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Fine mapping, physical mapping and development of diagnostic markers for the *Rrs2* scald resistance gene in barley

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Abstract

The *Rrs2* gene confers resistance to the fungal pathogen *Rhynchosporium secalis* which causes leaf scald, a major barley disease. The *Rrs2* gene was fine mapped to an interval of 0.08 cM between markers 693M6_6 and P1D23R on the distal end of barley chromosome 7HS using an Atlas (resistant) × Steffi (susceptible) mapping population of 9179 F_2-plants. The establishment of a physical map of the *Rrs2* locus led to the discovery that *Rrs2* is located in an area of suppressed recombination within this mapping population. The analysis of 58 barley genotypes revealed a large linkage block at the *Rrs2* locus extending over several hundred kb which is present only in *Rrs2* carrying cultivars. Due to the lack of recombination in the mapping population and the presence of a *Rrs2*-specific linkage block we assume a local chromosomal rearrangement (alien introgression or inversion) in *Rrs2* carrying varieties. The variety analysis led to the discovery of eight SNPs which were diagnostic for the *Rrs2* phenotype. Based on these SNPs diagnostic molecular markers (CAPS and pyrosequencing markers) were developed which are highly useful for marker assisted selection (MAS) in resistance gene pyramiding programmes for *Rhynchosporium secalis* resistance in barley.

Keywords *Hordeum vulgare – Rhynchosporium secalis* – scald – resistance gene *Rrs2*

Introduction

Leaf scald, also referred to as leaf blotch, is a foliar disease in barley (*Hordeum vulgare* L.) and in other members of the Poaceae, caused by the hemibiotrophic, haploid fungus *Rhynchosporium secalis* (Oudem.) J.J. Davis (Lehnackers and Knogge 1990; Shipton 1974; Zaffarano et al. 2006). Due to the potential high yield losses and decreased grain quality upon *R. secalis* infection, scald is an economically important barley disease worldwide in the cool and semi-humid barley growing areas (Beer 1991; Shipton 1974). Further information regarding *Rhynchosporium secalis* and leaf scald can be obtained from a recently published review article (Zhan et al. 2008).

The first studies of inherited resistance of barley cultivars to *Rhynchosporium secalis* were conducted 80 years ago by Mackie (1929). Since then several resistance genes (*R* genes) against *R. secalis* have been identified and mapped. There are four major resistance loci, the *Rrs1* complex on chromosome 3H with at least 11 known alleles (Bjørnstad et al. 2002), the *Rrs2* locus on 7HS (Schweizer et al. 1995), *Rrs13* on chromosome 6HS (Abbott et al. 1995; Genger et al. 2003b) and the *Rrs15* locus on 2HS (Schweizer et al. 2004). Some resistance genes originate from wild barley, for example from *H. vulgare* subsp. *spontaneum Rrs12 on 7HS, Rrs13* on 6HS, *Rrs14* on 1HS, and *Rrs15* on 7HL (Abbott et al. 1992; Abbott et al. 1995; Garvin et al. 1997; Garvin et al. 2000; Genger et al. 2003a; Genger et al. 2003b; Genger et al. 2005), or from *Hordeum bulbosum Rrs16* on 4HS (Pickering et al. 2006). Furthermore, a number of QTL studies revealed QTLs for scald resistance on several chromosomes whose loci often coincided with locations of known scald resistance genes (Backes et al. 1995; Bjørnstad et al. 2004; Cheong et al. 2006; Genger et al. 2003; Grønnerød et al. 2002; Jensen et al. 2002; Spaner et al. 1998; Thomas et al. 1995; von Korff et al. 2005; Wagner et al. 2008; Yun et al. 2005).

Several molecular markers for most of the known *Rhynchosporium* resistance genes have been described; for the *Rrs1* locus on 3H: HVS3 (Genger et al. 2003a), cMWG680 (Graner and Tekauz 1996), Bmac209 (Grønnerød et al. 2002), Falc666 (Penner et al. 1996); for *Rrs4* on 3H: HVM60 (Patil et al. 2003); for *Rrs12* on 7HS: Bmag7 (Genger et al. 2003b); for *Rrs13* on 6HS: *Cxp3* (Abbott et al . 1995), MWG916 (Genger et al. 2003b); for *Rrs14* on 1HS: *Hor2* (Garvin et al.

2000); for *Rrs15*$_{CI8288}$ on 2HS: (Schweizer et al. 2004); for *Rrs15* on 7HL: HVM49 (Genger et al. 2005). However, none of these markers have been described to be diagnostic.

Up to now, not much is known about the identity of the mapped resistance genes and only very few reports exist about possible functions and resistance mechanisms. For the *Rrs1* mediated resistance, prevention of subcuticular hyphae growth has been observed to be a major factor in the defence reaction against *R. secalis* (Lehnackers and Knogge 1990). The barley variety Atlas 46, which carries the *R* genes *Rrs1* and *Rrs2*, had been found to be resistant to four *R. secalis* isolates to which the otherwise resistant cultivar Atlas, carrying only the *Rrs2* gene, was susceptible (Lehnackers and Knogge 1990). Accordingly, the resistant reaction in Atlas 46 upon infection with those isolates must be mediated by *Rrs1*. Using this experimental set-up, NIP1, a small protein secreted by *R. secalis*, was found to elicit defence reactions specifically in barley plants expressing the *R* gene *Rrs1*. In fact, NIP1 is the product of the fungal avirulence gene *AvrRrs1*. Fungal strains lacking the *NIP1* gene or strains with specific point mutations in *NIP1* are able to overcome *Rrs1* resistance (Rohe et al. 1995). However, *Rrs1* does not encode for the NIP1 receptor. The *Rrs1* gene product presumably plays a role in recognizing the interaction of NIP1 with its receptor and upon this it activates defence reactions (Knogge et al. 2003).

