Theor Appl Genet (2008) 117:721–728
DOI 10.1007/s00122-008-0813-4

ORIGINAL PAPER

The use of high resolution melting (HRM) to map single nucleotide polymorphism markers linked to a covered smut resistance gene in barley

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Received: 2 January 2008 / Accepted: 22 May 2008 / Published online: 13 June 2008

Abstract Using an established genetic map, a single gene conditioning covered smut resistance, *Ruh.7H*, was mapped to the telomere region of chromosome 7HS in an Alexis/Sloop doubled haploid barley population. The closest marker to *Ruh.7H*, abg704 was 7.5 cM away. Thirteen loci on the distal end of 7HS with potential to contain single nucleotide polymorphisms (SNPs) were identified by applying a comparative genomics approach using rice sequence data. Of these, one locus produced polymorphic co-dominant bands of different size while two further loci contained SNPs that were identified using the recently developed high resolution melting (HRM) technique. Two of these markers flanked *Ruh.7H* with the proximal marker located 3.8 cM and the distal marker 2.7 cM away. This is the first report on the application of the HRM technique to SNP detection and to rapid scoring of known cleaved amplified polymorphic sequence (CAPS) markers in plants. This simple, precise post-PCR technique should find widespread use in the fine-mapping of genetic regions of interest in complex cereal and other plant genomes.

Communicated by F. Ordon.

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Introduction

High resolution melting (HRM) is a post polymerase chain reaction (PCR) technique which can be used for high throughput mutation scanning and genotyping (Gundry et al. 2003). The technique involves a standard PCR reaction and the use of a double stranded DNA binding dye. When melted, each PCR product will exhibit a characteristic melting or disassociation behavior (Montgomery et al. 2007). Specialized HRM instruments can plot the change in fluorescence that occurs when double stranded DNA amplicons melt to form single stranded DNA. Differences between homozygous samples can be distinguished by a simple shift in the melting temperature (T_m), whereas heterozygous samples can be distinguished by changes in the shape of the melt curve (Gundry et al. 2003; Wittwer et al. 2003; Reed and Wittwer 2004). Differences in melt curves arise from variations in amplicon sequences, lengths, and GC content, provided that the salt, buffer conditions and the volume of each sample remain constant (White and Potts 2006; Montgomery et al. 2007). A number of methods have been used to assay single nucleotide polymorphism (SNP) markers, including pyrosequencing (Huang and Röder 2005), digestion with restriction enzymes (Bulgarelli et al. 2004), denaturing high-performance liquid chromatography (Kota et al. 2001) and the use of allele-specific PCR markers (Bundock et al. 2006). For these methods, knowledge of the sequence and/or position of the SNP is required. In contrast the HRM technique can be performed in the absence of the SNP sequence information and does not require the separation of products by gel electrophoresis. HRM of PCR products has been employed in biomedical research for a number of years to detect mutations and distinguish single-nucleotide polymorphisms (SNPs) (Liew et al. 2004; Herrmann et al. 2007; Krypuy

et al. 2007), however, this is the first report of its application to mapping in plant genomes.

Covered smut is a seed borne disease of barley (*Hordeum vulgare* L.) caused by the fungus *Ustilago hordei* (Pers.) Lagerh. It is recognized by the appearance within smutted spikelets of masses of spores enclosed in a semi-persistent membrane that ruptures during harvesting (Langdon et al. 1976). The covered smut pathogen survives as teliospores (resting spores) on seed or in infested soil. Infection occurs as the seedlings emerge from the sprouting seed. The fungus enters the young seedling and grows intercellularly through the plant into the head (Willits and Sherwood 1999) to infect the developing florets.

This disease can cause serious problems for growers. Growers with infected grain face both yield loss and marketing problems since grain contaminated with covered smut is unacceptable for malting. Fortunately this disease can be effectively controlled by fungicidal seed treatments (Wildermuth 1988) and resistant lines are available. Nevertheless, molecular marker-assisted selection for covered smut is desirable, as screening for this disease is space-, labour-, and time- consuming with affected plants usually not showing symptoms until ear emergence (Willits and Sherwood 1999; Grewal et al. 2008).

