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ORIGINAL PAPER

Fine mapping and chromosome walking towards the *Ror1* locus in barley (*Hordeum vulgare* L.)

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Abstract
Key message **The *Ror1* gene was fine-mapped to the pericentric region of barley chromosome 1HL.**
Abstract Recessively inherited loss-of-function alleles of the barley (*Hordeum vulgare*) *Mildew resistance locus o* (*Mlo*) gene confer durable broad-spectrum disease resistance against the obligate biotrophic fungal powdery mildew pathogen *Blumeria graminis* f.sp. *hordei*. Previous genetic analyses revealed two barley genes, *Ror1* and *Ror2*, that are *Required for mlo-specified resistance* and basal defence. While *Ror2* was cloned and shown to encode a t-SNARE protein (syntaxin), the molecular nature or *Ror1* remained elusive. *Ror1* was previously mapped to the centromeric region of the long arm of barley chromosome 1H. Here, we narrowed the barley *Ror1* interval to 0.18 cM and initiated a chromosome walk using barley yeast artificial chromosome (YAC) clones, next-generation DNA sequencing and fluorescence in situ hybridization. Two non-overlapping YAC contigs containing *Ror1* flanking genes were identified. Despite a high degree of synteny observed between barley and the sequenced genomes of the grasses rice

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(*Oryza sativa*), *Brachypodium distachyon* and *Sorghum bicolor* across the wider chromosomal area, the genes in the YAC contigs showed extensive interspecific rearrangements in orientation and order. Consequently, the position of a *Ror1* homolog in these species could not be precisely predicted, nor was a barley gene co-segregating with *Ror1* identified. These factors have prevented the molecular identification of the *Ror1* gene for the time being.

Introduction

Powdery mildews are widespread obligate biotrophic pathogenic fungi, colonizing about 10,000 flowering plant species (Takamatsu 2004). In barley (*Hordeum vulgare*), monogenic resistance to *Blumeria graminis* f.sp. *hordei* (*Bgh*) is mediated by recessively inherited mutants of *Mildew resistance locus o* (*Mlo*) (Jørgensen 1992a). The *mlo* resistance is effective against all known natural *Bgh* isolates, and has been durable in the field despite extensive deployment in European cultivars for several decades (Jørgensen 1992b). Mutagenesis of the powdery mildew resistant *mlo*-5 null mutant resulted in the identification of two suppressor mutants designated as *Ror1* and *Ror2* (*Required for mlo-specified resistance*) (Freialdenhoven et al. 1996). In addition to partially impairing *mlo* resistance, *ror1* and *ror2* single mutants result in super-susceptibility to *Bgh* when present in a susceptible *Mlo* background, indicating that the two genes contribute to a moderate level of basal resistance present in wild-type plants (Collins et al. 2003). Moreover, the *ror1 ror2* double mutant permits higher colonization levels to *Bgh* than the respective single mutants (Collins et al. 2003), suggesting *Ror1* and *Ror2* control two non-redundant resistance pathways.

The *ror1* and *ror2* mutations also compromise the expression of other traits caused by *mlo* mutations, including an enhanced subcellular H_2O_2 (hydrogen peroxide) burst at attempted fungal entry sites, spontaneous or pathogen-induced formation of cell wall appositions, and mesophyll cell death (Freialdenhoven et al. 1996; Hückelhoven et al. 2000; Peterhänsel et al. 1997; Piffanelli et al. 2002), indicating overlap between processes regulated by *Mlo* and *Ror* genes. However, these genes do show some divergence in the direction or specificity of their effects relating to other phytopathogenic fungi. *Ror1* and *Ror2* contribute to a cell-wall-localized first line of defence of barley against the non-adapted wheat powdery mildew pathogen (*Blumeria graminis* f.sp. *tritici*) (Peterhänsel et al. 1997; Trujillo et al. 2004). Moreover, *Ror1* and *Mlo*, but not *Ror2*, were found to contribute to penetration resistance against the rice blast pathogen (*Magnaporthe grisea*) in barley epidermal cells (Jarosch et al. 1999, 2005). Plant lines with *mlo* and *ror1* mutations were also more sensitive to necrosis-inducing toxin from *Bipolaris sorokiniana* than wild-type (*Mlo Ror1*), but showed less disease symptoms than *mlo Ror1* parents (*ror2* not tested; (Kumar et al. 2001)). Furthermore, mutations in all three genes interfered with aspects of root colonization by the arbuscular mycorrhizal fungus *Glomus mosseae* (Ruiz-Lozano et al. 1999).