To our knowledge there is only one report published about the *Rrs2* mediated resistance reaction. Jørgensen et al. (1993) found that the resistance reaction in the cultivars Digger and Osiris, which both carry the *Rrs2* gene, is characterized by the prevention of fungal penetration of the cuticle through an accelerated and larger formation of papillae and halos in the cell walls compared to susceptible cultivars. The authors also suspect the involvement of chemical compounds in the inhibition process.

The gene *Rrs2* is still effective against most *Rhynchosporium secalis* isolates. Overall, only a few cases are reported in the literature where fungal isolates were shown to break the *Rrs2* mediated resistance of cultivar Atlas in Europe. The study of Schürch et al. (2004) showed that two isolates (AU2 and RS88CA27) out of 41 tested were able to break the resistance. Lehnackers and Knogge (1990) identified four races which were able to complete the developmental cycle from spore germination to sporulation on Atlas. However,

three of these isolates (CV3, UK7, and UK8) only caused microscopically visible necrotic spots, even though the sporulation was strong. One strain (US238.1) also produced the typical lesions. Salamati and Tronsmo (1997) identified 4 out of 42 isolates to which Atlas was susceptible. Furthermore, since the year 1990 the *Rrs2* mediated resistance has been observed to be effective in the *Rhynchosporium secalis* test fields of the Bayerische Landesanstalt für Landwirtschaft in Freising, Germany. From our point of view, the *Rrs2* resistance gene is therefore valuable for barley breeding programmes, especially if it is pyramided with other *R* genes against *R. secalis*. For this, the molecular identity or at least closely linked markers for *Rrs2* are required. A first study on the development of molecular markers for the dominantly inherited *Rrs2* gene (formerly called *Rh2*) was undertaken by Schweizer et al. (1995) who mapped the gene to the distal part of the short arm of barley chromosome 7H. The RFLP marker CDO545 co-segregated with the resistance gene in a population of 85 doubled-haploid plants of a cross of the resistant cultivar Atlas (CI 4118) and Steffi. Schmidt et al. (2001) established a high-resolution genetic map of the *Rrs2* region and could delimit the position of *Rrs2* between RFLP markers MWG2018 and MWG555a.

In this article we report the further fine mapping of *Rrs2*, the establishment of two physical BAC contigs flanking the *Rrs2* locus, a variety analysis and the development of diagnostic markers applicable for marker-assisted selection.

Materials and methods

Plant material

The F_2-mapping population used in this work was derived from a cross between the varieties Atlas and Steffi. Atlas (CI 4118) is a 6-rowed American spring barley variety carrying the resistance gene *Rrs2*. Steffi is a 2-rowed Bavarian spring barley variety (Saatzucht Ackermann, Irlbach) which is susceptible to *Rhynchosporium secalis* (Oudem.) J.J. Davis.

For the variety study a set of 58 different barley accessions was chosen based on their reported resistance phenotypes from personal communications or literature data. The varieties Atlas (CI 4118), Atlas 46, Coast (CI 2235), Escaldadura 15, Gloria LB Iran x Harrington, Osiris (CI 1622), Pewter, and

PI452395 were confirmed as *Rrs2* carrying varieties by mapping experiments performed at the Bayerische Landesanstalt für Landwirtschaft (Bavarian State Research Institute) in Freising, Germany (unpublished results). Cultivars Digger and Livet were reported to carry *Rrs2* by William Thomas (SCRI, Dundee, UK) and the variety Forrest (AUS) by Hugh Wallwork (SARDI, Adelaide, Australia). A complete list of all varieties including pedigree, origin, resistance reaction and literature citations is available in Supplemental File S1. Seeds of the accessions as well as DNA samples of several varieties were obtained from following sources: Genebank Gatersleben, Germany; Australian Winter Cereal Collection, Tamworth, Australia; BBSCR Cereals Collection, John Innes Centre, Norwich, UK; Bayerische Landesanstalt für Landwirtschaft, Freising, Germany. Some accessions were acquired from two sources resulting in a redundant set of 72 accessions which were used in the analysis.

Scald resistance test

The resistance test was performed at the Bayerische Landesanstalt für Landwirtschaft in Freising, Germany. For each recombinant plant line or barley accession four seedlings were tested in two replicates per resistance test. Resistance tests were repeated at least twice per recombinant plant line. Inoculation of *R. secalis* was carried out as described in Schweizer et al. (1995) with the following modifications. A spore suspension of the single spore isolate "Sachs 271" (collected at Straßmoos, Bavaria, Germany) was diluted to concentrations of 2 x 10^5 spores/ml. Seedlings at the 3-leaf stage were sprayed uniformly with inoculum (approximately 0.25 ml per plant) and left for 20 min to dry. Inoculated plants were kept for 48 h in a dark moist chamber at 18°C. Symptoms developed after 14 days and plants were scored on a scale from 0 to 4 according to Jackson and Webster (1976). The cultivars Atlas (resistant) and Steffi (susceptible) were used as reference cultivars.

Bacterial artificial libraries

For the establishment of the physical map, mainly the HVVMRXALLhA BAC library of barley cultivar Morex (Yu et al. 2000) was used. Additional BAC libraries of which each contributed one clone to the BAC contig included HVVCCALLhA (Isidore et al. 2005) of cultivar Cebada Capa, HVVMRXALLhB

(Langridge et al., unpublished data) and HVVMRXALLeA (Langridge et al., unpublished data) of cultivar Morex. BAC library naming follows an agreed general nomenclature for Triticeae BAC libraries (http://wheat.pw.usda.gov/GG2/BAClibrary/BAC_naming.html). Clones of the last two libraries were identified through high information content fingerprinting in frame of the establishment of a physical map of barley (Schulte et al. 2009) at the lab of Nils Stein (Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany). The Cebada Capa library HVVCCALLhA was kindly screened by Beatrice Scherrer at the lab of Beat Keller (Institute of Plant Biology, University Zurich, Switzerland).

Sequence analysis

Sequence alignments and manipulations of nucleotide sequences were performed with the programmes Sequencher™ version 4.5 (Gene Codes Corporation, Ann Arbor, MI, USA). Homologous sequences were identified using BLAST tools (Altschul et al. 1990) from the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) under default parameter settings. Repetitive elements were identified through BLAST against the database for Triticeae repetitive elements (TREP, wheat.pw.usda.gov/ITMI/Repeats).

