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"Perfect" markers for the *Rht-B1b* and *Rht-D1b* dwarfing genes in wheat

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Abstract PCR-based markers were developed to detect the point mutations responsible for the two major semi-dwarfing genes *Rht-B1b* (*Rht1*) and *Rht-D1b* (*Rht2*) in wheat. These markers were validated by testing 19 wheat varieties of known *Rht* genotype. They included *Rht-B1b* and *Rht-D1b* dwarfs, double-mutant varieties and tall wheats. These were correctly genotyped with the *Rht-B1b* and *Rht-D1b*-specific primers, as well as markers specific for the tall alleles *Rht-B1a* and *Rht-D1a*. Using a family of doubled-haploid lines segregating for *Rht-B1b* and *Rht-D1b*, the markers were mapped to the expected homoeologous regions of chromosomes 4B and 4D, respectively. Both markers were strongly correlated with a reduction in height, accounting for 23% (*Rht-B1b*) and 44% (*Rht-D1b*) of the phenotypic variance in the population. These markers will have utility in marker-assisted selection of the *Rht-B1b* and *Rht-D1b* genes in wheat breeding programs.

Keywords Wheat · Reduced height · GA insensitivity · PCR markers

Introduction

The *Rht-B1b* (*Rht1*) and *Rht-D1b* (*Rht2*) semi-dwarfing genes were introduced into commercial wheat cultivars from the Japanese variety Norin10 in the 1960s as part of wheat improvement programs in the USA and at CIMMYT, Mexico. A reduction in plant height improved lodging resistance and partitioning of assimilates to the developing grain (Evans 1993). The large increases in yield that followed the introduction of these dwarfing genes led to widespread adoption of the dwarfing genes throughout the world (Gale et al. 1985).

Recently, the homoeologous genes *Rht-B1b* and *Rht-D1b* were isolated from wheat (Peng et al. 1999). They are orthologous to the Arabidopsis GAI gene, a de-repressible modulator of gibberellic acid (GA) response (Peng et al. 1997). Both the *Rht-B1b* and *Rht-D1b* mutations are associated with a single base-pair change leading to a TAG stop codon shortly after the start of translation (Peng et al. 1999). These mutations reduce the plant's ability to respond to GA, so that exogenous application of this hormone does not restore wild-type plant height. Hence the presence of these dwarfing genes can be determined by testing seedlings for the lack of responsiveness to GA (Gale and Gregory 1977; Richards 1992). Although relatively easy, this test is time-consuming, not always reliable, and does not discriminate between *Rht-B1b* and *Rht-D1b*.

These limitations can be overcome by using molecular markers for these dwarfing genes. We developed PCR-based markers aimed at discriminating between mutant (dwarf) *Rht-B1b* and *Rht-D1b* and their wild-type (tall) alleles. We tested the specificity and the robustness of these markers on a range of wheat varieties of known *Rht* genotypes as well as in a family of doubled-haploid (DH) lines segregating for the *Rht-B1b* and *Rht-D1b* dwarfing genes.

Materials and methods

Plant material

A range of both bread (*Triticum aestivum* L.) and durum wheat (*Triticum turgidum* L. var. durum) varieties were obtained from the Australian Winter Cereal Collection or from wheat breeders.

DNA extraction and PCR analysis

Genomic DNA was extracted from samples of five seeds using a FastDNA isolation kit (Bio101, Vista, USA) according to the manufacturer's instructions for plant samples, with the exception that

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an ethanol precipitation step was added at the end of the procedure.

The following oligonucleotide primers were used:

- BF (5′-GGTAGGGAGGCGAGAGGCGAG-3′),
- DF (5′-CGCGCAATTATTGGCCAGAGATAG-3′),
- DF2 (5′-GGCAAGCAAAAGCTTCGCG-3′),
- MR1 (5′-CATCCCCATGGCCATCTCGAGCTA-3′),
- WR1 (5′-CATCCCCATGGCCATCTCGAGCTG-3′),
- MR2 (5′-CCCCATGGCCATCTCGAGCTGCTA-3′),
- WR2 (5′-GGCCATCTCGAGCTGCAC - 3′).

