Theor Appl Genet (1995) 90: 920–924

G. F. Schweizer · M. Baumer · G. Daniel · H. Rugel
M. S. Röder

RFLP markers linked to scald (*Rhynchosporium secalis*) resistance gene *Rh2* in barley

Received: 11 October 1994 / Accepted: 8 December 1994

Abstract *Rhynchosporium secalis* is the causal organism of barley scald disease. A number of resistance genes against the fungus are well known; one of them, the single dominant *Rh2* resistance gene, has been mapped on the linkage map of barley using RFLP (restriction fragment length polymorphism) markers. The *Rh2* gene was located on the distal part of chromosome arm 1S co-segregating with the RFLP marker CDO545 in 85 doubled-haploid progeny plants. The spring barley test population used was a cross between the 6-rowed American spring barley cv Atlas, C.I. 4118, carrying the *Rh2* resistance gene, and a Bavarian 2-rowed malting barley cv Steffi, susceptible for *R. secalis*. The assessment of resistance versus susceptibility was based on artificial infections with a one-spore inoculum in greenhouse tests and with pathotype mixtures in field tests. By testing a pathotype mixture of German origin good resistance was found for the *Rh2* gene in the field.

Key words *Hordeum vulgare* · *Rhynchosporium secalis* · Scald · Resistance gene *Rh2* · Restriction fragment length polymorphism (RFLP)

Introduction

Barley (*Hordeum vulgare* L.) is an ideal crop for gene mapping and has some special advantages for genetic studies: it is a diploid inbreeder, the in vitro haploid technique is well established, the genetic map comprises more than 150 phenotypic markers, which define seven linkage groups (von Wettstein-Knowles 1992), and several extensive RFLP maps have been constructed which cover a total of 1453 cM (Graner et al. 1991; Heun et al. 1991; Kleinhofs et al. 1993). A major disadvantage – especially for map-based cloning – is the large genome which contains approximately 5.5×10^9 bp of DNA (Bennett and Smith 1976). However, RFLP markers can easily be used for gene tagging and, because of their environmental stability and nearly unlimited availability, have become an essential tool for plant breeding. In addition to this, the RFLP procedure is easily standardized for all DNA probes.

Scald in barley, caused by the fungus *Rhynchosporium secalis* (Oudem.) J. J. Davis, is an economically important disease that is found worldwide in cool, semi-humid areas of barley production (Beer 1991; Xue and Hall 1992). In humid years, the losses caused by scald are greater than those caused by powdery mildew. Since chemical control in agriculture is limited, the introduction of resistance into cultivars is of considerable interest.

The pathogen *R. secalis* is a fungus imperfectus and lives sub-cuticulary, primarily above anticlinal epidermal cell walls of leaves, during early stages of pathogenesis (Wevelsiep et al. 1993). Resistance of barley to the pathogen is often controlled by a gene-by-gene interaction, a gene for resistance in the plant corresponding to a gene for avirulence in the pathogen (McDonald et al. 1989; Hahn et al. 1993). Studies on the inheritance of resistance to *R. secalis* in barley led to the definition of several major and minor resistance genes, such as *rh11* (Habgood and Hayes 1971), *Rh3* and *Rh4* (Dyck and Schaller 1961a, b). Many of these resistance genes segregate independently and show no coupling with the resistance gene *Rh2*. The *Rh2* gene in cv "Atlas" gave resistance against 11 out of 35 pathotypes of *R. secalis* in Australia (Ali 1976). Consequently, depending on the *R. secalis* pathotype, Atlas C.I. 4118 was described as either susceptible or resistant to scald (Riddle and Briggs 1950). Good resistance was found in cv "Atlas" for a pathogen mixture representing the Bavarian pathovars (Baumer, unpublished results).

The objective of the present study was to indentify molecular markers linked to the *Rh2* gene for resistance to

Communicated by G. Wenzel

G. F. Schweizer (✉) · M. Baumer · G. Daniel · H. Rugel
Bayerische Landesanstalt für Bodenkultur und Pflanzenbau (LBP), Biotechnologie, Vöttingerstraße 38, 85354 Freising, Germany

M. S. Röder
Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Abteilung Cytogenetik, Corrensstr. 3, 06466 Gatersleben, Germany

scald which can be used for a marker-based breeding program for *R. secalis*-resistance in barley.

