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New CAPS Marker for Selection of a Barley Powdery Mildew Resistance Gene in the *Mla* Locus

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In PI466495, a powdery mildew resistance source of wild barley (*Hordeum vulgare* ssp. *spontaneum*), one gene conferring powdery mildew resistance was identified in the *Mla* locus. In this paper, the *RGH1a* gene sequence was used as source for the development of a cleaved amplified polymorphic sequence (CAPS) marker. Co-segregation between this marker and powdery mildew resistance was analysed by specific DNA fragments associated with each allele of the gene using 286 F_2 plants derived from a cross between winter barley (*H. vulgare* L.) variety 'Tiffany' and PI466495. For the co-dominant marker *RGH1aI1a,* three fragments, 370 bp, 82 bp and 59 bp in size, were amplified from F_2 plants exhibiting resistance reaction types 0 and 0–1 to powdery mildew; whereas two fragments, 429 bp and 82 bp in size, were amplified in susceptible plants. Simple procedures based on polymerase chain reaction and restriction enzyme digestion allowed for identifying the plants susceptible to powdery mildew (*Blumeria graminis* f. sp. *hordei*) and plants homozygous or heterozygous for the resistance allele. The *RGH1aI1a* marker was positioned 0.85 cM to the resistance gene and the efficiency of marker-assisted selection (MAS), evaluated as the probability of crossing-over between the marker and the targeted gene, was 99%. The CAPS marker *RGH1aI1a* is a valuable candidate for MAS and gene transfer into barley varieties susceptible to powdery mildew.

Keywords: *Blumeria graminis* f. sp. *hordei,* DNA markers, *Hordeum vulgare* ssp. *spontaneum,* resistance gene homolog

Introduction

Environmentally insensitive genetic markers permit following the inheritance of important agronomic traits. In particular, DNA markers have the potential to enhance the effi-

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ciency of plant breeding (Peleman and van der Voort 2003). Marker-assisted selection (MAS) includes the construction of a genetic map for a crop, the high resolution mapping of a gene conferring an important agronomic trait, and the identification of tightly linked markers that co-segregate with the trait.

Blumeria graminis DC. f. sp. *hordei* Ém. Marchal is the obligate biotrophic fungus that causes powdery mildew in barley (*Hordeum vulgare* L.). Simple sequence repeat (SSR) markers turned out to be very useful for rough mapping. In barley, Varshney et al. (2007) constructed an SSR consensus map by joining six individual maps. In this map all chromosomes are saturated with a sufficient number of microsatellites. Nevertheless, there have been only a few reports of DNA markers being successfully used to select for powdery mildew resistance genes in barley. The *Mla*-resistance cluster on barley chromosome 1H was saturated with molecular markers for high-resolution mapping (Schwarz et al. 1999; Wei et al. 1999). This enabled to identify a physical contig of YAC and BAC clones spanning the *Mla* cluster and, in addition, three resistance gene homologue (RGH) families with the NBS-LRR motif were revealed (Wei et al. 1999). Three tightly linked AFLP markers were identified for the *Mla* region and one sequence-tagged site (STS) marker *(MWG2197.2)* was mapped distal to the *Mla* locus by Schwarz et al. (1999). Previously, three closely linked genomic RFLP clones were located around this locus at a distance of 0.7 to 5.1 cM (Schüller et al. 1992). However, for barley breeders, RFLP, AFLP and STS markers are difficult to use in routine selection of barley genotypes resistant to powdery mildew.

In the cross 'Tiffany' × PI466495, we found a resistance gene associated with *Bmac0213* SSR marker on chromosome 1H (Řepková et al. 2006), which corresponds to the *Mla* gene. In this paper, we describe the development of a tightly linked cleaved amplified polymorphic sequence (CAPS) marker of the *Mla* resistance gene in the wild barley PI466495. Efficiency of the marker for marker-assisted selection is tested by marker – resistance co-segregation.