PCR conditions were as follows (20 µl total volume): 1 × Hotstar Buffer, 1 × Hotstar Q solution, 100 ng of template DNA, 4 nmol of dNTPs, 10 pmol each of the forward and reverse primers, 1 unit of Hotstar *Taq* polymerase (Qiagen, Hilden, Germany). Amplification was carried out on a Hybond polymerase chain reaction (PCR) express cycler running the following program: 5 min at 94 °C; seven "touchdown" cycles of 30 s at 94 °C, 30 s at 65 °C, 1 min 20 s at 72 °C with a 1 °C drop in annealing temperature at each cycle; 30 cycles of 15 s at 94 °C, 15 s at 58 °C, 50 s at 72 °C.

For the primer combination DF2-WR2, slightly different conditions were used. For a 20 µl total volume: 1 × Hotstar Buffer, 100 ng of template DNA, 4 nmol of dNTPs, 20 pmol each of the forward and reverse primers, 2 units of Hotstar *Taq* polymerase (Qiagen, Hilden, Germany). Amplification was carried out on a Hybond PCR express cycler running the following program: 5 min at 95 °C, 42 cycles of 20 s at 94 °C, 30 s at 58 °C, 10 s at 72 °C, and a final step of 72 °C for 2 min.

PCR products were separated on 2% agarose gels and visualised after ethidium bromide staining using standard procedures (Sambrook et al. 1989).

Mapping analysis

Associations between plant height and presence of the *Rht-B1b* and *Rht-D1b* markers was assessed on a random set of 157 Sunco × Tasman doubled-haploid progeny (Kammholtz et al. 2001). Plant height for each line was obtained from field measurements at Roma, Queensland, in 1999 (John Sheppard, unpublished) and correlated with the marker genotype. Existing molecular markers mapped in this population were used to confirm the chromosomal location of the *Rht-B1b* and *Rht-D1b* markers. Regression procedures (Zeng 1994) were used in MapManager QT to estimate the phenotypic variance accounted for, and additive genetic effects associated with each marker. Permutation tests (Doerge and Churchill 1996) were also carried out to obtain likelihood-ratio statistics for plant height with each marker.

Results

PCR markers specific for *Rht-B1b* and *Rht-D1b*

PCR markers specific for the wheat *Rht-B1b* and *Rht-D1b* dwarfing mutations were designed as illustrated in Fig. 1. MR1 and MR2 are reverse primers with the 3′ terminal base pair located on the TAG stop codon in the *Rht-B1b* and *Rht-D1b* mutations, respectively. The "wild type" primer WR1 is identical to MR1, with the exception of the single base pair responsible for the mutation (its 3′ end covers the CAG Q62 codon in the wild-type tall allele *Rht-B1a*). Primer WR2 was designed to be specific for the tall allele *Rht-D1a*. These primers were used in conjunction with B- and D- genome specific primers (BF, DF and DF2) that bind to a region of the promoter with no sequence homology between *Rht-B1b* and *Rht-D1b* (N. Harberd, unpublished.)

Validation of PCR markers on selected wheat varieties

A range of wheat varieties of known *Rht* genotype was selected (Table 1) and DNA samples from these lines were analysed by PCR using the *Rht-B1b*- and *Rht-D1b*-specific markers (Fig. 2). Varieties known to contain the *Rht-B1b* mutation gave an amplification product of the expected size (237 bp) with the *Rht-B1b*-specific (BF-MR1) primer combination (Fig. 2A). These included seven wheat semi-dwarfs (Janz, Inia 66, Cranbrook, Siete Cerros, Baxter, Avocet and Veery 5), a durum semi-dwarf (Tamaroi) and the double-dwarf varieties (Norin10 and Yecora). As expected, the BF-WR1 primer combination gave results complementary to BF1-MR1 (Fig. 2B), by amplifying PCR products from all varieties known to contain the tall allele *Rht-B1a* (the *Rht-D1b* dwarfs and the tall wheats). Five *Rht-D1b* dwarf varieties (Pavon, Sonora 64, Ciano 67, Westonia and Trident) scored positive with the *Rht-D1b*-specific (DF-MR2) primers (Fig. 2C), as did the double-dwarf varieties. No PCR amplification was observed in the *Rht-B1b* dwarfs or the tall wheats. The primer combination DF2-WR2, specific for the tall allele *Rht-D1a*, gave complementary results (Fig. 2D). No amplification was observed in *Rht-D1b* or double dwarfs, or obviously from either of the durum wheats.