The barley *Mlo* and *Ror2* genes have been cloned using map-based approaches. The product of *Mlo* is a seven-transmembrane (7-TM) domain protein whose biochemical activity is unknown (Büschges et al. 1997; Devoto et al. 1999). Based on its coexpression with a large set of defence-related genes, *Mlo* is inferred to play a role in plant immunity (Humphry et al. 2010). *Ror2*, isolated with the assistance of gene synteny between barley and the sequenced rice genome, encodes a t-SNARE protein (syntaxin; Collins et al. 2003). Ror2 and its functional counterpart in *Arabidopsis thaliana* (PEN1) were shown to cooperate with other components of the secretion machinery to provide powdery mildew resistance (Collins et al. 2003; Kwaaitaal et al. 2010; Kwon et al. 2008; Meyer et al. 2009), most likely by facilitating delivery of physical or chemical defence factors to the site of attempted pathogen entry. Arabidopsis *PEN1* plays an analogous role to barley *Ror2*, being required for full expression of the powdery mildew resistance arising from mutations in the Arabidopsis *MLO2* gene (Consonni et al. 2010).

In Arabidopsis, results of genetic epistasis analysis suggested a decisive role for lineage-specific secondary metabolites in *mlo2*-conditioned antifungal defence against biotrophic powdery mildews (Consonni et al. 2010; Bednarek et al. 2009, 2011). This raises the question if the immunity of barley *mlo* plants also involves accumulation of specific defence-related secondary metabolites, whose biosynthesis or secretion might be dependent on *Ror1*. A previous study (von Röpenack et al. 1998) revealed *p*-coumaroyl-hydroxyagmatine, which is biosynthetically linked with barley-specific antimicrobials (hordatines; Stoessl and Unwin 1970), as a *Bgh*-inducible metabolite in barley leaves. Of note, *p*-coumaroyl-hydroxyagmatine accumulated to higher levels in leaves of infected *mlo*-5 seedlings as compared to infected wild type (*Mlo* genotype) plants. However, accumulation of this compound was unaffected by mutation of *Ror1* (von Röpenack et al. 1998). Despite these observations, the possibility remains that *Ror1*-mediated resistance involves production of an as yet unidentified antifungal compound.

The isolation of the *Ror1* gene promises to broaden our understanding of the molecular factors and processes necessary for effective *mlo* resistance and basal resistance towards certain biotrophic and hemibiotrophic fungi. As a first step towards cloning this gene, *Ror1* was localized to a 0.2–0.5 cM interval near the centromere on chromosome 1HL (Collins et al. 2001). Here, we report further efforts

to isolate *Ror1* from the large (5.1 Gb) barley genome, using strategies based on fine mapping, gene synteny, chromosome walking with a barley yeast artificial chromosome (YAC) library and fluorescence in situ hybridization (FISH). Comparative genomics of the *Ror1* region in barley with three other model grasses reveals genomic reorganization in the *Ror1* pericentric region of chromosome 1HL.