The Integrated Map 04/16/08 tool of the HarvEST: Barley (Assembly 35) version 1.68 database (http://harvest.ucr.edu) was used to identify unigenes which map to the *Rrs2* locus.

Homologous sequences of rice were identified using the MSU Rice Genome Annotation Project BLAST search (http://rice.plantbiology.msu.edu/blast.shtml). All results are based on Release 6 (January 30, 2009) of the rice genome.

Variety analysis

Genotypic data of 72 barley accessions was obtained by sequencing six PCR fragments located within or near the co-segregating area of *Rrs2* on barley chromosome 7HS (Supplemental File S2). Primers used for amplification of the PCR fragments are listed in Table 1.

For performing the cluster analysis the programme MEGA version 4 (Tamura et al. 2007) was employed. The cladogram was calculated using the

neighbour-joining method with the maximum composite likelihood model based on the total SNP set of all six analyzed PCR fragments. Bootstrapping was carried out with 800 repetitions and random seed was set to 64,238.

Associations were calculated assuming the general linear model (GLM) using the software package TASSEL version 2.0.1 (Bradbury et al. 2007; http://www.maizegenetics.net). The calculations were performed for each PCR fragment separately using either the SNP data or haplotype data. Phenotypic data was divided into four groups (resistant, *Rrs2*, susceptible, intermediate) which were represented by numerical values for the association calculations.

Molecular marker development

Nucelotide sequences were aligned with the programme Sequencher™ version 4.5 (Gene Codes Corporation, Ann Arbor, MI, USA). The multiple alignments were analyzed for presence of restriction sites at SNP positions with the programme SNP2CAPS (Thiel et al. 2004). Restriction digestions of PCR products were carried out in a 25 µl volume using 7 µl of PCR product, 3 U of the respective restriction endonuclease (Fermentas, St. Leon-Rot, Germany) and an incubation time of minimum 3 hours. The restricted fragments were separated on 2.5 to 3% 1 x TAE agarose gels.

Pyrosequencing primers were developed using the Assay Design Software, version 1.0.6 (Biotage AB, Uppsala, Sweden). After PCR with genomic DNA, pyrosequencing was carried out according to the manufacturer's standard protocol with Pyro Gold Reagents (Biotage AB) on a Pyrosequencer PSQ^{TM} 96HS96A 1.2 machine (Biotage AB). The detected SNPs were analyzed with the manufacturer's software.

Markers for the *Rrs2* phenotype are listed in Table 2.

Results

Fine mapping and physical mapping of the *Rrs2* locus

For fine mapping of the *Rrs2* gene a F_2-mapping population with 9179 F_2-plants of the cross Atlas × Steffi was used. In total, 41 recombination events were identified in this population between markers AFLP14 and P1D23R which

corresponds to a genetic distance of 0.22 cM (Fig. 1). The location of the *Rrs2* gene could be delimited to an interval of 0.08 cM flanked by the markers 693M6_6 and P1D23R on the distal end of barley chromosome 7HS. The first co-segregating markers with the *Rrs2* gene are 693M6-71 and A-EST (Fig. 1). Any additional markers developed for this interval also co-segregated with the *Rrs2* phenotype.

Two BAC contigs covering parts of the *Rrs2* locus were constructed using four different BAC libraries. The BAC clones were identified primarily by chromosome walking, but two clones also through high information content fingerprinting (Luo et al. 2003). The minimal tiling path of the distal and proximal BAC contig consisted of eight and four BAC clones, respectively. The two contigs were overlapping partly with the co-segregating area of *Rrs2*, but do not overlap with each other (Fig. 1).

Identification of genes in the *Rrs2* co-segregating area

Since any of the genomic sequences originating from the *Rrs2* co-segregating area could potentially harbour the *Rrs2* gene, the available sequence information of both BAC contigs as well as sequence information of BAC clones MRXhA_668A17 and MRXhA_348I22 was annotated concerning the genic DNA content. For the distal BAC contig 62,313 bp of sequence information starting at bp position 196,570 of Acc. AY853252 (position of marker 693M6_6) until the end of the distal BAC contig (Acc. AY853252 + Acc. FJ974009) was considered for sequence annotation. For the proximal BAC contig 77,380 bp of sequence information (Acc.No. xxxxxxxx, until position of marker P1D23R) was annotated. The annotation was performed using blastn and blastx searches in the NCBI database. Additionally, the HarvEST Barley Integrated Map 04/16/08 (Version 1.68, Assembly #35; http://harvest.ucr.edu/) proved to be a valuable source for further information regarding mapped genes at the *Rrs2* locus. In total, eleven putative genes were identified in the available sequence information for the *Rrs2* co-segregating region (Table 3). Ten of them showed high similarity to barley ESTs and nine sequences contained coding regions for proteins with predicted functions. The remaining two had significant homology to barley ESTs, but no hit to a characterized protein. They are therefore referred to as 'hypothetical proteins'. Two genes of the co-segregating region could be

identified indirectly through mapped genes of the HarvEST Barley Integrated Map 04/16/08 (Assembly #35). In this map, five unigenes map to map position 3.3 on chromosome 7HS. Three of them are present in the sequence information available for the *Rrs2* locus. HarvEST unigene #15670 corresponds to gene No. 4 in Table 3, #2643 corresponds to gene No. 5 in Table 3, and #3681 is found on the proximal barley BAC contig (gene No. 8 in Table 3). The fourth unigene, #1552 which corresponds to gene No. 6 in Table 3 was localized on BAC clone ME194H14 by PCR. The remaining unigene #2649 could not be identified in the sequence data obtained up to date, but since all five unigenes map to the same position, it is very likely that #2649 (gene No. 7 in Table 3) originates from within the gap between the two BAC contigs flanking the *Rrs2* co-segregating area.