The varieties used in this study were mainly from Mexico and Australia. However, we found that the markers would also correctly genotype wheat varieties from Europe or Russia (data not shown).

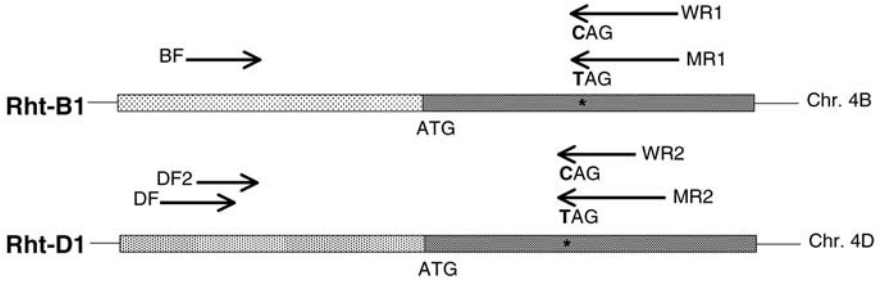

Fig. 1 Diagram of the PCR primers relative to the *Rht-B1b* and *Rht-D1b* genes

Table 1 List of cultivars of known *Rht* genotype. The numbers in the "source" column refer to Australian Winter Cereal Collection AUS accession numbers. The genotype of the varieties was derived from the references listed, from unpublished allelism tests or pedigree information

Variety	Source	Country of origin	Release Date	Rht Genotype	Reference
Janz	24794	Australia	1993	*B1b, D1a*	Rebetzke et al. 1999
Inia 66	356	Mexico	1966	*B1b, D1a*	Gale et al. 1981
Cranbrook	22660	Australia	1984	*B1b, D1a*	Gale and King 1988
Siete Cerros	10684	Mexico	1966	*B1b, D1a*	Gale et al. 1981
Baxter	27694	Australia	1998	*B1b, D1a*	Rebetzke (unpublished)
Avocet	20601	Australia	1979	*B1b, D1a*	Gale and King 1988
Veery 5	20785	Mexico	1977	*B1b, D1a*	Rebetzke (unpublished)
Pavon	Breeders Seed	Mexico	1975	*B1a, D1b*	Gale et al. 1981
Sonora 64	12276	Mexico	1964	*B1a, D1b*	Gale et al. 1981
Ciano 67	125	Mexico	1967	*B1a, D1b*	Gale et al. 1981
Westonia	Breeders Seed	Australia	1997	*B1a, D1b*	Rebetzke (unpublished)
Trident	25924	Australia	1993	*B1a, D1b*	Rebetzke (unpublished)
Thatcher	Breeders Seed	Canada	–	Tall	Rebetzke (unpublished)
Vig18	Breeders Seed	Australia	–	Tall	Richards (unpublished)
Halberd	11612	Australia	1969	Tall	Rebetzke et al. 1999
Norin10	12053	Japan	1935	*B1b, D1b*	Gale et al. 1981
Yecora	17964	Mexico	1971	*B1b, D1b*	Sayre et al. 1997
Tamaroi (D)	27663	Australia	–	*B1b*	Unpublished
Sel. 49 (D)	Breeders Seed	Middle East	–	Tall	Unpublished