Although a large number of mapped microsatellite-based markers are available in the public domain (Graingenes; http://wheat.pw.usda.gov/GG2/index. shtml), to date characterised microsatellites are still sparsely distributed in some genomic regions, including the telomeric region of chromosome 7HS investigated in this study. Over 1,000 expressed sequence tag (EST) markers have been mapped in barley (Stein et al. 2007) and 478,682 barley EST sequences are currently (May 2008) available from public websites (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). The increasing availability of EST sequences provides a valuable source of new DNA markers, primarily based on SNPs. In this study we report on the successful application of HRM to the identification and mapping of SNPs in a population derived from the barley cross Alexis/Sloop in order to obtain EST markers closely linked to the covered smut resistance gene, *Ruh.7H*.

Materials and methods

Plant material

The doubled haploid (DH) barley population, Alexis/Sloop, consists of 111 lines and was developed by the Australian National Barley Molecular Marker Program (NBMMP) (Barr et al. 2003; D. B. Moody et al. unpublished data; Lehmensiek et al. 2007). Sloop is resistant to covered smut while Alexis is susceptible. Sloop was bred by R. C. M. Lance, D. H. B. Sparrow, and A. R. Barr in South Australia. It was reselected in F_6 from the F_2-derived breeders line WI2785 and was previously known as WI2875–22 (Barr et al. 2003). The German malting variety Alexis was bred by Saatzucht Joseph Breun GDBR from the cross Br1622d54/Trumpf (Friedt et al. 2000). Three seeds of each barley line of the Alexis/Sloop population were germinated in a tissue culture rack in a 24°C incubator. Leaf material (about 50 mg) was taken from one week old seedlings and extracted using the Wizard® Genomic DNA Purification Kit (Promega Corporation). The DNA was quantified using an Implen Nanophotometer™.

Covered smut screening and analysis of data

Individual barley lines were planted in 3-m single row plots (ten plots per row) with a row spacing of 50-cM. Each line was artificially inoculated using the spore suspension method (Tapke and Bever 1942). Spores were obtained from the previous year's smutted heads. Unpublished testing indicated that this is a Race 5 isolate as per Tapke (1945). Fungus-inoculated seeds of 81 lines of the Alexis/Sloop population were planted in the field at Wellcamp, QLD and screened for covered smut in 2001 (three replicates). Due to space constraints only 50 of the same 81 lines were randomly selected and planted at the same location in 2002 (one replicate). A further 20 lines of this population, which had not been rated in previous years, were screened in 2003 (one replicate) and again in 2004 (two replicates). After harvest, plants were rated for percentage incidence of plants with smutted heads per experimental plot.

To convert the covered smut ratings (% incidence of smutted heads) data to a classification of resistant or susceptible, the following procedure was followed. The standard error of the covered smut rating for the resistant parent Sloop was calculated and converted into a one-sided 95% confidence interval. Lines which had scores within this confidence interval for each trial in which they were rated were designated as resistant and all remaining lines were scored as susceptible.

The Chi square test for goodness-of-fit was used to test for deviations of observed and expected segregation ratios.

Linkage map

A linkage map had previously been constructed using restriction fragment length polymorphism (RFLP), simple sequence repeat (SSR), and amplified fragment length polymorphism (AFLP) markers (Barr et al. 2003; Willsmore et al. 2006). Maps were curated using the methods discussed in Lehmensiek et al. (2005). Three new EST markers and *Ruh.7H* were added to the existing Alexis/Sloop

Table 1 Primer sequences of markers tested for linkage to covered smut resistance on chromosome 7HS. Sequences for primers 7–12 were obtained from http://earth.lab.nig.ac.jp/~dclust/cgi-bin/barley_map_pub/index.html and primer sequences for marker 13 were obtained from Bulgarelli et al. (2004). Expected and observed fragment sizes for the markers are listed