Materials and methods

Plant material

Genetic data are based on 85 doubled-haploid (DH) barley lines derived from a cross between the spring malting barley cv "Steffi"(Saatzucht Ackermann, Irlbach), 2-rowed and very susceptible to scald, and cv "Atlas" C.I. 4118 (Harlan and Martini 1936; Dyck and Schaller 1961 a), 6-rowed and carrying the single dominant *Rh2* gene for resistance to scald. "Atlas" is a widely grown malting barley in California and originated from a pure-line selection made from Coast barley in 1917 (Riddle and Briggs 1950). The in vitro regeneration of anthers was conducted as described by Daniel and Baumann (1990).

Resistance testing

The reaction of the 85 DH-lines against *R. secalis* was assessed in a seedling test in the greenhouse in seven replications. For evaluating resistance, two different single-spore isolates were used in combination in seven replicates. One isolate was collected in Uffenheim, Bavaria, the other one in Kaltenberg, Thuringia.

The DH-lines were planted in plastic pots, each line being represented by five seedlings, and were grown in the greenhouse at 15–20°C. At the two-to-three leaf-stage, a spore suspension adjusted to 500 000 spores per ml was sprayed onto the plants with a chromatography sprayer. After inoculation the barley plants were placed into a growth chamber at 16°C and 100% relative humidity in the dark. After 48 h the plants were returned to the greenhouse, where symptom expression permitted disease evaluation 21 days after inoculation. For disease rating the scale described by Jackson and Webster (1976) was used to score the foliar symptoms on the following basis: 0=no visible symptoms; 1=very small lesions confined to the leaf margins; 2=small lesions not confined to leaf margins; 3=large coalescing lesions, involving a majority of the leaf area; and 4=total collapse of the leaf. Reaction types 0, 1 and 2 were recorded as resistant, 3 and 4 as susceptible.

DNA extraction

DNA extractions were performed according to Dellaporta et al. (1983), Tai and Tanksley (1990) and Anderson et al. (1992) with the following modifications. Plant tissue (6 g of leaf) was frozen in liquid nitrogen, powdered with a mortar and pestle and transferred into 50-ml Falcon tubes with 20 ml of extraction buffer [100 mM Tris-HCl pH 8.0, 50 mM EDTA, 500 mM NaCl, 1.25% SDS (w/v), with 0.38 g/100 ml of sodium bisulphite added immediately before use]. The mixture was incubated at 65°C for 30 min, chloroform/isoamylalcohol (24:1) extracted, and centrifuged (15 min, 6000 g, 4°C). The aqueous phase was transferred into a new tube and mixed with 2 µg of RNase (Boehringer) and incubated at 20°C for 30 min. The probes were then centrifuged at 40 000 g for 30 min and the supernatant was transferred into a new tube. The nucleid acids were precipitated with 1/10 vol (v/v) 3 M sodium acetate pH 5.2 and 2 vol (v/v) of ice-cold ethanol for 30 min at –20°C. The DNA was hooked out and washed in 1 ml of cold 70% ethanol, dried under vacuum for 6 min at 20°C and then dissolved in 1TE pH 8.0. We obtained about 20–40 mg DNA/g fresh weight.

RFLP analysis

The RFLP probes used in this study were from barley cDNA (BCD), oat cDNA (CDO), and wheat genomic (WG) libraries and have been developed and mapped by the group of Tanksley and Sorrells, as previously described by Heun et al. (1991). RFLP probes originating from several barley *Pst*I libraries (prefix "MWG") were kindly provided by the group of Fischbeck, Wenzel, Herrmann and are described in Graner et al. (1991).

The RFLP analyses were performed as described in Anderson et al. (1992) using four restriction enzymes (*Eco*RI, *Eco*RV, *Dra*I, *Hin*dIII; Boehringer-Mannheim).