For nine of the eleven predicted coding sequences, a homologous rice gene could be identified. For barley chromosome 7HS a high degree of microsynteny to rice chromosome Os6 has been reported (Kilian et al. 1995). Even though five of the homologous rice genes originated from rice chromosome Os6, only two were actually from the orthologous region of the *Rrs2* region (underlined genes in Table 3).

Three putative genes, which were identified in the sequence information of the co-segregating area, show an annotation that hints to an involvement in pathogen defence related processes. Those genes are coding for a putative serine-threonine protein kinase and two pectinesterase inhibitor domain containing proteins. They are regarded as possible candidate genes for *Rrs2* because of their co-segregation with the *Rrs2* phenotype.

Analysis of varieties

By analysing a set of differing varieties it is possible to reach a much higher marker resolution due to the higher diversity and recombinational history of the samples, which contain many more informative meioses than classical mapping populations (Gaut and Long 2003). Therefore, 58 different genotypes were chosen based on their reported resistance phenotype. Since some accessions with the same name were acquired from more than one source, in total a redundant set of 72 accessions was used in this study. Six genomic regions distributed across the co-segregating region of *Rrs2* and neighbouring regions on

chromosome 7HS were chosen for obtaining genotypic data of all accessions (Supplemental File S2).

The analysed fragment sizes ranged from 252 bp to 666 bp, totalling 2,583 bp of sequence information. Overall, 132 polymorphic sites, comprising 129 SNPs and 3 INDELs, were identified (Supplemental File S3). About half of the observed single nucleotide polymorphisms, 69 SNPs, had an allele frequency <10% and were therefore regarded as minor SNPs. These SNPs mainly originated from the 16 landraces which were among the 72 accessions. The average number of SNPs per kb for major SNPs (allele frequency >10%) was 24 which is equivalent to 1 SNP per 42 bp.

A cluster analysis was performed with the summarized SNP data (major and minor SNPs) of all 72 accessions and all six genomic regions. Interestingly, the vast majority of accessions carrying *Rrs2* (all except two) clustered in one subgroup of the resulting neighbour-joining tree (Fig. 2). This clustering was due to the fact that all those accessions shared the same SNP pattern for all six genomic regions (Supplemental File S3). The two exceptions were variety Osiris and one accession of Atlas 46 (HOR20489). However, the data of Osiris should be regarded with caution, since the sample of Osiris used in the study seemed to be heterozygous and sequence data probably only originated from one of the two alleles present. Furthermore, it is questionable whether accession HOR20489 represents the true genotype of Atlas 46, since it showed a different SNP pattern in two of the analysed fragments compared to all other accessions of Atlas 46.

Three resistant varieties, which previously were not reported to carry the *Rrs2* gene, also appeared in the *Rrs2* subgroup of the cluster. The varieties Atlas 54 (CI 9556), Turk × Atlas (CI 7189), and Wisconsin Winter × Glabron (CI 8162) always shared the same SNP pattern as the *Rrs2* accessions for all six analysed regions. It is likely that these varieties carry the *Rrs2* gene, also since two of them are directly related to Atlas. However, this assumption needs to be confirmed through mapping studies.

Accessions belonging to the *Rrs2* subgroup in the cluster shared the same haplotype (group of genotypes with identical SNP/INDEL pattern) for all analysed regions whereas other accessions generally belonged to different haplotypes (Supplemental File S3). The presence of the haplotype block was only observed in varieties carrying the *Rrs2* gene hinting to a recombinationally

inactive chromosomal fragment extending over several hundred kb in those varieties. As a result, it was not possible to delimit the location of the *Rrs2* gene to a narrower interval by using the differential set of varieties. However, through this analysis, several SNPs only present in the *Rrs2* subgroup could be identified. Due to the presence of the haplotype block in the *Rrs2* varieties, these SNPs are highly diagnostic for the *Rrs2* gene and suitable for developing molecular markers.

Development of diagnostic markers

Comparing the SNP patterns of all accessions for all analysed genomic regions of the *Rrs2* locus, four regions can be identified which carry diagnostic SNPs for *Rrs2* (Supplemental File S3, grey highlighted SNPs). To evaluate the SNPs by statistical means a test for significant associations of SNPs with the *Rrs2* phenotype assuming the General Linear Model (GLM) was conducted. The analysis was performed for all SNPs of all six genomic regions and all 72 accessions also taking the population structure of the samples (summer/winter cultivars, 2-row/6-row genotypes) into account. Associations with a p-value $<5\times10^{-2}$ were regarded significant.

As expected, genomic regions 2 (Acri), 4 (668A17_g1-3), 5 (668A17_e11-2), and 6 (134N7_con5-3) contained SNPs which were significantly associated with the presence of the *Rrs2* gene (Table 4, Supplemental File S3). All SNPs specific for *Rrs2* showed p-values $<10^{-14}$. All SNPs with a p-value between 10^{-10} and 10^{-13} were significant, but not 100% correlated with *Rrs2*. In case of SNP 25 of region 2 the diagnostic allele for *Rrs2* was also shared by two non-*Rrs2* accessions. In case of all SNPs of region 6, one additional variety carried the diagnostic alleles. SNPs of region 6 were therefore not used for marker development. All other significant SNPs (p-values between 10^{-9} and 5×10^{-2}) could not be correlated with the *Rrs2* phenotype and can be regarded as spurious associations. Additionally to the SNPs identified through the association calculations regarding individual SNPs, two SNPs of region 2 (SNPs 14 and 15) were significantly associated with *Rrs2* if regarded as a haplotype (p-value 2.9887×10^{-16}, value not included in Table 4). These SNPs are only specific for *Rrs2* if the diagnostic alleles occur together (Supplemental File S3).