Marker number	GenBank accession number	Expected fragment size (bp)	Observed fragment size (bp)	Primer sequence
1	CK123008	191	191	5′-AGGGGAGTGGTTCTGTTGTG-
				5′-CAGTGGCACTTCTACCAGCA-
2	CA003755	137	137	5′-CAAGCCAATCGAGAGTCACA-
				5′-GATCTTGGTCTTGGCCTTCA-
3	AL506064	290		5′-CTTGTTAGTGGGCGAACCAT-
				5′-CATAATAGGGCAGGCAAGGA-
4	CA030150	197	197	5′-AGCCCGAGCAAATTGTCTAA-
				5′-ATGGTCGATTTGGATGGGTA-
5	BQ471402	243		5′-TTAGGCCTTGGGTTGATGTC-
				5′-GTAGCAGAAGCTGCCGAAGT-
6	BQ468427	220		5′-TCTGAACTGCAATGGTGAGC-
				5′-TAGCCCCGTATCCTTTGTTG-
7	AV836787	172	150, 172	5′-TAATGGCAGGACCTCTCCAC-
				5′-CGGTAAGACAGAGCCGCTAC-
8	AV911272	209	209	5′-CAGATTCAGGGACCAAGGAA-
				5′-GTGGACGCGTTTGACTACAC-
9	AV834214	180	300	5′-GTCGGTGATGGCCTGTATCT-
				5′-TTCTCGACGTATCCCAGGAC-
10	AV913712	181		5′-CCAGGACGATGCTCTCTAGG-
				5′-CCTTTTATGGCAGGAACGAA-
11	AV913519	205		5′-TAATGCACAGCCCAACTCTG-
				5′-CTGCCAAACTTTCCACCAAT-
12	AV938293	223	340	5′-CAGCAGCAGCATCAATCAAT-
				5′-AACCATCCGAGACAAACCTG-
13	BV078160	90	90	5′-CCAAGCTAGAAGGAAACCTTCCACTCT-
				5′-GCATGATGACGACACGTGGCTT-

map using the ‘links report’ function in MapManager version QTXb20 (Manly et al. 2001) to find the best location for these markers.

Development of EST markers and primers

Expressed sequence tag (EST) markers were added to the region containing the covered smut gene. The RFLP marker psr160 on barley chromosome 7HS and on rice chromosome 6 was used as the common marker to align a barley consensus map with a rice map in Gramene (http://www.gramene.org). The physical map of the rice chromosome was then obtained from the international rice genome sequencing project (IGRSP; http://rgp.dna.affrc. go.jp/E/IRGSP/download.html) and individual rice clones identified (IGRSP; http://rgp.dna.affrc.go.jp/cgi-bin/statusdb/status.pl). Sequences of these clones were downloaded and used in BLAST searches to retrieve orthologous barley (EST) sequences from the Gramene or TIGR Genome Annotation Database (TIGR; http://www.tigr.org/tdb/e2k1/osa1). Primers were designed using Primer3 version 0.4.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (markers 1–6; Table 1). Primers were designed in such a way that the PCR products were less than 400 base pairs (bps) in size (Table 1). Primer sequences for six additional barley ESTs were obtained from the barley EST project http://earth.lab.nig.ac.jp/~dclust/cgi-bin/barley_map_pub/index.html (markers 7–12; Table 1). Primer sequences for a derived cleaved amplified polymorphic sequence (dCAPS) marker were obtained from Bulgarelli et al. (2004) who had previously developed this marker for fine mapping of the chromosome 7HS region (marker 13; Table 1). To make sure that EST markers produce single fragments they were amplified on the parental DNA using the standard PCR protocol described below and visualized using a Gel-Scan 2000™ (Corbett Life Sciences, Sydney, Australia).