Materials and methods

Plant material

Barley lines used in this study include cv. Ingrid (*Mlo*), landrace Grannenlose Zweizeilige (*mlo*-11; Jørgensen 1976), mutant *mlo*-3 in the background of cv. Malteria Heda (Favret 1965), BCIngrid *mlo*-5 lines containing mutations in *ror1* (A39, *ror1*-1; A89, *ror1*-2; C36, *ror1*-3; C69, *ror1*-4; C88, *ror1*-5 (Freialdenhoven et al. 1996); C33, *ror1*-6 and C82, *ror1*-7 (this work)), and *ror2* (A44, *ror2*-1; Freialdenhoven et al. 1996), and a panel of recombinants for the *Ror1* interval (C473, 74-2, 51, 77-5, 111, 21-2, 26-3, C487 and 102-1; Collins et al. 2001) derived from a cross between the partially susceptible *ror1* mutant line A89 (*mlo*-5 *ror1*-2; Freialdenhoven et al. 1996) and the fully resistant *mlo*-3 mutant (genotype *mlo*-3 *Ror1*) in the background of cv. Malteria Heda.

Initial fine genetic mapping

The two mapping crosses, segregating for partial resistance at the *Ror1* locus (C69 × Grannenlose Zweizeilige and A89 × Malteria Heda), have been described (Collins et al. 2001). Fine mapping was performed using a panel of 16 recombinants for the *Ror1* interval (markers *CDO1173* to *ABG452*), that had been marker-selected from 1,301 to 1,399 F_2 plants from the first and second cross, respectively. This panel comprised seven previous recombinants (Collins et al. 2001), and nine recombinants that were identified in the current study by screening 1,087 A89 × Malteria Heda F_2 plants. The *Ror1* locus genotypes of F_2 recombinants were determined using resistance assays on recombinant-derived $F_{2:3}$ or $F_{3:4}$ families, as described previously (Collins et al. 2001).

The known relationship between rice chromosome 10 and the middle of barley chromosome 1H (Stein et al. 2007) was used to identify barley genes for generation of new molecular markers potentially linked to *Ror1*. Rice genes in the interval ~18–19 Mb on chromosome 10 were used in BLASTn searches at NCBI (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) to identify barley expressed sequence tags (ESTs) from putative orthologs, which were then used to derive polymerase chain reaction (PCR)-based barley cleaved amplified polymorphism (CAPS) or direct-sequencing markers, using established procedures (Chen et al. 2009). Alternatively, PCR amplicons representing barley gene fragments were cloned and used as probes to map restriction fragment length polymorphisms (RFLPs). Markers were mapped by scoring them on the 16 recombinants. Because the markers were not scored on the whole population, genetic distances obtained between the outermost markers on the maps may not be representative of those that might have otherwise been obtained using the whole population.

Brachypodium sylvaticum BAC library and screening

A bacterial artificial chromosome (BAC) library of *Brachypodium sylvaticum* with 30,228 clones and an average insert size of 102 kb (Foote et al. 2004) was screened by PCR for the presence of clones containing the *Ror1* closest predicted genes, *Con* and *Pol*. *Pol* encodes a RNA polymerase I subunit 2, *Con* a protein of unknown function. The fragment across the gap between *Pol* and *Con* was amplified using the primer pair MB49-CONS-16 (5′-AGCTATACCGGCTCATGCCGATGG-3′) and MB44-POL-2 (5′-GTATAATTGGTGACAAGTTCAGCA-3′).

Screening of the barley YAC library and chromosome walking

The initial barley YAC library from cv. Ingrid, comprised 40,400 clones with an average insert size of 480 kb, representing approximately four genome equivalents (Simons et al. 1997). DNA from 330 YAC pools (primary pools), each representing 96 yeast clones, was prepared according to Schmidt et al. (2001) and screened by two consecutive rounds of PCR (using sets of nested primers). Positive primary pools were re-screened by columns and rows and the positive YAC clone was confirmed by colony PCR.

To isolate the end fragments from the YAC inserts, the PCR-based "bubble oligonucleotide" approach (Chaplin and Brownstein 2001a) with the exception of some primers from Ogilvie and James (1996) was used. For clones YHV87-A3R and YHV82-B11L the restriction enzymes *Rsa*I (Fermentas GmbH, St. Leon-Rot, Germany) and *Pvu*II (New England Biolabs GmbH, Frankfurt, Germany) were used, respectively. For YHV158-C12L restriction enzymes *Pvu*II and *Bgl*II (Roche, Mannheim, Germany) were selected. The YAC end sequences were used to design primer pairs (Table S1) for re-screening of the YAC library to identify overlapping clones. Because of the presence of highly repetitive regions in some of the YAC ends, the second round PCR product was digested with restriction enzymes to identify unique (informative) repeat units present in the positive YAC clone, which could be used for

comparison to the products obtained from the putatively overlapping clones. For clones YHV87-A3R and YHV82-B11L the restriction enzymes *Hph*I (Fermentas GmbH, St. Leon-Rot, Germany) and *Dra*I (New England Biolabs GmbH, Frankfurt, Germany), respectively, were selected.