CAPS markers and pyrosequencing markers were developed based on five SNPs originating from three analysed genomic regions (Table 4). For region 2

(Acri) in total three markers were developed, a CAPS and pyrosequencing marker for SNP position 9 and a CAPS marker based on SNPs 14 and 15. For SNP 4 of region 4 (668A17_g1-3) two CAPS markers and one pyrosequencing marker could be generated. For region 5 (668A17_e11-2) one pyrosequencing marker based on SNP 5 and one CAPS marker based on SNP 16 were developed. All markers were tested with *Rrs2* genotypes and non-*Rrs2* genotypes and found to correctly predict the genotype of the varieties. As example, CAPS marker Acri_SNP14+15 is shown in Fig. 3. The diagnostic SNPs identified in this study and the developed markers will be useful to assist breeding programmes for *Rhynchosporium secalis* resistance in barley.

Discussion

Fine mapping and physical mapping of the *Rrs2* gene

The *Rrs2* gene was fine mapped to an interval of 0.08 cM on the genetic map of the Atlas × Steffi population. The outermost markers which co-segregate with the *Rrs2* phenotype (693M6-71 and A-EST) are physically located on different BAC contigs (Fig. 1). An estimation of the physical size for the co-segregating area is difficult due to the co-segregation of markers. According to the updated map of Künzel et al. (2000) at http://pgrc.ipk-gatersleben.de/kuenzel/image7h.gif the recombination rate at the distal end of barley chromosome 7HS corresponds to 1 Mb/cM. The average observed recombination rate of the regions flanking the *Rrs2* co-segregating area was 2.8 Mb/cM. Assuming these recombination rates, the distance between markers 693M6_6 and A-EST_2_EST should be between 80 and 224 kb. In total, 139 kb of contiguous sequence information have been obtained for this interval. The physical size of the unsequenced BAC clones (MRXhA_668A17, MRXhA_348I22, MRXhB_068J02, and MRXeA_194H14) can be estimated to 200 kb presuming an average insert size of 100 kb and an overlap of 50%. In addition to that an unknown size for the gap has to be taken into account. With a minimum size of 339 kb + gap the physical distance for the interval 693M6_6 – A-EST is therefore considerably larger than what can be estimated based on observed recombination rates for this region.

The co-segregation of markers located on the non-overlapping BAC contigs had already been detected after screening only 4721 F_2-plants. In order to identify recombination events in the immediate vicinity of the *Rrs2* gene and break the co-segregation of markers, the size of the mapping population was increased by additional 4458 F_2-plants to its final size. This increase did not result in a higher genetic resolution of the *Rrs2*-locus, nor did it lead to a more accurate positioning of the *Rrs2* gene. All identified recombination events were redundant and not a single recombination between any of the co-segregating markers was identified. The data strongly suggests that the *Rrs2* gene is located within an area of suppressed recombination which exists within the Atlas × Steffi mapping population. As a consequence, neither the precise calculation of the recombination rate and estimation of the physical distance for the co-segregating marker interval, nor the exact determination of the location of the *Rrs2* gene is possible in absence of recombination.

Possible reasons for suppressed recombination at the *Rrs2* locus

Introgressions of alien chromatin and structural rearrangements (inversions, insertions, deletions) have been hypothesized or reported to be responsible for suppressed recombination surrounding many disease resistance loci of several plant species like tomato, poplar, wheat and barley. In tomato, suppression of recombination was observed in the vicinity of the *Tm-2a* gene, conferring resistance to tobacco mosaic virus, which was introgressed into cultivated tomato from the wild species *L. peruvianum* (Ganal et al. 1989; Ganal and Tanksley 1996). Messeguer et al. (1991) obtained similar results for the introgressed root knot nematode resistance gene *Mi-1* in tomato from *L. peruvianum*. The reduced homology between the introgressed segment and the receptor genome had originally been hypothesized to be responsible for the severe reduction in recombination (Ho et al. 1992; Messeguer et al. 1991). Later it was shown by Seah et al. (2004) that the lack of recombination events at the *Mi-1* locus was due to a paracentric inversion between resistant and susceptible tomato lines.

A more than 25-fold reduction of the recombination frequency for the region which harbours the *MXC3* gene in poplar conferring resistance to *Melampsora* leaf rust was reported by Stirling et al. (2001). Further studies

revealed that the suppression of recombination was most likely caused by large hemizygous rearrangements (insertions/deletions) in one of the parents used for establishing the mapping population (Yin et al. 2004).

The *Lr20-Sr15-Pm1* resistance locus on chromosome 7AL in wheat confers resistance to leaf rust, stem rust and powdery mildew. A lack of recombination was observed in two mapping populations for the *Lr20* and *Pm1* genes. Neu et al. (2002) concluded that this might be caused by an alien introgression. However, other possibilities, for example an inversion, could not be excluded. Additionally, suppressed recombination in the vicinity of resistance gene loci which had been introgressed from wild relatives into hexaploid wheat were reported for stem rust resistance gene *Sr22* (Paull et al. 1994), and leaf rust resistance genes *Lr9* (Schachermayr et al. 1994), *Lr24* (Schachermayr et al. 1995), and *Lr35* (Seyfarth et al. 1999).

The barley *Mla* powdery mildew resistance cluster on chromosome 1HS showed a 10-fold reduction in recombination compared to flanking regions which was attributed to a high degree of polymorphism between the parents of the mapping cross and additionally also to the introgressed *Mla6* allele which originates from *H. spontaneum* (Wei et al. 1999). Görg et al. (1993) observed suppressed recombination near the *Mlg* locus in barley, which also confers resistance to powdery mildew.

As for the *Rrs2* locus, both possibilities of either an introgressed chromosomal segment or a chromosomal inversion, present only in the varieties carrying the *Rrs2* gene, seem plausible explanations for the suppressed recombination observed in the Atlas × Steffi mapping population. The crossing of a plant with a rearranged chromosome segment with a plant with the "wild type" chromosome would result in incomplete chromosome pairing in meiosis and prevent crossing over events in the area of sequence dissimilarity. However, an introgression looks to be less likely, since all PCR primers developed from Morex sequence information binding within the area of suppressed recombination also gave amplification results with plants carrying the *Rrs2* gene. If the sequence dissimilarity of the putative introgressed segment was high enough to suppress recombination, it would be expected that at least some primer pairs would not amplify DNA fragments from plants carrying the introgressed segment containing the *Rrs2* gene.