EST amplification and HRM analysis

EST amplification and HRM analysis was performed with a Rotor-Gene™ 6000 (Corbett Life Science, Sydney, Australia). A standard PCR protocol was used to amplify the ESTs. The reaction mixture consisted of 30 ng DNA, 5 µM of each primer, 100 µM of each dNTPs, 1.5 mM $MgCl_2$, 1× of PCR buffer (Bioline Pty Ltd., Australia), 0.25 U Immolase™ DNA polymerase (Bioline Pty Ltd., Australia) and 0.75 µl (18.75 µM) SYTO® 9 (Invitrogen Pty Ltd., Australia) in a total volume of 20 µl. A negative control containing all reagents minus DNA was included in each run. The following PCR cycle profile was used: 7 min at 95°C, followed by 40 cycles of 15 s at 95°C, 20 s at 55–60°C (depending on annealing temperature) and 20 s at 72°C. The melt analysis was performed once amplification was completed by ramping the temperature from 75°C to 95°C, rising by 0.1° each step with continuous acquisition of fluorescence. The automated genotype calling software (Corbett Life Science, version 1.7) was used to determine the genotypes of individual lines. For the HRM analysis the fluorescence versus temperature graphs were normalized to 100 to allow all the curves to be compared, thus having the same starting and ending fluorescent signal level. The raw data graph was used to adjust the regions of normalization. This was done according to the protocol provided by the supplier (Corbett Life Science).

Results

Phenotypic data

To classify DH lines as resistant or susceptible the standard error of the covered smut ratings for the resistant parent Sloop were converted into a one-sided 95% confidence interval for each trial. Lines which had scores within this confidence interval (Table 2) across both years were designated as resistant. Only four lines were classified as resistant in one trial but susceptible in the other. Of the 31 lines tested only in 2001, 12 were rated as resistant and all three field replicates fell within the confidence interval. Based on this classification, lines of the Alexis/Sloop population segregated in a 1:1 ratio ($\chi^2 = 0.16$; $P = 0.69$) of susceptible to resistant lines, indicating a single major gene. Covered smut scores ranged between 0 and 76.6%, with a mean of 4.1 to 18.6 across different years (Table 2).

Mapping of covered smut gene

The covered smut resistance gene was designated *Ruh.7H* and was mapped 7.5 cM distal to RFLP marker abg704 (Fig. 1a). As abg704 is not a PCR-based marker and no flanking marker was available for the distal side of the gene, barley consensus maps were examined for potential markers closer to the gene. Microsatellites or other PCR-based markers known to map to this region and polymor-

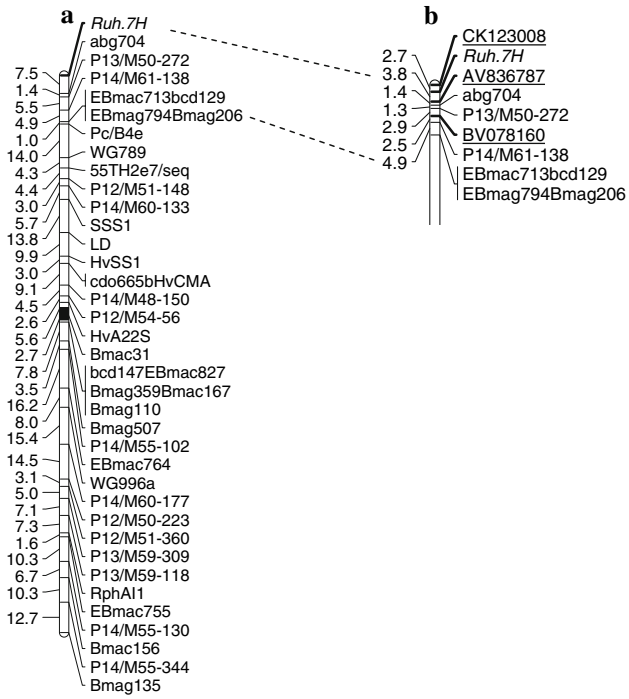

Fig. 1 Location of the covered smut gene, *Ruh.7H* on chromosome 7H of the barley cross Alexis/Sloop. Map distances (cM) are given on the *left*; **a** indicates the original map without EST markers (the centromere region is indicated in *black*); the region containing the *Ruh.7H* gene has been enlarged in **b** and the EST markers have been underlined

