and coleoptile length ($P = 0.425 > 0.05$) (Figure 1B). The Een No. 1 and Chinese Spring showed significant response for both the first leaf length ($P = 0.007$, 5.43E-05 < 0.05) and coleoptile length ($P = 0.000$, $0.027 < 0.05$).

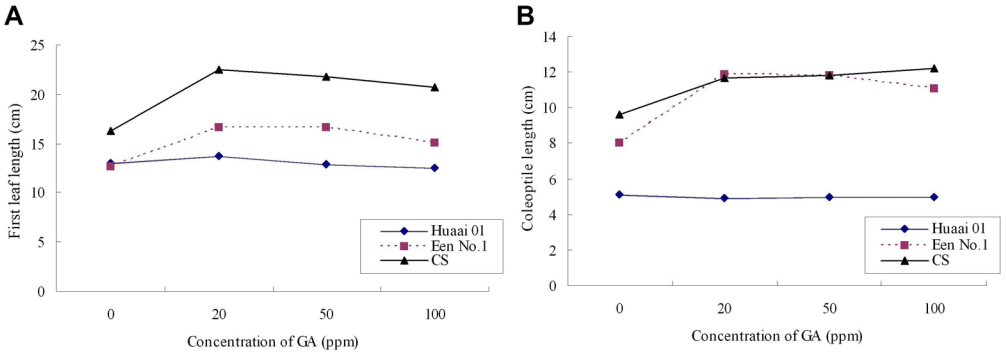

Figure 1. Effects of gibberellin acid (GA) on the first leaf length (**A**) and coleoptile length (**B**) in the seedling stage. CS = Chinese Spring.

Genetic analysis of the dwarfing gene in Huaai 01

The plant height and its components of Huaai 01 and Een No. 1 were evaluated in Wuhan, Hubei Province of China for 3 years. The averaged plant height of Huaai 01 was 44 ± 1.71 cm across all 3 years, which was significantly shorter than the tall parent Een No. 1 (97.6 ± 2.25 cm) (Table 1 and Figure 2). No significant difference between the 3 years for plant height was detected for both Huaai 01 and Een No. 1. The length of internodes of Huaai 01 was also significantly shorter than that of the tall parent Een No. 1. However, the spike length of Huaai 01 was similar to that of Een No. 1 (Table 1).

Table 1. Evaluation of plant height and its components of Huaai 01 and Een No. 1 in 2007-2010.

Parental line	Year	Plant height (cm)	Spike length (cm)	Length of internode (cm)				
				Uppermost internode	1st	2nd	3rd	4th
Huaai 01	2007-2008	43.2	10.1	17.3	8.2	4.1	3	0.5
	2008-2009	44.2	10.8	17.1	8.3	4.4	3	0.6
	2009-2010	44.6	10.3	17.4	8.3	4.9	3.1	0.5
	Average	44	10.5	17.2	8.3	4.5	3	0.6
	SD	1.71	0.59	1.56	1.14	0.7	0.7	0.97
P value		0.429	0.074	0.916	0.992	0.127	0.921	0.966
Een No. 1	2007-2008	98.5	9.2	36.4	24.9	14.4	10	3.6
	2008-2009	97.6	9.7	34.6	23.9	14.3	11.1	3.9
	2009-2010	97.2	9.1	34.6	24.5	14.1	11.3	3.6
	Average	97.6	9.4	34.9	24.3	14.2	11	3.7
	SD	2.25	0.87	2.72	1.35	0.89	1.19	0.63
P value		0.741	0.338	0.616	0.574	0.833	0.295	0.667

Figure 2. Plant height of Huaai 01 (right), Een No. 1 (middle), and their F_1 hybrid (left).

The relative length of each internode to the plant height in Huaai 01 and Een No. 1 was calculated using the length of each internode divided by the length of the plant. The relative length of the 1st, 2nd, 3rd internodes, and spike showed significant differences between the 2 parents. The relative length of the uppermost internode was not significant ($P > 0.05$)

between the 2 parents in years 2007-2008 and 2009-2010. The relative length of 4th internode was significantly different between the 2 parents in years 2007-2008 and 2008-2009 (P = 0.001, 0.006 < 0.01), but not significantly different in year 2009-2010 (P = 0.011 > 0.01). Comparing the relative length of each internode in Huaai 01 and Een No. 1, we found that the relative lengths of spike and uppermost internodes of Huaai 01 were longer than those of Een No. 1, while all other internodes were shorter (Table 2 and Figure 3).