Variety analysis

By analysing a collection of cultivars instead of a segregating population it is possible to obtain a much higher marker resolution as was demonstrated by the study of Tommasini et al. (2007), who observed a 390-fold higher marker resolution with a collection of 44 winter wheat cultivars compared to a RIL population.

In our study this approach was anticipated to reveal markers which could break the co-segregation of the flanking markers 693M6-71 and A-EST leading to a smaller interval for *Rrs2*. In total, 72 diverse barley accessions (58 genotypes) were analysed concerning their SNP patterns for six genomic regions originating from within or near the co-segregating area of *Rrs2*. The haplotype analysis revealed a large linkage block extending over several hundred kb which seems to be inherited as such in all varieties carrying the *Rrs2* gene. No evidence of a linkage block at the investigated region was found for all other, non-*Rrs2,* varieties. Due to the linkage block the break up of the co-segregation of markers at the *Rrs2* locus was not possible, however these findings support the hypothesis that there exists an introgressed or inverted segment of the chromosome at the *Rrs2* locus in varieties which show the *Rrs2* phenotype.

Probable origin of *Rrs2*

Since all varieties possessing the *Rrs2* gene contain an identical stretch of sequence, it is very likely that this sequence originates from a single common ancestor, but the pedigree of *Rrs2* carrying varieties does not show any obvious relationship between the varieties. Three different groups of common geographical origin or pedigree can be distinguished, but the links between the groups are missing (Fig. 4). There is a slight hint that the *Rrs2* gene could have originated in Northern Africa. The varieties Coast and Osiris are the oldest varieties from the set of 14 varieties studied (introduction year unknown for two varieties) and both varieties are reported to originate from Northern Africa. However, the geographical origin data of Coast was found not to be consistent between different sources, therefore the possible origin of *Rrs2* from Northern Africa remains only a guess (Fig. 4).

Microsynteny to rice and putative candidate genes for *Rrs2*

Sequence analysis of the co-segregating region of *Rrs2* led to the identification of eleven putative genes which show reliable hits to known unigene clusters or ESTs. Homologous rice genes were identified for nine out of the eleven genes. However, only two of them originated from the orthologous region in rice on chromosome Os6 (underlined genes in Table 3). The regions flanking the co-segregating area showed a slightly higher microsynteny (data not shown), however, the whole *Rrs2* locus underwent extensive rearrangements compared to rice. Such large rearrangements are often observed at the telomeric ends of chromosomes (Caldwell et al. 2004) and at disease resistance loci (Ramakrishna and Bennetzen 2003) which are particularly unstable (Devos 2005) due to their rapid reorganization (Leister et al. 1998).

Among the eleven putative genes identified in the *Rrs2* co-segregating region a putative serine-threonine protein kinase and two genes coding for pectinesterase inhibitor domain containing proteins could be identified. Those genes show a functional annotation that hints to an involvement in disease resistance processes.

There are several examples where products of disease resistance genes belong to the serine-threonine kinase family, for example Xa21 and Xa26 of rice conferring resistance to bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* (Song et al. 1995; Sun et al. 2004), *Pi-d2* of rice against the fungal pathogen *Magnaporthe grisea* (Chen et al. 2006), the Pto gene for race-specific resistance to *Pseudomonas syringae* in tomato (Martin et al. 1993) or the barley *Rpg1* gene against *Puccinia graminis* f. sp. *tritici* (Brueggeman et al. 2002).

In many plant-pathogen interactions the cell wall functions as a physical barrier which first must be overcome for further colonization of the plant. One strategy of phytopathogenic fungi and bacteria is to secrete cell wall degrading enzymes which enable penetration (Esquerré-Tugayé et al. 2000). Apart from cellulose and hemicellulose, pectin is the main and the most complex polysaccharide of plant cell walls. Pectinolytic enzymes are among the first to be secreted by plant pathogens and include, polygalacturonases, pectate and pectin lyases, as well as pectinesterases (Alghisi and Favaron 1995; Valette-Collet et al. 2003). Plants in turn produce enzymes which can inhibit the activity of microbial enzymes. Known inhibitor proteins for pectinolytic enzymes include

polygalacturonase inhibiting proteins (PGIPs) (Ferrari et al. 2006; Ferrari et al. 2003; Lorenzo and Ferrari 2002; Powell et al. 2000) and a pectinmethylesterase inhibitor (PMEI) from pepper (*Capsicum annuum*) (An et al. 2008). Additional to their co-segregation with *Rrs2*, there is another good reason to consider the genes coding for pectinesterase inhibitor domain containing proteins as possible candidate genes for *Rrs2*. Histological studies of the infection process of *Rhynchosporium secalis* have shown that the fungus often initiates penetration of the cuticle in the groove between two epidermal cells, a region rich in pectin (Jones and Ayres 1974; Lehnackers and Knogge 1990; Xi et al. 2000). This led to the hypothesis that *Rhynchosporium secalis* produces pectin degrading enzymes for penetration of the cuticle (Xi et al. 2000). The activity of pectin degrading enzymes in later stages of the *R. secalis* infection is suggested by Jones and Ayres (1974) due to observations of structural changes in pectic substances near subcuticular hyphae.

All sequence information obtained of the *Rrs2* co-segregating region originates from the susceptible barley cultivar Morex. It can be argued that the *Rrs2* gene possibly is not present in cultivar Morex and hence can also not be identified in the sequence information. Furthermore, not all barley BAC clones of the *Rrs2* co-segregating region have been fully sequenced and there also remains a sequence gap of unknown size spanning this region. Therefore, further work is needed to close this gap and to obtain all the missing sequence information, preferentially from a *Rrs2* carrying cultivar, so that all possible candidate genes can be identified. Ultimately, a confirmation of candidate genes, for example by gene silencing or transformation experiments, is needed.