Table 2 Descriptive statistics of the Alexis/Sloop covered smut trials indicating the number of DH lines screened (N), the minimum (min) and maximum (max) covered smut percentages, the mean and standard error (SE) for each trial and the mean parental line scores (% infected heads) for each year. N (Sloop) is the number of Sloop plots used to calculate the one-sided 95% confidence interval (CI) for the resistant parent

Year	N	Min	Max	Mean	SE	Alexis	Sloop	N (Sloop)	CI (%)
2001	81	0.0	70.6	18.6	2.3	52.4	4.4	3	0, 10.0
2002	50	0.0	76.6	16.6	2.8	36.2	1.8	5	0, 2.6
2003	23	0.0	27.7	4.1	1.4	20.0	0.9	2	0, 2.3
2004	20	0.0	48.6	9.4	3.1	25.9	1.9	4	0, 3.6

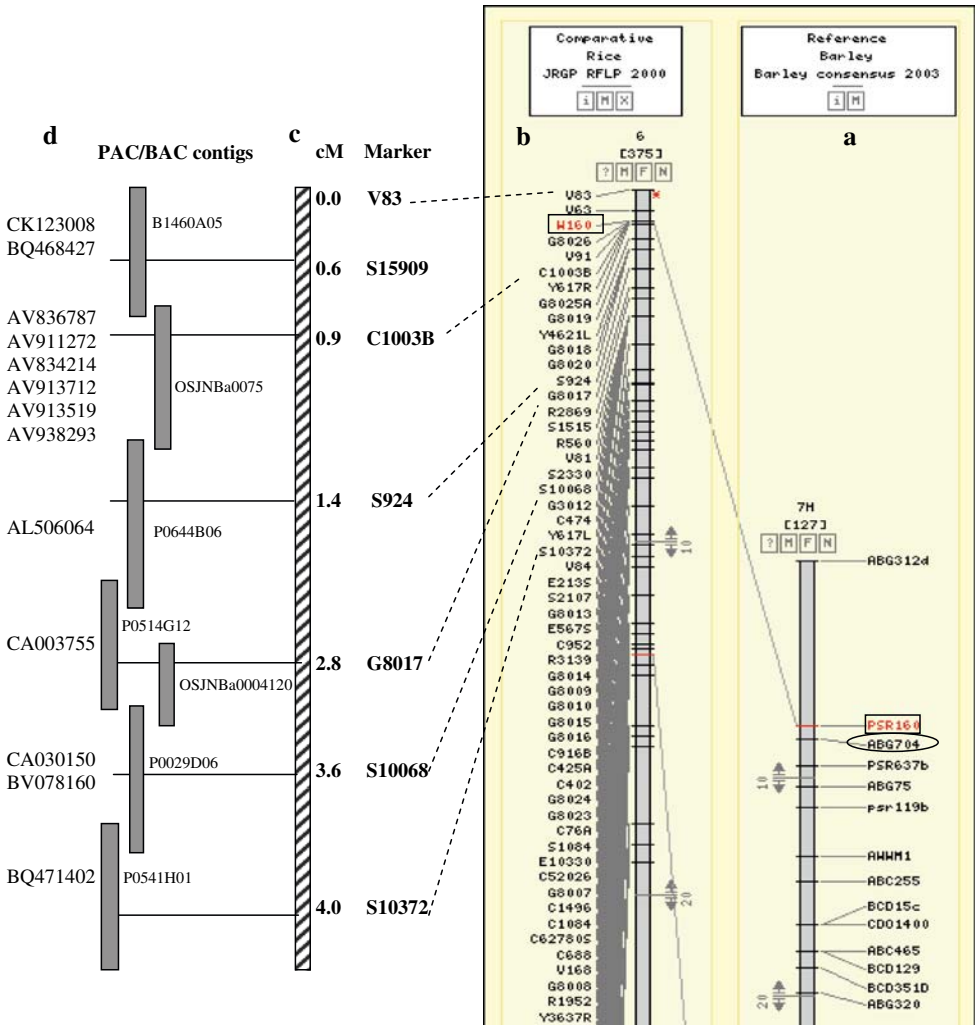

Fig. 2 Alignment of the 7H barley consensus 2003 map (**a** Gramene: http://www.gramene.org) with the rice JRGP RFLP 2000 chromosome 6 map (**b** Gramene). The RFLP marker abg704 in the region of *Ruh.7H* is circled. Marker psr160 was used to align the two maps (marker w130 is orthologous to psr160). Markers located in a 10 cM region on the rice map were aligned to the rice physical map downloaded from IGRSP (**c** http://rgp.dna.affrc.go.jp/E/IRGSP/ download.html). **d** Orthologous barley ESTs used in this study are listed and their expected locations on the rice PAC/BAC contigs indicated

phic in this population could not be found. The colinearity of the barley and rice genomes was therefore investigated to identify potential EST markers from this region.

Identification and linkage analysis of EST/SNP markers