Table 2. Relative length of each internode to the plant height in Huaai 01 and Een No.1.

Internode	2007-2008			2008-2009			2009-2010			Average		
	Huaai 01 (%)	Een No. 1 (%)	P value	Huaai 01 (%)	Een No. 1 (%)	P value	Huaai 01 (%)	Een No. 1 (%)	P value	Huaai 01 (%)	Een No. 1 (%)	P value
Spike	24.4	9.3	0.000	24.5	10	0.000	23.1	9.3	0.000	23.8	9.6	0.000
Uppermost internode	38.4	37	0.091	38.6	35.5	0.049	39.1	35.6	0.057	39.2	35.8	0.002
1st	19.6	25.3	0.002	18.9	24.5	0.000	18.6	25.2	0.000	18.8	24.9	0.000
2nd	10.8	14.7	0.001	9.9	14.7	0.000	11.1	14.5	0.001	10.1	14.6	0.000
3rd	6.8	10.2	0.001	6.7	11.3	0.000	7	11.6	0.000	6.8	11.3	0.000
4th	0	3.7	0.001	1.4	4	0.006	1.1	3.7	0.011	1.2	3.8	0.000

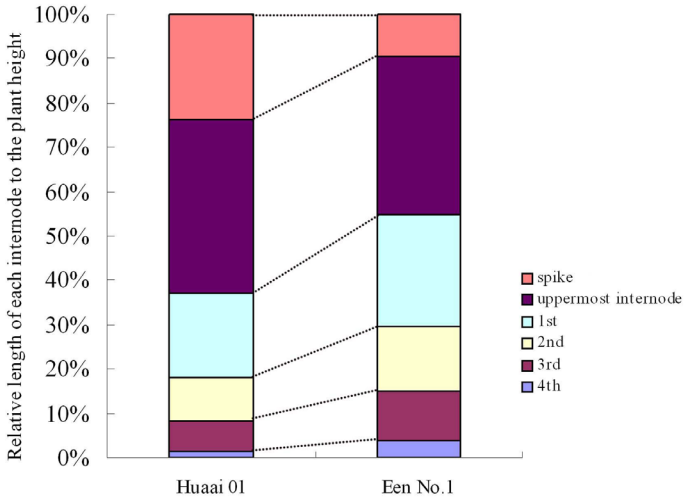

Figure 3. Schematic presentation of the relative length in each internode of Huaai 01 and Een No. 1.

To analyze the inheritance of the dwarfing gene in Huaai 01, this cultivar was crossed with tall varieties Huamai No. 9, Een No. 1, and Huamai 2152. Plant heights in all F_1 plants were intermediate between the 2 parents, and close to the tall parent in all crosses (Table 3). This suggested that the dwarfing gene is an incompletely recessive gene. Plant heights in the F_2 population of each cross showed a trimodal distribution from 35 to 100 cm, with 3 peaks (Figure 4). All F_2 individuals could be roughly classified into 3 groups corresponding to putative homozygous dwarf, heterozygous, and homozygous tall groups. The χ^2 test indicated a 1:2:1 segregation ratio (Table 3), suggesting that a single gene controlled the dwarf phenotype. The gene was named *Rht-B2*.

Table 3. Plant height in the F_2 population derived from the cross between Huaai 01 and several tall cultivars in 2008-2009.

Cross	Plant height of Huaai 01 (cm)	Plant height of tall parent (cm)	Plant height of F_1 (cm)	No. of F_2 plants	No. of putative homozygous dwarf[a]	No. of putative heterozygous	No. of putative homozygous tall	$\chi^2_{(1:2:1)}$
Huaai 01/Huamai No. 9	44.2	87.6	71.7	184	50	93	41	0.9
Huaai 01/Een No. 1	44.2	97.6	86.6	333	84	169	80	0.17
Huaai 01/Huamai 2152	44.2	86.8	77.2	142	39	77	26	3.39

$\chi^2_{2,0.05} = 5.99$. [a]Cutting points of the 3 populations were around 57-63 and 76-83 cm.