DNA transfer and labelling

The DNA transfer for non-radioactive labelling was conducted with a vacuum blotting apparatus (Millipore in Eschborn) and the following conditions: 6 min 0.25 M HCl; 25 min 0.5 M NaOH, 1.5 M NaCl; 30 min 1.5 M NaCl, 0.5 M Tris-HCl pH 7.2; 40 min vacuum transfer (90 mbar) with 20×SSC on a Nylon membrane (positively charged, Boehringer-Mannheim). Fixation of the DNA was carried out for 15 min at 120°C. The southern transfer for radioactive labelling and the hybridization procedures are described in Heun et al. (1991). The non-radioactive DNA-labelling with digoxigenin-dUTP was done by random priming; hybridization and detection were performed according to the user manual of Boehringer-Mannheim with Lumi-PPD as substrate.

Linkage analyis

Linkage analyis was conducted using MAPMAKER computer software (Lander et al. 1987).

Results

Since the chromosomal location of *Rh2* was unknown, previously mapped RFLP probes which were evenly distributed in the barley genome were used to screen for loose linkage. For this purpose, 40 RFLP clones were surveyed for polymorphism between the parents of the test cross "Atlas" and "Steffi" using three enzymes (*Eco*RI, *Eco*RV, *Hin*dIII) and the radioactive-labelling method. Polymorphism with at least one enzyme was found for 28 clones (70%). The polymorphic clones were tested for linkage with the *Rh2* resistance gene on filters containing seven susceptible and five resistant plants of the progeny. Linkage was detected for clone BCD130 which maps to the distal region of chromosome arm 1S (short arm or plus arm) of barley. The coupling of *Rh2* to BCD130 was confirmed by using more progeny plants, the recombination fraction was 10.6%.

Neighbouring markers were identified from the published RFLP maps (Graner et al. 1991; Heun et al. 1991) and mapped on 85 doubled-haploid lines of the test cross using both the radioactive- and the digoxigenin-labelling methods. In total, ten RFLP markers from barley chromosome 1S were placed on the test cross (Fig. 1) with genetic distances to *Rh2* of between 0 and 21.4 cM and a LOD score of >3. The closest linkage was obtained with clone CDO545 which had no recombination to *Rh2*. Polymorphism for CDO545 was obtained after digestion with *Sac*I or *Dra*I (Fig. 2). A size polymorphism was obtained after digestion with *Dra*I with a 3.6-kb fragment in "Atlas" and a 4.0-kb fragment in "Steffi". Only one recombination

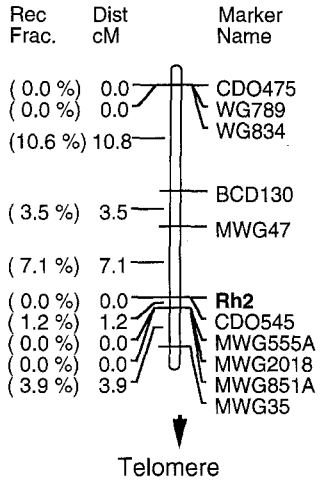

Fig. 1 RFLP map of the distal region of barley chromosome 1S around the *Rh2* gene. The map is depicted in Kosambi (1944) units. The LOD score of the map presented is >3

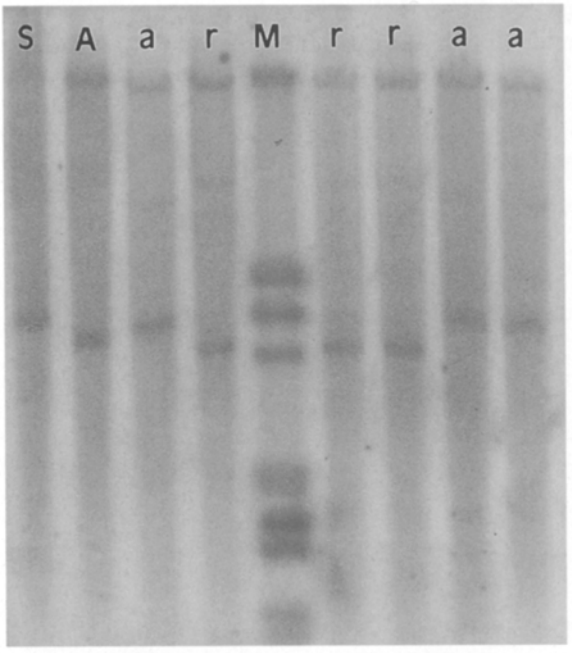

Fig. 2 Southern analysis of *Dra*I-digested DNA from Atlas (*A*), Steffi (*S*) and from susceptible (*a*) and resistant (*r*) DH-lines. The DNA was separated on a 0.8% agarose gel and hybridized with the digoxigenin-labelled probe CDO545. DNA size (*S*=4.0 kb; *A*=3.6 kb; *M*=DNA molecular-weight marker III of Boehringer Mannheim)

event was found between *Rh2* and MWG555A and two co-segregating markers, MWG2018 and MWG851A.