The RFLP marker abg704 was used to identify the location of *Ruh.7H* on a barley consensus map constructed in 2003 (obtained from Gramene; Fig. 2a). The psr160 marker which is closely linked to abg704 (about 1 cM distal to abg704) was in turn used to align this region with a region in rice using the rice JRGP RFLP 2000 chromosome 6 map (obtained from Gramene; Fig. 2b). Markers located in a 10 cM region on the rice map (including markers V83, C1003B, S924, G8017, S10068, and S10372) were then used to align this map to the rice physical map downloaded from IGRSP (http://rgp.dna.affrc.go.jp/E/IRGSP/ download.html (Fig. 2c). Six rice EST markers located on PAC/BAC contigs B1460A05, P0644B06, P0514G12, P0029D06, and P0541H01 were analysed in BLAST (http://www.gramene.org and http://www.tigr.org/tdb/e2k1/osa1) to obtain orthologous barley EST sequences (Fig. 2d). Sequences for a further six barley ESTs located on contig OSJNBa0075 were obtained from the barley EST project (http://earth.lab.nig.ac.jp/~dclust/cgi-bin/barley_map_pub/index.html; Fig. 2d). A primer set used to amplify a dCAPS marker located on PAC contig P0029D06 to produce a fragment (BV078160) of around 90 bps (Bulgarelli et al. 2004) was also investigated (Fig. 2d; Table 1).

Seven (CK123008, CA003755, CA030150, AV911272, AV834214, AV938293, and BV078160) of the 13 markers produced a single fragment of the same size in both parents, while one marker (AV836787) was co-dominant. The five remaining EST markers did not produce any PCR products across a range of annealing temperatures. Most of the amplified markers had the expected size, with the exception of markers AV834214 and AV938293 which were 300 and 340 bps in length, respectively (Table 1).

The seven non-polymorphic markers were amplified with the Rotor-Gene™ 6000 and HRM analysis was subsequently performed. A difference in T_m indicating a SNP was identified in two of the seven ESTs, CK123008, and