Diagnostic molecular markers

The variety analysis led to the discovery of eight SNPs, originating from three different genomic regions. The SNPs were exclusively present in lines which carry the *Rrs2* gene and therefore diagnostic for the *Rrs2* phenotype. In a statistical test assuming the General Linear Model (GLM) these SNPs were also shown to be highly associated with the presence of *Rrs2* (Table 4). Based on five SNPs, eight diagnostic molecular markers for the *Rrs2* phenotype (five CAPS markers and three pyrosequencing markers) were developed. These high-

throughput markers can now be applied to marker assisted selection (MAS) in breeding programmes for resistant cultivars against *Rhynchosporium secalis*. MAS makes it possible to select traits with greater accuracy and to develop new varieties quicker and therefore more cost effectively than with conventional selection methods (Collard and Mackill 2008). *Rhynchosporium secalis* is a highly variable fungus which is able to overcome single resistance genes quickly (Williams et al. 2001). Therefore, it is generally not considered worthwhile to base breeding programmes for *Rhynchosporium* resistance on single major genes (Jørgensen et al. 1995; Tekauz 1991; Salamati and Tronsmo 1997). Rather it is thought that the durability of resistance against the fungus could be greatly prolonged by pyramiding several *Rhynchosporium secalis* resistance genes in one cultivar (Tekauz 1991). Tightly linked molecular markers are especially useful for pyramiding resitance genes, since those genes often can not be differentiated by their phenotypic reaction and could only be discriminated by extensive progeny tests (Collard and Mackill 2008).

The successful pyramiding of disease resistance genes or resistance QTLs and the effectiveness of this strategy has been reported for example for stripe rust (Castro et al. 2003) or barley yellow mosaic virus resistance (Okada et al. 2004, Werner et al. 2005) in barley, leaf rust (Kloppers and Pretorius 1997) or powdery mildew resistance (Liu et al. 2000) in wheat, as well as leaf blast (Hittalmani et al. 2000) or bacterial blight resistance (Shanti et al. 2001; Singh et al. 2001) in rice. A successful combination of pairs of different *Rhynchosporium* resistance genes was also reported by Brown et al. (1996).

Several molecular markers more or less closely located near different *Rhynchosporium* resistance genes have been reported, however, the markers presented in this paper are the first truly diagnostic markers described for any *Rhynchosporium secalis* resistance gene in barley. Their tight linkage to *Rrs2* makes them especially suitable for employing in gene pyramiding programmes. Furthermore, the markers can also be used for identification of the *Rrs2* gene in germ plasm collections.

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Figure legends:

Fig. 1 Physical and genetic map of the *Rrs2* locus

Fig. 2 Cluster analysis of 72 barley accessions. The unrooted neighbour-joining tree is based on SNP patterns of six genomic regions of the *Rrs2* locus on chromosome 7HS. Numbers on the branches indicate the frequency (%) with which a clade appeared in 800 bootstrap samples. Row number (2 or 6), and growing type (S or W) as well as resistance phenotype of each accession are given. The resistance reaction is indicated with 'resistant' for those varieties which were not reported to carry the *Rrs2* gene, but which were found to be resistant against *Rhynchosporium secalis*

Fig. 3 CAPS marker based on SNPs 14 and 15 of region 2 (Acri). The 324 bp PCR fragment of region 2 is digested by *Eco*32I (*Eco*RV) into 145 and 179 bp long fragments in cultivars which carry *Rrs2*, but not in non-*Rrs2* varieties. Sample of Osiris was heterozygous

Fig. 4 A possible common ancestor of varieties carrying *Rrs2* might originate from Northern Africa.

Table 1 Primers used for PCR amplification of 72 barley accessions

Locus	BAC clones	Forward primer (5'→3')	Reverse primer (5'→3')	PCR product [bp]
RGH-3	MRXha_693M06	ATT TTG ATG TGC TTT TCA GTA GAG G	ATT TTA GCT GTT GAA TCT CAT CTG G	459 bp
Acri	MRXha_693M06	AGT CTT GTG GAA GGG GAG GT	GCA CCC TAT CCC TAT GAG CA	324 bp
FST-2	MRXha_693M06	ATA CAT CGT TGA GGC AAT GGA G	ATA CAC TTC TTG GCA AAC ATC CAC	402 bp
668A17_g1-3	MRXhA_668A17	ACG AAC TCA AGG TGG TGG AC	TGT TGA GCT CCT GGC TTT CT	480 bp
668A17_e112	MRXhA_668A17	CTT GAT GTG GTG CCT CCT CT	GGT GGG AGC TAC AGA ACC AA	252 bp
134N7_con5-3	MRXhA_134N07	AAA AAT CTC AAC CCT CGC ACA TCA	TAC CAA GAA GGC CCA ACC AAC AGT	666 bp

Table 2 Diagnostic markers for the *Rrs2* phenotype. Bold highlighted polymorphisms are indicative for the *Rrs2* allele. Primers marked with * are biotinylated at the 5' end

Marker	Assay type (restriction enzyme)	Polymorphism	Sequence to analyse	Forward primer	Reverse primer	Sequencing primer	PCR product [bp]
Acri_SNP9	Pyro	**G**/C	CC/G ATA AAC CAC AAC AAC AAA CAC CGC G	*TTG CCA TTT TAC GCG GTG T	ACT GTC CCT CTA ACC CGT TGG T	TAA CCC GTT GGT TCG	63 bp
Acri	CAPS SNP9 (*Bsp*68I)	**uncut**/(151, 173 bp)	–	AGT CTT GTG GAA GGG GAG GT	GCA CCC TAT CCC TAT GAG CA	–	324 bp
	CAPS SNPs14+15 (*Eco*RV)	**(145, 179 bp)**/uncut	–				
668A17_g1-3_SNP4	Pyro	**C**/T	C/TG GAG CTC CTT TTG TCC GAC CGG AGA	*CGC AAC AGG CAC CTG AAC	GAT GAG CGA CAG GTC AAG G	GGA GGT CCT TGG CCC	102 bp
668A17_g1-3	CAPS SNP4 (*Bcn*I)	**uncut**/(128, 352 bp)	–	ACG AAC TCA AGG TGG TGG AC	TGT TGA GCT CCT GGC TTT CT	–	480 bp
	CAPS SNP4 (*Gsu*I)	**(278, 108, 94 bp)/** (278, 202 bp)	–				
668A17_e11-2_SNP5	Pyro	**A**/C	C/AG GTG GAA CCA CCA CTA GAG GTC GTC	GAA AAG GCG GGC CGA GAG	*TCA ACG CGC TTT GTT GCC	GGA GGG AGC ATC GTA G	119 bp
668A17_e11-2	CAPS SNP16 (*Nla*III)	**(47, 62, 63, 80 bp)/** (47, 80, 125 bp)	–	CTT GAT GTG GTG CCT CCT CT	GGT GGG AGC TAC AGA ACC AA	–	252 bp