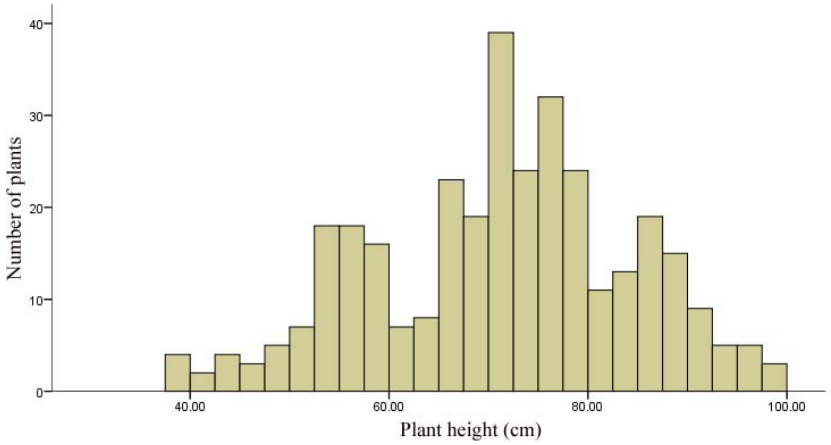

Figure 4. Plant height distribution of F_2 population derived from the cross of Huaai 01 and Een No. 1. The distribution showed a trimodal distribution from 35 to 100 cm with 3 peaks.

Allelic relationship between *Rht-B2* and other dwarfing genes in wheat

To test whether Huaai 01 shared the same allele with other known dwarfing genes, we made 3 test crosses. All progeny in the 3 crosses were tall (Table 4), suggesting that the gene *Rht-B2* controlling plant height in Huaai 01 was nonallelic with *Rht-B1b* in these 3 dwarf varieties.

Table 4. Segregation of plant height in the test crosses between F_1 of Huaai 01 x Een No. 1 and 3 dwarf varieties.

Test cross	Generation	No. of total plants	No. of dwarf plants	No. of tall plants[a]	χ^2 (1:1)
(Huaai 01/Een No. 1)/Chuannongmai No. 1	TC_1	147	0	147	-
(Huaai 01/Een No. 1)/Emai No. 11	TC_1	171	0	171	-
(Huaai 01/Een No. 1)/Yangmai 158	TC_1	152	0	152	-

$\chi^2_{1, 0.05} = 3.841$. [a]Plant height in these test crosses were all normally distributed with a height range of 75-100, 80-110, 80-105 cm, respectively.

Genetic mapping of the dwarfing gene in the Huaai 01

Five of the 904 SSR markers showed polymorphism between the bulked-DNA pools. The 5 polymorphic markers were used to genotype 333 individuals from the F_2 population. Linkage analysis mapped all 5 markers to the same linkage group. The markers barc1096 and xgwm495 were closely linked to the dwarfing gene at a distance of 2.9 and 3.3 cM, respectively (Figure 5). These 5 markers, barc1096, xgwm495, wmc657, xgwm513, and wmc459, were all mapped to chromosome 4B based on 4 reference maps (Somers et al., 2004; Song et al., 2005; Sourdille et al., 2005; Xue et al., 2008), and the flanking markers barc1096 and xgwm495 of *Rht-B2* were mapped to the 4B long arm (Figure 5), suggesting that the dwarfing gene *Rht-B2* in Huaai 01 was located on the long arm of chromosome 4B.