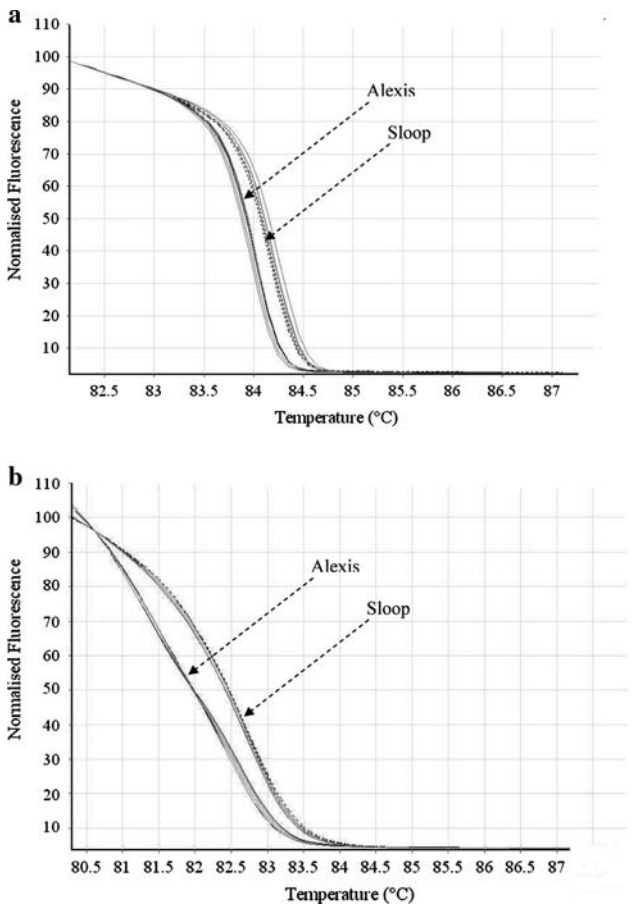

Fig. 3 Normalized HRM curves for marker CK123008 **a** and marker BV078160 **b** two independent samples of parent Alexis (*black solid lines*) and Sloop (*black dashed lines*) and eight progeny (*gray*) are shown

BV078160. The normalized HRM graphs for the parents Alexis (two independent copies) and Sloop (two independent copies) and eight of the progeny are shown in Fig. 3. Alexis and Sloop had a T_m of 84.03°C and 84.22°C, respectively, with marker CK123008 (Fig. 3a) and a T_m of 81.96°C and 82.38°C, respectively, with marker BV078160 (Fig. 3b). Three EST markers, co-dominant marker AV836787 and the SNP markers CK123008 and BV078160, were mapped on the Alexis/Sloop map. Marker AV836787 mapped between *Ruh.7H* and marker abg704, 1.4 cM distal to marker abg704 and 3.8 cM proximal to *Ruh.7H* (Fig. 1b). CK123008 was located 2.7 cM distal to *Ruh.7H* (Fig. 1b) and marker BV078160 mapped 4.2 cM proximal to abg704.

Discussion

Based on an available map of the Alexis/Sloop DH population (Barr et al. 2003; Willsmore et al. 2006), we have identified a major covered smut gene, *Ruh.7H* near the end of chromosome 7HS. Subsequent targeted mapping has led to the addition of two EST markers distal to abg704 which flank the gene. Only one other published study has mapped a locus for covered smut resistance (Ardiel et al. 2002). Using a bulked segregant analysis approach, a randomly amplified polymorphic DNA (RAPD) marker tightly linked to the covered smut resistance gene, *Ruhq* was identified in the barley line Q21861 (Ardiel et al. 2002). This marker was mapped to chromosome 1HS in a Harrington/TR306 mapping population and together with two more closely linked markers has been validated and applied in marker-assisted introgression (Grewal et al. 2008).

Other disease resistance genes have been mapped to the 7HS region, including the leaf stripe (*Rdg2a)* and stem rust resistance (*Rpg1)* genes, and high resolution mapping of this region has been undertaken (Ayliffe et al. 2000; Brueggeman et al. 2002; Bulgarelli et al. 2004; Brueggeman et al. 2006). *Rdg2a* and *Rpg1* are located proximal to RFLP abg704 (approximately three and 1 cM, respectively).