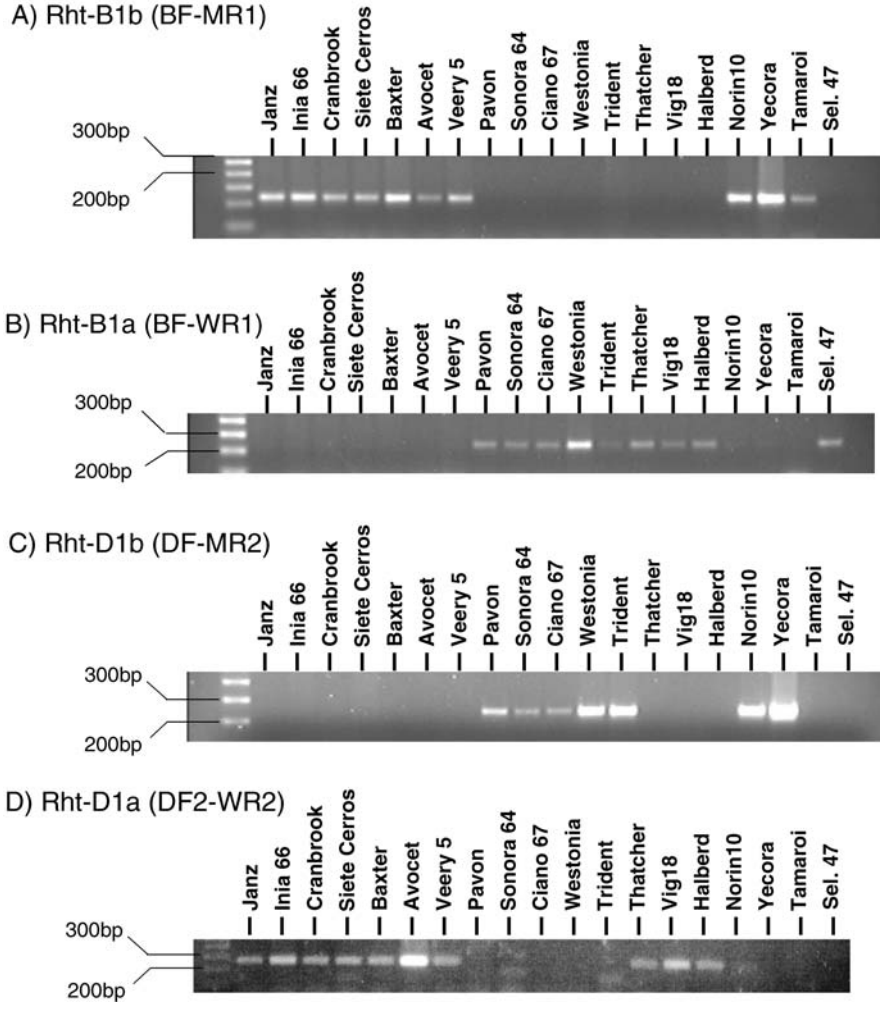

Fig. 2A–D PCR analysis of selected wheat varieties. PCR products were separated on 2% agarose gels after amplification with the following primer sets: **A**) BF-MR1, **B**) BF-WR1, **C**) DF-MR2 and **D**) DF2-WR2. The expected product sizes are 237 bp for BF-MR1 and BF-WR1, 254 bp for DF-MR2, and 264 bp for DF2-WR2

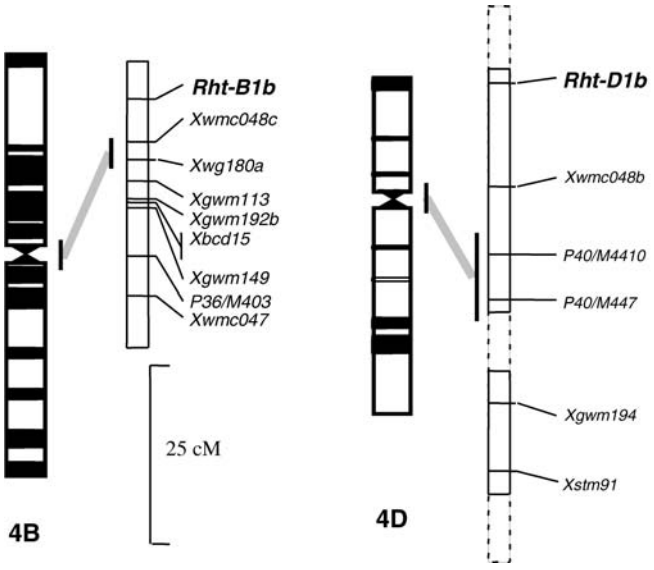

Fig. 3 Map location of the *Rht-B1b* and *Rht-D1b* markers. The genetic map has been aligned to the C-banded karyotype using published literature (Kammholtz et al. 2001), and a scale of genetic distance (cM = centimorgans) is provided