Materials and Methods

Plant material

Wild barley accession with resistance to powdery mildew (Dreiseitl and Bockelman 2003; Dreiseitl and Dinoor 2004) and the two-row winter barley variety 'Tiffany', possessing powdery mildew resistance genes *Mla7* and *MlaMu2* (Dreiseitl 2007) were crossed (Řepková et al. 2006) and the F_2 population was established.

Resistance tests

A virulent *(Va7, VaMu2)* pathotype 0323 of the pathogen was used for the resistance tests described by Řepková et al. (2006). Reaction types (RTs) of individual F_2 plants were scored on the 0–4 scale (Torp et al. 1978). Reaction types 2–3 and lower were considered resistant.

CAPS analysis and molecular procedures

Genomic DNA extraction from leaves of parental and 286 F_2 plants was done using the Gene Elute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich Co.). The CAPS marker was developed from the *RGH1a* gene sequence (*R*-gene homolog; accession number AF427791, http://www.ncbi.nlm.nih.-gov/) from chromosome 1H. A pair of primers for the first intron was designed by the PRIMER3 program: 5'-GCCTCATCTGACC-GTTGATT-3' and 5'-GCATCACTGCCATCTCTATGC-3' (Fig. 1). PCR analysis was performed in a volume of 10 µl with 1x reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pmol of each primer, 0.5 units of *Taq* polymerase and 100 ng of barley genomic template DNA. The following program was designed for the amplification of the 490 bp long DNA fragment: one cycle for 3 min at 94 ºC; 35 cycles for 1 min at 94 ºC, 1 min at 60 ºC, 1 min at 72 ºC, and a final extension of 5 min at 72 ºC for amplification. To identify polymorphic sites, the restriction enzymes *Afl* III, *Alu* I, *Bsa*J I, *Dde* I, *Dra* I, *Hpa* II, *Mse* I, *Nla* III, *Pst* I, *ScrF* I, *Taq* I and *Xba* were used to digest parental DNAs. For CAPS analysis, 5 µl of the amplification products were digested for 3 h in a volume of 10 µl with 1x restriction enzyme buffer and 5 units of restriction enzyme. The resulting fragments were subsequently detected on 3% agarose gels.

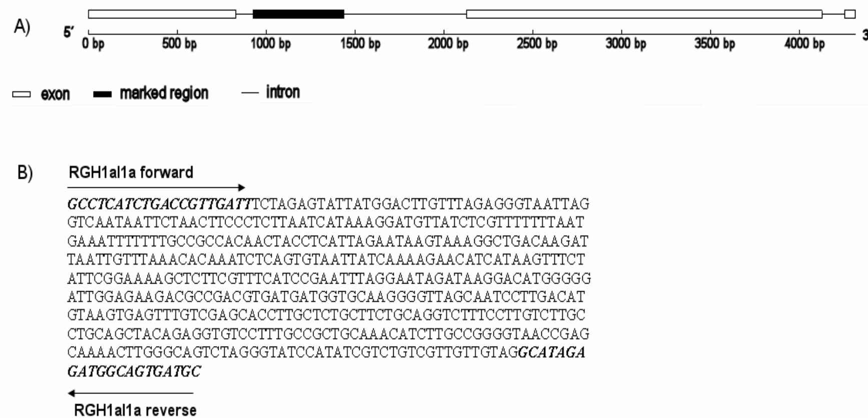

Figure 1. Region (black box) of the first intron of the *RGH1a* gene used for *RGH1aI1a* marker development (A) and nucleotide sequence of the *RGH1a* gene used for primer design and amplification (B)

Linkage analysis

For the CAPS marker, linkage was evaluated by recombination frequency (*r* %) between the gene of interest and the DNA marker (number of recombinant chromosomes / total number of tested chromosomes). The molecular typing was restricted to only recessive F_2 segregants *rr,* that is susceptible plants, because of their informative character. The estimate of map distance (*D*, cM) was given by the Kosambi's mapping function $D = 25\ln(100 + 2r/100 - 2r)$ with standard error s_D (Kosambi 1944).

Co-segregation of DNA marker and powdery mildew resistance

Phenotypic values for reaction types (RTs) of leaf segments of individual F_2 plants scored on a 0–4 scale were compared with a particular fragment amplified for the tightly linked CAPS marker.