A net form of net blotch (NFNB) resistance QTL, *QNFNBAPR.A/S-7Ha* contributed by Alexis, has been identified in the Alexis/Sloop cross in the same region as *Ruh.7H* (Lehmensiek et al. 2007). Reanalysis of the NFNB data, after the addition of the two EST markers distal to abg704, has indicated that abg704 is still the closest marker to *QNFNBAPR.A/S-7Ha* (data not shown), suggesting that this locus is located a few cM proximal to *Ruh.7H*. One of the two lines in the Alexis/Sloop population with a cross-over between marker abg704 and AV836787 possesses the resistant marker alleles for both the covered smut and the NFNB resistance genes and may be a useful selection for backcrossing into elite backgrounds.

The dCAPS marker BV078160, mapped by Bulgarelli et al. (2004), was examined in our study. dCAPS markers are initially amplified by standard PCR, after which the product is digested with a restriction enzyme overnight and the fragments compared on an agarose gel. Use of HRM analysis immediately after the PCR step eliminates the need for restriction enzyme digestion and gel electrophoresis, allowing results to be obtained within 2 h of commencing PCR. The BV078160 marker mapped 4.2 cM proximal to marker abg704, a distance which is similar to the estimate of 5.6 cM for this interval indicated by Bulgarelli et al. (2004) using 93 recombinant lines of a Thibaut/Micro population.

Of the 13 putative ESTs investigated on the distal end of 7HS, only one of the eight ESTs that produced PCR products in our study indicated a size-based polymorphism by standard PCR. However, by applying the HRM technique we identified two additional ESTs with sequence-based polymorphisms (SNPs). Five ESTs could not be amplified even though a range of annealing temperatures was tested. The lack of amplification may be due to variations at the

primer binding sites. Bulgarelli et al. (2004) similarily found that only 8 of the 19 putative ESTs, for which they had produced primers, amplified successfully.

In this paper we have illustrated the usefulness of HRM analysis to identify and map SNP markers in barley. This technique clearly has applications for trait mapping across a wide range of crops. HRM analysis is a rapid way of mapping SNPs without sequence knowledge or electrophoresis and is also useful as an alternative method for scoring known CAPS markers. To obtain reproducible results with HRM, several criteria apply. PCR conditions must be optimized to amplify only a single fragment, since the presence of non-specific bands and primer dimers can significantly reduce HRM performance (White and Potts 2006). Studies which have examined the effect of PCR product size on sensitivity and specificity of HRM, have concluded that amplicons with sizes greater than 300 bps may produce more errors (Reed and Wittwer 2004; White and Potts 2006; Montgomery et al. 2007). In this study, high quality DNA was essential, as partially degraded DNA produced inconclusive results when using HRM, while 20 µl PCR reaction volumes produced clearer, more consistent results than 10 µl reaction volumes (data not shown).

The HRM technique can detect all single base changes, with the A/T conversions being the most difficult to detect (Gundry et al. 2003; Liew et al. 2004), requiring instrumentation of high precision with regard to temperature control. The difference in melt temperature is >0.8°C for C/T and G/A base changes and decreases to <0.2°C for A/T base changes (Liew et al. 2004). Marker BV078160 indicated a T_m difference of 1.4°C between the two parents suggesting that this polymorphism is due to a C/T or G/A base change. Sequencing of this fragment has indicated that a G/A base change is present (data not shown). Marker CK123008 indicated a T_m difference of 0.19°C between the two parents. This small difference may indicate that the polymorphism is due to an A/T base change.

This study demonstrates the successful application of the HRM technique to fine mapping of the barley genome and indicates that the EST markers AV836787 and CK123008 are closely linked to the covered smut resistance locus *Ruh.7H*, present in the cultivar Sloop. These markers are currently being screened across elite barley lines known to be resistant to this disease, to determine their usefulness for marker assisted selection in Australian breeding populations.

Acknowledgments This project was funded by the Grains Research & Development Corporation's Australian Winter Cereals Molecular Marker Program. We would like to thank Dr Emma Mace and Noel Knight for valuable discussions, Dr Graham Wildermuth and Greg Platz for their advice on phenotyping, and Dr Christine McDonald and Dr Ashley Plank for assistance with statistical analysis. Maria Harris assisted RBM with the covered smut phenotyping.

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