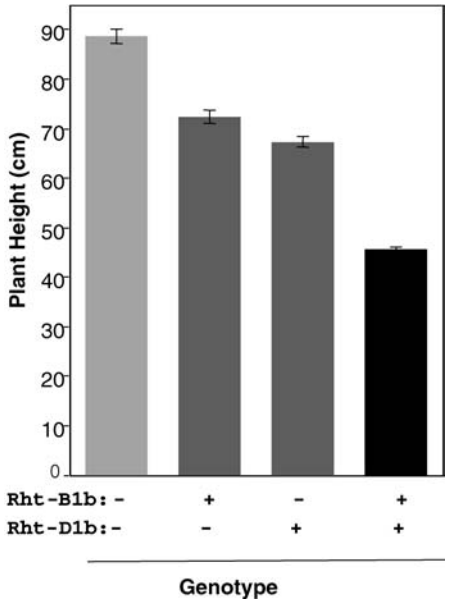

Fig. 4 Height distribution the Sunco × Tasman DH lines. The heights were averaged on the basis of the PCR screening results for individual lines: +, product amplification from the markers; –, no amplification. Error bars: SEM

Table 2 Summary statistics and genetic estimates for *Rht-B1b* and *Rht-D1b* markers and their association with plant height

Chromosome	Marker	Likelihood ratio statistic	% Height phenotypic variance	Additive genetic effect (cm)
4Bs	Rht-B1b	44**	23	–8.4
Ds	Rht-D1b	70**	44	–11.3

Genetic mapping of the *Rht-B1b* and *Rht-D1b* markers

The *Rht-B1b* and *Rht-D1b* markers were scored in a family of 157 DH lines derived from a cross of Sunco (*Rht-B1b*) and Tasman (*Rht-D1b*). As a control for the quality of the template DNA, lines which scored negative for both primers were re-screened with the marker for the tall allele *Rht-B1a*. All lines scored positive for either the tall or the semi-dwarf allele. The genotypes were incorporated in an existing genetic map (Kammholz et al. 2001). The markers were located to homoeologous regions of chromosomes 4B and 4D, respectively (Fig. 3) which were consistent with previously determined map positions for *Rht-B1b* and *Rht-D1b* in wheat (Konzak 1987; Börner et al. 1997).

The four genotypes determined by the PCR markers were strongly correlated with plant height measured in the field (Fig. 4). DH lines classified as "tall" by PCR analysis (negative for both markers) were on average 88.7 cm tall (n = 41, SEM = 1.5 cm). The *Rht-B1b* lines averaged 72.4 cm (n = 34, SEM = 1.3 cm), and the *Rht-D1b* lines 67.3 cm (n = 53, SEM = 1.1 cm). Lines containing both mutations only grew to an average height of 45.5 cm (n = 43, SEM = 0.6 cm).

QTL analysis revealed a highly significant association with height of both markers (Table 2), together accounting for 67% of the phenotypic variance in height. No other significant height QTL was observed in this population (data not shown). Of the two dwarfing genes, the *Rht-B1b* mutation had a smaller effect, reducing height by an average of 8.4 cm per allele. The *Rht-D1b* allele accounted for a larger proportion of the phenotypic variance and had a larger effect on height (a reduction of 11.3 cm, Table 2).

Discussion

We developed PCR markers specific for the *Rht-B1b* and *Rht-D1b* semi-dwarfing genes in wheat. These markers can be described as "perfect markers", in the sense that they are specific for the base pair change responsible for the semi-dwarf phenotype.

Here, we present evidence that validates the specificity of these markers. First, a range of selected wheat varieties of known *Rht* status was correctly genotyped by these markers.

Secondly, the polymorphisms generated by these markers mapped to the expected chromosomal location of *Rht-B1b* and *Rht-D1b*. Thirdly, the markers were strongly correlated with reduced plant height in a family of DH lines segregating for *Rht-B1b* and *Rht-D1b*.

Unlike the test for GA responsiveness, the PCR markers are specific for each dwarfing gene and hence can discriminate between *Rht-B1b* and *Rht-D1b*. These markers will facilitate the monitoring of *Rht-B1b* and *Rht-D1b* in segregating populations and assist in selecting parental varieties with identical *Rht* genotype to avoid height segregation in later generations.

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