All PCR primers were designed using the FastPCR software (Kalendar et al. 2009). PCR fragments were purified using the Nucleo Spin Extract II Kit (Macherey–Nagel GmbH & Co. KG, Düren, Germany) before Sanger sequencing on ABI Prism 377 and 3700 sequencers (Applied Biosystems, Weiterstadt, Germany) using BigDye-terminator v3.1 chemistry. Premixed reagents were from Applied Biosystems. The software BioEdit (Hall 1999) or Lasergene 7/SeqMan (DNA star Inc., Madison, WI, USA) was used to analyze the sequence data.

Next-generation sequencing of YAC clones

YAC DNA from clones YHV87-A3, YHV82-B11 and YHV158-C12 was isolated using the protocol described by Chaplin and Brownstein (2001b). For clones YHV72-C11, YHV354-G1, YHV305-A11 and YHV66-H11 the Yeast DNA Isolation Kit E.Z.N.A. (Omega Bio-tek, Norcross, GA, USA) was used. For samples YHV87-A3, YHV82-B11, YHV158-C12, YHV72-C11, YHV354-G1 and YHV305-A11, 3 µg of DNA was fragmented with a Covaris S2 focused ultrasonicator (Covaris, Woburn, MA, USA). Fragments were purified using Ampure Beads (Beckman Coulter Genomics, Danvers, MA, USA). Libraries were prepared following Illumina standard protocols. Each sample was sequenced on one lane of a Genome Analyzer IIx (GAIIx; Illumina, San Diego, CA, USA), using paired-end reads (2 × 36 bp), following the manufacturer's recommendations. Library construction and DNA sequencing were performed by the Cologne Center for Genomics at the University of Cologne. For sample YHV66-H11, 1 µg of DNA was fragmented on the Covaris S2 (Covaris, Woburn, MA, USA) and purified with the Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany). A TruSeq DNA library was prepared according to recommendations of the supplier (TruSeq DNA sample preparation v2 guide, Illumina). The library was quantified by fluorometry, immobilized, barcoded and processed onto a flow cell with a cBot (Illumina, San Diego, CA, USA) followed by sequencing-by-synthesis with a paired-end 2 × 100 bp reads on a HiSeq2000 system. Sequencing data was processed with standard Illumina pipeline software, CASAVA 1.8. Library construction and DNA sequencing were performed by the Max Planck Genome Centre in Cologne.

Reads were filtered against the yeast (*Saccharomyces cerevisiae*) genome, downloaded from the comprehensive yeast genome database (CYGD, http://ftpmips.gsf.de/yeast/sequences) at the Munich Information Center for Protein Sequences (MIPS, http://mips.helmholtz-muenchen.de). The aligner software Bowtie (Langmead et al. 2009) was used in paired-end mode, with a maximum insert size of 500 nucleotides. The remaining reads that did not align to the yeast genome were aligned to barley sequences in two databases: HarvEST (version 1.77, assembly No. 35, http//harvest.ucr.edu) and a barley full length cDNA (FLcDNA) collection (Matsumoto et al. 2011), DDBJ accessions AK353559-AK377172. The alignments were visualized using the integrative genomics viewer (IGV, Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA, USA; Robinson et al. 2011). Furthermore, the reads were partially assembled using the software ABySS (Simpson et al. 2009) with parameters $k = 20$, $n = 10$. The resulting contigs were used in BLAST searches (Altschul et al. 1990) against either the NCBI database or sorted chromosome arms at the IPK Gatersleben database (barley BLAST server, http://webblast.ipk-gatersleben.de/barley/).