All markers on chromosome arm 1S mapped on the test cross were skewed towards the Atlas allele. The skewing decreased from the proximal markers towards markers located near the telomere (Table 1). Maximum skewing was observed for WG834 with a ratio of 2.36:1=A:S ("Atlas" towards "Steffi" alleles). For *Rh2*:*rh2* the ratio was 1.43:1, which was below the 5% significance level for distorted segregation in a chi^2 test.

Table 1 Distorted segregation ratios

Marker	Ratio "A":"S"	χ^2	Significance level
MWG35	1.33	1.57	–
MWG555	1.37	2.04	–
CDO545	1.43	2.65	–
Rh2	1.43	2.65	–
MWG47	1.83	7.02	1.0%
BCD130	2.0	9.89	0.5%
WG834	2.36	13.76	0.1%

Discussion

The *Rh2* resistance gene was mapped to the telomeric region of the short (plus) arm of chromosome 1. A tight linkage with RFLP marker CDO545 was found. Diallele analysis with other barley lines which are resistant against *Rhynchosporium secalis* (Rugel in preparation) confirm the independent heredity of *Rh2* from other described scald resistance genes (Dyck and Schaller 1961a, b; Habgood and Hayes 1971). One of these, the *Rh* locus on barley chromosome 3L, was tagged with RAPD markers (Barua et al. 1993).

The anther-culture-derived mapping population showed a skewed segregation around the *Rh2* resistance locus. In the short arm of chromosome 1 significantly more "Atlas" than "Steffi" alleles were detected. Distorted segregation ratios have been observed in several DH populations of barley (Kintzios et al. 1994). One possible explanation for the skewing in this region is a indirect selection during the in vitro phase in the production of the DH lines (Powell et al 1986; Graner et al. 1991; Thomson et al. 1991). The occurrence of a gametophytic factor *Ga*, leading to a distorted segregation, was reported for chromosome 1 near the waxy locus *wy* in barley (Tabata 1961; Konishi et al. 1990). It is possible that such a factor is present in the Steffi × Atlas cross; however, further data will be needed to test this possibility.

The comparison between (^{32}P)- and digoxigenin-labelled single-copy probes shows a limited storage life for radioactive-labelled probes but a good re-probing quality of the Southern-blot filters; therefore, radioactive labelling is more useful for surveying numerous clones. The major problem encountered in using the non-radioactive method is the higher background involved, but sufficient signal intensity can be achieved for single-copy probes. Non-radioactive detection is especially useful for screening plant material with labelled probes for which close linkage to the gene of interest has been established.

The aim of this work is the application of marker-based selection in plant breeding. Marker-based selection allows screening for resistance at early stages of development for

a specific resistance locus. It does not require biotests if a good correlation exists, and is independent of the genetic background. Furthermore, if appropriate markers are available, it offers the possibility to screen the same plants simultaneously for several resistance genes. A number of resistance genes in cereals have already been mapped and tagged with RFLP markers. For example, powdery mildew resistance *ml-o* on chromosome 4 (Hinze et al. 1991) and powdery mildew resistance *Ml-a* on chromosome 5 in barley (Jahoor and Fischbeck 1993), leaf-scald resistance *Rh* on chromosome 3 in barley (Barua et al. 1993), virus resistance *ym4* on chromosome 3 in barley (Graner and Bauer 1993), stem-rust resistance *Rpg1* on chromosome 1 in barley (Jin et al. 1993; Kilian et al. 1994), powdery mildew resistance *Pm3* on chromosome 1 in wheat (Hartl et al. 1993), leaf-rust resistance *Lr9* on chromosome 6B in wheat (Schachermayr et al. 1994), steam-rust resistance *Pg3* in oat (Penner et al. 1993 a) and crown-rust resistance *Pc68* in oat (Penner et al. 1993 b). It was found that resistance genes are not randomly distributed over the genome, but frequently occur clustered on particular chromosomal regions (Islam et al. 1989; Hartl et al. 1993; Kreike et al. 1993).