Results

CAPS analyses and molecular procedures

The marker named *RGH1aI1a* (*I1* – intron1, *a* – particular sequences used for amplification are given in Fig. 1) amplified a 511 bp fragment in both ‘Tiffany’ and PI466495. Polymorphism between parents was revealed after *Dra* I and *Hpa* II digestion. Only *Hpa* II was used for CAPS analysis. DNA fragments of 429 bp and 82 bp were found in ‘Tiffany’ and 370 bp, 82 bp and 59 bp in PI466495. In heterozygotes all fragments were detected (Fig. 2). Only the longest fragments (429 bp and 370 bp), determined after digestion of the particular PCR product, were used for subsequent linkage and co-segregation analysis. *Hpa* II cleavage of a small fraction of 511 bp fragments in susceptible plants is blocked by CpG methylation. Those fragments were excluded from analyses.

Linkage analysis

The frequency of recombination and map distance were evaluated directly from susceptible plants and their molecular typing. One recombinant was identified among 59 susceptible plants of RT3 to RT4 (118 chromosomes tested). The frequency of recombination between the resistance gene and *RGH1aI1a* was 0.85±0.03% and the map distance was 0.85±0.03 cM.

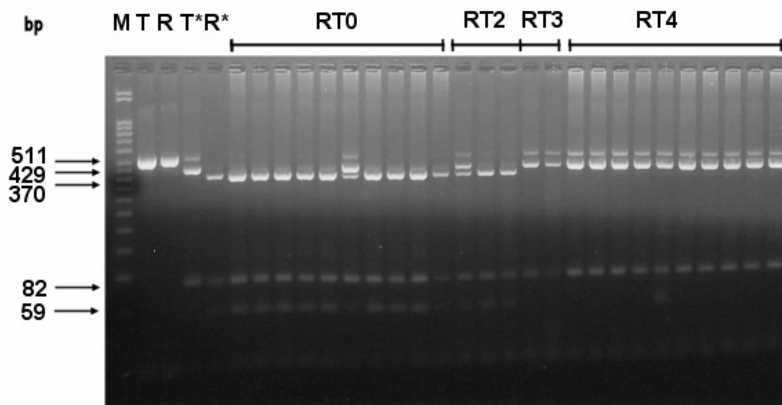

Figure 2. Testing for polymorphism and co-segregation analysis for CAPS marker *RGH1aI1a:* Size marker 0.5 kb DNA ladder (M), DNA fragments (bp) obtained after polymerase chain reactions (T, R) and after digestion with *Hpa* II on ‘Tiffany’ (T*) and PI466495 (R*); Reaction types (RT) of individual F_2 plants from the cross ‘Tiffany’ × *Hordeum vulgare* ssp. *spontaneum* PI466495: RT0 and RT2 – resistant plants, RT3 and RT4 – susceptible plants

Table 1. Evaluation of co-segregation of resistance, conferred by a powdery mildew resistance gene originating from wild barley (*Hordeum vulgare* ssp. *spontaneum*) accession PI466495, using *RGH1aI1a* marker, in individual F_2 plants

RT[a]	No. of plants	Genotypes[b]		
		RR	*Rr*	*rr*
0	27	22	5	0
0–1	39	30	8	1
1	53	16	37	0
1–2	59	1	56	2
2	40	2	36	2
2–3	9	0	9	0
3	3	0	0	3
3–4	1	0	0	1
4	55	0	1	54

[a] reaction type, determined as phenotypic manifestation of powdery mildew resistance after inoculation of tested plants with virulent pathotype 0323
[b] genotypic constitution of plants determined using *RGH1aI1a*

Co-segregation of DNA marker and powdery mildew resistance

In 52 F_2 plants scored with resistance reaction types 0 and 0–1 to the pathotype 0323 of the pathogen, 370 bp and 120 bp fragments were amplified from the co-dominant marker *RGH1aI1a* (Table 1). Besides, 13 plants of the same RTs were heterozygous and both alleles of the resistance gene were detected. Fragments in the heterozygous state were amplified largely in resistant plants with RT1 to RT2-3 (138 plants). In susceptible plants with RT3 to RT4, 429 bp fragments were amplified (58 plants), with one exception in which one crossing-over occurred between the resistance gene and the *RGH1aI1a* marker (Table 1). This DNA marker is useful for selecting against plants with the susceptible allele of the gene (Fig. 2). The efficiency of MAS was 99.2% and was evaluated as the probability of a recombination event between the marker and the target gene among 118 chromosomes tested.