Table 3 Genes identified in the co-segregating region of *Rrs2* with homology to barley ESTs and rice genes

No.	Homologous proteins	BAC	Barley EST	E-value	Best rice hit
1	Electron transfer flavoprotein, beta-subunit	MRXhA_693M06	CB882327	$3e^{-114}$	Os04g10400
2	Flavonol-sulfotransferase	MRXhA_693M06	BY842569	0.0	Os09g38239
3	**Putative serine-threonine protein kinase**	CChA_Pool002	–	–	Os06g36270
4	**Pectinesterase inhibitor domain containing protein**	CChA_Pool002	BI950063	0.0	Os11g03750
5	Hypothetical protein	MRXhA_668A17	BU968635	0.0	Os06g19800
6	**Pectinesterase inhibitor domain containing protein**	MRXeA_194H14	BQ472845	–[a]	–
7	ß-1,3-glucan synthase	–[b]	CK567361	–	Os06g02260
8	High-affinity zinc uptake system membrane protein ZnuB	MRXhA_246J13	BM099166	0.0	–
9	Hypothetical protein	MRXhA_246J13	CA007927	$2e^{-45}$	Os12g01250
10	Ent-kaurenoic acid oxidase	MRXhA_524N03	AF326277	0.0	Os06g02019
11	Eyes-absent like protein	MRXhA_524N03	CB864317	0.0	Os06g02028

Rice hits with conserved synteny to the '*Rrs2* orthologous region' are underlined. Homologous proteins in bold show similarity to known disease resistance genes and are regarded as candidate genes for *Rrs2*.

[a] E-value not possible to determine, since no sequence information of BAC clone available; information is based on HarvEST unigene 1552 (Assembly 35), which was mapped to the *Rrs2* locus in the HarvEST Integrated Map 04/16/08 and could be amplified by PCR from BAC clone MRXeA_194H14

[b] Based on mapping data of HarvEST unigene 2649 (Assembly 35) in HarvEST Integrated Map 04/16/08; EST maps to the *Rrs2* locus, but could not be identified on any known BAC clone, possibly located in the gap between both flanking BAC contigs

Table 4 Results of association calculations for SNPs of four genomic regions of the *Rrs2* locus on barley chromosome 7HS. SNPs which are correlated with the *Rrs2* phenotype in 100% of the cases are highlighted in bold. The SNPs depicted in bold + italics did not show a significant association, however as a combination (haplotype) they are 100% diagnostic for *Rrs2*. Even though highly associated, the underlined SNPs are not 100% specific for *Rrs2* (Supplemental File S3).

SNP no.	p-value			
	region 2 (Acri)	region 4 (668A17_g1-3)	region 5 (668A17_e11-2)	region 6 (134N7_con5-3)
1	0.7818	0.316	0.3161	0.0023
2	0.3884	0.4187	0.3161	$\underline{1.61}\times\underline{10^{-10}}$
3	0.7818	0.4187	0.0486	$\underline{1.61}\times\underline{10^{-10}}$
4	0.3884	**2.99×10^{-16} (2 CAPS + Pyro)**	0,2848	0.2848
5	0.3884	0.3623	**2.99×10^{-16} (Pyro)**	0.7308
6	0.3884	0.5494	3.38×10^{-04}	0.7308
7	0.135	0.2418	0.3623	0.7308
8	0.3884	0.4962	0.3623	0.0363
9	**2.9887×10^{-16} (CAPS+Pyro)**	0.5494	0.1895	0.7308
10	0.3342	0.2418	0.3623	$\underline{1.61}\times\underline{10^{-10}}$
11	0.5494	0.5494	3.38×10^{-04}	3.38×10^{-04}
12	0.135	0.5494	7.82×10^{-04}	0.7308
13	0.3884	0.4187	**2.99×10^{-16}**	0.7308
14	***0.0048*** } **(CAPS)**	0.3387	0.7308	0,7308
15	***1.12×10^{-04}*** }	3.38×10^{-04}	0.7308	0,7308
16	0.135	3.38×10^{-04}	**2.99×10^{-16} (CAPS)**	0.7308
17	0.135	0.2418	**2.99×10^{-16}**	0.7308
18	0.1311		0.2409	0.7308
19	0.3884		0.0486	0.7308
20	0.5494			0.5089
21	0.3074			6.66×10^{-04}
22	0.135			$\underline{8.55}\times\underline{10^{-12}}$
23	0.135			
24	0.1311			
25	$\underline{7.84}\times\underline{10^{-13}}$			
26	0.2848			
27	5.53×10^{-09}			
28	**3.97×10^{-15}**			

Supplemental Files:

Supplemental File S1 Information about accessions used in the variety study including donor number, source, origin, resistance reaction and described resistance genes in the literature

Supplemental File S2 Map of the *Rrs2* locus on barley chromosome 7HS indicating the location, names and sizes of six genomic regions which were analysed from 72 barley accessions concerning their SNP patterns. Flanking markers of the *Rrs2* co-segregating area and of the whole interval which delimits the location of *Rrs2* are indicated. As far as known, exact or estimated distances between the analysed regions are given

Supplemental File S3 SNP patterns of 72 barley accessions obtained from four genomic regions of the *Rrs2* locus