The isolated probe CDO545 used in the present study provides a new tool for the successful identification of the *Rh2* resistance gene, irrespective of the genetic background and independent of the presence of other resistance genes against the same pathogen. The use of this probe and the RFLP mapping of further resistance genes will facilitate specific selection in backcross programs and the pyramiding of resistance genes. The co-segregating RFLP marker with the *Rh2* gene provides a good base for cloning and for a more detailed molecular characterization of the scald resistance gene.

Acknowledgements We thank I. Wiedenmayer, M. Haase and S. Buonamico for excellent technical assistance and Dr. E. Sachs from the BBA in Kleinmachnow for providing single-spore isolates.

References

Ali SM (1976) Pathogenicity of 203 isolates of *Rhynchosporium secalis* on 21 barley cultivars. Physiol Plant Pathol 9:135–143

Anderson JA, Ogihara Y, Sorrells ME, Tanksley SD (1992) Development of a chromosomal-arm map for wheat based on RFLP markers. Theor Appl Genet 83:1035–1043

Barua UM, Chalmers KJ, Hackett CA, Thomas WTB, Powell W, Waugh R (1993) Identification of RAPD markers linked to a *Rhynchosporium secalis* resistance locus in barley using near-isogenic lines and bulked segregant analysis. Heredity 71: 177–184

Beer W (1991) Leaf blotch of barley (*Rhynchosporium secalis*). Zentralbl Mikrobiol 146:339–358

Bennett MD, Smith JB (1976) Nuclear DNA amounts in angiosperms. Phil Trans R Soc Lond Ser B 274:227–274

Daniel G, Baumann A (1990) Einfluß verschiedener Faktoren auf die Pflanzenregeneration in der Antherenkultur bei Sommer- und Wintergerste. Bayer Landw Jahrbuch 64:609–617

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: Version II. Plant Mol Biol Rep 1 (4):19–21

Dyck PL, Schaller CW (1961 a) Inheritance of resistance in barley to several physiologic races of the scald fungus. Can J Genet Cytol 3:153–164

Dyck PL, Schaller CW (1961 b) Association of two genes for scald resistance with a specific barley chromosome. Can J Genet Cytol 3:165–169

Graner A, Bauer E (1993) RFLP-mapping of the *ym4* virus-resistance gene in barley. Theor Appl Genet 86:689–693

Graner A, Jahoor A, Schondelmaier J, Siedler H, Pillen K, Fischbeck G, Wenzel G, Herrmann RG (1991) Construction of an RFLP map in barley. Theor Appl Genet 83:250–256

Habgood RM, Hayes JD (1971) The inheritance to *Rhynchosporium secalis* in barley. Heredity 27:25 37

Hahn M, Jüngling S, Knogge W (1993) Cultivar-specific elicitation of barley defense reactions by phytotoxic peptide NIP1 from *Rhynchosporium secalis*. Mol Plant-Microbe Interact 6:745–754

Harlan HV, Martini ML (1936) Problems and results in barley breeding. US Dept Agric Yearbook: 303–346

Hartl L, Weiss H, Zeller FJ, Jahoor A (1993) Use of RFLP markers for the identification of alleles of the *Pm3* locus conferring powdery mildew resistance in wheat (*Triticum aestivum* L.). Theor Appl Genet 86:959–963

Heun M, Kennedy AE, Anderson JA, Lapitan NLV, Sorrells ME, Tanksley SD (1991) Construction of a restriction fragment length polymorphism map for barley (*Hordeum vulgare*). Genome 34:437–447

Hinze K, Thompson RD, Ritter E, Salamini F, Schulze-Lefert P (1991) Restriction fragment length polymorphism-mediated targeting of the *ml-o* resistance locus in barley (*Hordeum vulgare*). Proc Natl Acad Sci USA 88:3691–3695

Islam MR, Shepherd KW, Mayo GME (1989) Recombination among genes at the L group in flax conferring resistance to rust. Theor Appl Genet 77:540–546

Jackson LF, Webster RK (1976) Race differentiation, distribution, and frequency of *Rhynchosporium secalis* in California. Phytopathology 66:719–725