Discussion

The number of DNA markers tightly linked with known powdery mildew resistance genes in barley is slowly growing and their utilization for marker-assisted selection in barley breeding is extremely valuable. The drawback of breeding barley for resistance to powdery mildew is the frequent occurrence of virulent mutations of the pathogen, which can result in the overcoming of race-specific resistance genes in new varieties within a few years. Considering the limited number of new resistance genes in cultivated barley, related potential sources have been screened for effective resistance genes to powdery mildew. There appears to be a large pool of resistance genes in the progenitor of cultivated barley *H. vulgare* ssp. *spontaneum* (Jahoor and Fischbeck 1987; Dreiseitl and Bockelman 2003; Řepková et al. 2006). As a starting point, we performed genetic analyses of selected powdery mildew resistance accessions containing alleles of the *Mla* locus and several addi-

tional genes (Dreiseitl et al. 2007). The number of resistance genes was determined and confirmed. Understanding the genetic basis of this important agronomic character will be beneficial for further research. Molecular mapping of the cross derived from PI466495 showed that the resistance gene coincided with the known *Mla* locus and differed from the *Mla7* gene (Řepková et al. 2006). SSRs turned out to be very useful for rough mapping; however, they did not exhibit sufficiently tight linkage. Generally, it is difficult to choose such markers in barley, in spite of more than 700 SSRs known for its genome, because the markers were, until recently, located in several linkage maps created using different mapping populations. Consensus map of barley (Varshney et al. 2007) offers a better saturation of the chromosomal regions of interest. Applicability of markers and their polymorphism in diverse populations remains to be proven.

AFLP or STS markers developed from coding sequences, were found to be the most useful for high resolution mapping. Comparative genomics and knowledge of conservative gene motifs also offers a solution for cloning putative resistance genes of a variety of plant species and locating them on a genetic map. Only some of these genes have been functionally active, but they represent a considerable resource as molecular markers for disease resistance genes in barley and other cereals (Madsen et al. 2003). *RGH* genes were located on barley chromosomes by Wei et al. (1999), Collins et al. (2001), Madsen et al. (2003) and Mammadov et al. (2006).

The new co-dominant CAPS marker *RGH1aI1a,* developed from the known *RGH1a* gene sequence, simplified our research focused on identification of a tightly linked marker and proved to be diagnostic for a gene that is a part of the *Mla* locus. The expected sizes of marker fragments, associated with each allele of the gene, were present in resistant or susceptible plants. Thus, the marker made elimination of susceptible plants with RT3 and RT4 reliable, even though marker co-segregation with the susceptible allele was not absolute. In addition, one or two distinct amplified fragments of the marker could reveal the homozygous or heterozygous state of resistant plants. Precise selection of true homozygous plants for particular gene, secures genetically identical progenies. The marker, used in this study as diagnostic, was successful in identifying resistant plants with RTs0 and 0–1, where only a few heterozygotes were present. This is in contrast to plants with RTs1 up to 2–3, where heterozygotes were in the majority. This result indicates the necessity of plant genotyping over two successive generations to secure selection of plants homozygous for the powdery mildew resistance allele. In other words, a method is now available which allows assessing the allelic variation at this important gene locus.

Tightness of linkage is directly correlated with the efficiency of selection. High effectiveness of MAS is necessary for gene pyramiding, which is the prospective method to combine favourable alleles of several loci to develop superior varieties. The marker *RGH1aI1a* is a valuable candidate for powdery mildew resistance gene pyramiding, which may ensure durability of resistance in barley.

Acknowledgements

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