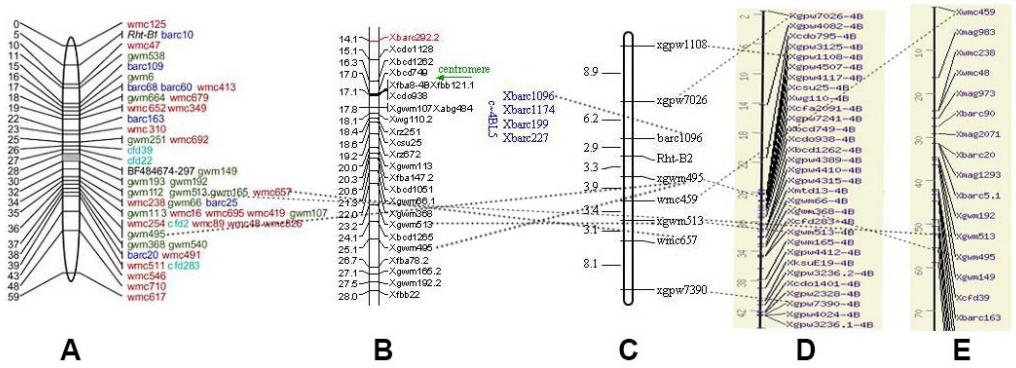

Figure 5. Partial linkage maps of *Triticum aestivum* L. indicated the relative location of the *Rht-B2* gene on chromosome 4B of the 4 reference maps. **A.** A microsatellite consensus map of chromosome 4B from 4 independent genetic maps (Somers et al., 2004). **B.** Partial genetic and physical map of chromosome 4B of wheat, Synthetic x Opata, BARC (Song et al., 2005). **C.** Linkage map of the region surrounding the *Rht-B2* on chromosome 4B. **D.** Partial genetic map of chromosome 4B of wheat, Synthetic x Opata, GPW (Sourdille et al., 2005). **E.** Partial genetic map of chromosome 4B of wheat, Nanda2419 x Wangshuibai (Xue et al., 2008).

DISCUSSION

Based on the sensitivity of wheat seedlings to GA treatment, the *Rht* genes in wheat can be classified into 2 main categories: GA-insensitive and -responsive dwarfing genes. These GA-insensitive dwarfing genes have a pleiotropic effect on plant growth, causing reduction in coleoptile length and seedling leaf area (Allan et al., 1962; Whan, 1976). In this study, both the first leaf length and coleoptile length in Huaai 01 were insensitive to exogenous GA_3 treatment (Figure 1). In wheat, *Rht-B1b* and *Rht-D1b* are GA insensitive and defective in the GA response pathway (Fridborg et al., 1999; Peng et al., 1999). Also, *Rht-B1b* and *Rht-D1b* genes are orthologs of *GAI* and *RGA* in *Arabidopsis* (Sun, 2000). Since the *Rht-B2* mutant did not respond to GA treatment, it is most likely defective in the GA response pathway. Alternatively, it may contain a mutation in a target gene of the GA response pathway.

Plant height is composed of the lengths of various internodes and the spike. In our study, plant height and the length of each internode of both parents over 3 years showed no significant difference ($P > 0.05$) (Table 1). This suggested that inheritances of these traits were stable, and not affected by environment. Relative lengths of spike and uppermost internodes of Huaai 01 were longer, but all the other internodes were shorter than those of Een No. 1 (Table 2 and Figure 3). Previous reports have suggested that the length of the spike is a qualitative character affected by one main allele with some modifying factors, and that the lengths of various internodes are quantitative characters, each having their own independent genetic patterns and controlled by polygenes (Wei and Wu, 1990). Our study supports this suggestion.

The plant height of F_1 plants derived from 3 different cross combinations were all intermediate between the 2 parents, and close to the tall parent, indicating an incompletely recessive dwarfing gene. The segregation ratio (1:2:1) of putative homozygous dwarf, heterozygous, and homozygous tall in each F_2 population suggested that a single gene with partially recessive controlled the dwarf phenotype in Huaai 01.

The allelism test of Huaai 01 with 3 dwarf varieties, which all contained *Rht-B1b*, showed that all progeny derived from test crosses between the F_1 of Huaai 01 x Een No. 1 and those 3 varieties were tall (Table 4). Since the dwarfism of Huaai 01 was controlled by a recessive allele, the F_1 of Huaai 01 x Een No. 1 was tall. If Huaai 01 were allelic with *Rht-B1b*, the progeny would be expected to have a tall:dwarf ratio of 1:1. Testcross results showed that the *Rht-B2* in Huaai 01 is nonallelic with the gene *Rht-B1b*.

Although more than 21 types of dwarfs or semi-dwarfs have been found so far, only 4 or 5 dwarfing genes of the spontaneous mutations have been widely used in breeding programs (Börner et al., 1993; Ahmad and Sorrells, 2002). Exploring a new dwarfing gene is of practical value. The newly discovered dwarf germplasm Huaai 01 is a spontaneous mutant with desirable agronomic traits: shortened stature (44 cm) and early maturity. The application of this valuable dwarfing gene can greatly enhance wheat breeding.

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