Jahoor A, Fischbeck G (1993) Identification of new genes for mildew resistance of barley at the *Ml-a* locus in lines derived from *Hordeum spontaneum*. Plant Breed 110:116–122

Jin Y, Steffenson BJ, Franckowiak JD (1993) Linkeage between the *Rpg1* gene for stem-rust resistance and the f5 locus on barley chromosome 1. Crop Sci 33:642–634

Kilian A, Steffenson BJ, Saghai Maroof MA, Kleinhofs A (1994) RFLP markers linked to the durable stem-rust resistance gene *Rpg1* in barley. Mol Plant-Microbe Interact 7:298–301

Kintzios S, Islam RM, Fischbeck G (1994) Distorted segregation for mildew resistance in doubled-haploid lines of spring barley. Plant Breed 112:248–251

Kleinhofs A, Kilian A, Saghai Maroof MA, Biyashev RM, Hayes P, Chen FQ, Lapitan N, Fenwick A, Blake TK, Kanazin V. Ananiev E, Dahleen L, Kudrna D, Bollinger J, Knapp SJ, Liu B, Sorrells M, Heun M, Franckowiak JD, Hoffman D, Skadsen R, Steffenson BJ (1993) A molecular, isoenzyme and morphological map of the barley (*Hordeum vulgare*) genome. Theor Appl Genet 86:705–712

Konishi T, Abe K, Matsuura S, Yano Y (1990) Distorted segregation of the esterase isozyme genotypes in barley (*Hordeum vulgare* L.). Jpn J Genet 65:411–416

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 12:172–175

Kreike CM, de Koning JR, Vinke JH, van Ooijen JW, Gebhardt C, Stiekema WJ (1993) Mapping of loci involved in quantitatively inherited resistance to the potato cyst-nematode *Globoderea rostochiensis* pathotyp Ro1. Theor Appl Genet 87:464–470

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

McDonald BA, McDermott JM, Allard RW, Webster RK (1989) Coevolution of host and pathogen populations in the *Hordeum vulgare-Rhynchosporium secalis* pathosystem. Proc Natl Acad Sci USA 86:3924–3927

Penner GA, Chong J, Wight CP, Molnar SJ, Fedak G. (1993 a) Identification of a RAPD marker for the crown-rust resistance gene *Pc68* in oats. Genome 36:818–820

Penner GA, Chong J, Levesque-Lemay M, Molnar SJ, Fedak G. (1993 b) Identification of a RAPD marker linked to the oat stem-rust gene *Pg3*. Theor Appl Genet 85:702–705

Powell W, Borrino EM, Allison MJ, Griffiths DW, Asher MJC, Dunnwell JM (1986) Genetical analysis of microspore-derived plants of barley (*Hordeum vulgare*). Theor Appl Genet 72:619–626

Riddle OC, Briggs FN (1950) Inheritance of resistance to scald in barley. Hilgardia 20:19–27

Schachermayr G, Siedler H, Gale MD, Winzeler H, Winzeler M, Keller B (1994) Identification and localization of molecular markers linked to the *Lr9* leaf-rust resistance of wheat. Theor Appl Genet 88:110–115

Tabata M (1961) Studies of gametophytic factor in barley. Jpn J Genet 36:157–167

Tai TH, Tanksley SD (1990) A rapid and inexpensive method for isolation of total DNA from dehydrated plant tissue. Plant Mol Biol Rep 8 (4):297–303

Thompson DM, Chalmers K, Waugh R, Fordter BP, Thomas WTB, Caligari PDS, Powell W (1991) The inheritance of genetic markers in microspore-derived plants in barley (*Hordeum vulgare* L.). Theor Appl Genet 81:487–492

Wettstein-Knowles P von (1992) Cloned and mapped genes: Current status. In: Shewry PR (ed) Barley: genetics, biochemistry, molecular biology and biotechnology. CAB International, Wallingford, UK, pp 73–98

Wevelsiep L, Rüpping E, Knogge W (1993) Stimulation of barley plasmalemma H^+-ATPase by phytotoxic peptides from the fungal pathogen *Rhynchosporium secalis*. Plant Physiol 101:297–301

Xue G, Hall R (1992) Effects of surface-wetness duration, temperature, and inoculum concentration on infection of winter barley by *Rhynchosporium secalis*. Phytoprotection 73:61–69