Analysis of *Ror1* candidate genes

Genomic DNA from seven-day-old barley seedlings from the panel of recombinants for the *Ror1* interval, *ror1* mutants and mapping parents was isolated using the urea-phenol method (Shure et al. 1983). The presence of each gene in a respective YAC clone was confirmed by two consecutive rounds of PCR using DNA from the individual YAC clone as initial template. PCR primers (Table S2) were designed using the FastPCR software (Kalendar et al. 2009) on the basis of the respective EST or FLcDNA sequences. For each gene, the first round PCR product, amplified from Malteria Heda and Ingrid genomic DNA, was sequenced and aligned to identify DNA polymorphisms between the two barley parental lines. Each marker was scored on the panel of recombinants for the *Ror1* interval. Its orientation with respect to each recombination event was then used to locate it on the high resolution map. In case of *Ppr* and *PR17c* (accession number EU131174), the coding sequence was PCR-amplified and sequenced directly from the *ror1* mutants. For *Ppr* we used the primers shown in Table S2, for *PR17c* we used the primer pair PR17cF (5′-CTCAGC GACACAGGCACGTA-3′) and PR17cR (5′-ACCGTCAA GTAGGCTAATCGTG-3′). Purification and sequencing of PCR products was performed as described above.

Fluorescence in situ hybridization (FISH)

Mitotic chromosome spreads of barley cv. Ingrid were prepared using the spreading technique described by Kato et al. (2006). Probes were generated using unique sequences in the *Con*, *Pol*, *Myo* and *Unk* genes, which were PCR-amplified from either genomic DNA of barley cv.

Ingrid or YAC DNA of clones YHV87-A3, YHV82-B11, YHV158-C12 or YHV305-A11 using the primers shown in Table S3. PCR products were purified by agarose gel slices using the MinElute Gel Extraction Kit with an additional cleanup step employing the QIAquick PCR purification kit, both from Qiagen (Hilden, Germany). The chromosome 1HL-specific probe pHv-1112 has been described before (Kato 2011). The probes were labeled with Texas red-dUTP (Perkin Elmer, Waltham, MA, USA), Alexa-488-dUTP (Invitrogen, Karlsruhe, Germany) or Cy-5-dUTP (GE Healthcare Life Science, Piscataway, NJ, USA) by nick translation as described by Kato et al. (2006). For FISH, the procedure of Ma et al. (2010) was followed. The monochromatic images were pseudo-colored and merged using Adobe Photoshop.

Comparative genomic analysis

To enable identification of potential homologs and to examine gene order conservation between grass species, barley ESTs or FlcDNAs from the genes present in the barley YACs were used as queries for BLAST searches and synteny analysis with *Orzya sativa* (http://rice.plantbiology.msu.edu/), *Brachypodium distachyon* (http://gbrowse.brachypodium.org) and *Sorghum bicolor* (http://genome.jgi.doe.gov/Sorbi1) genomes through the web-based resource EnsemblPlants (http://plants.ensembl.org/index.html).

ESTs and FlcDNAs were also used to search whole genome shotgun (WGS) sequence assemblies, high confidence (HC) gene sequences, and sequences of sorted chromosome arms available for barley by performing BLAST searches on the IPK barley BLAST server http://webblast.ipk-gatersleben.de/barley/. Integrated metadata sets of the different anchoring strategies and synteny "genomic stratification" of the barley genome were obtained via FTP download from ftp://ftpmips.helmholtz-muenchen.de/plants/barley/public_data/ (The International Barley Genome Consortium 2012).

Secondary metabolite analysis

First barley leaves—either non-inoculated or inoculated with a high density of *Bgh* (isolate K1) conidiospores—were collected at 24 and 48 h after inoculation and frozen in liquid nitrogen. In a compatible interaction (*Mlo* genotype), at these time points the pathogen has succeeded in haustorium formation at the majority of interaction sites (24 h post inoculation) and continues its development by elongating secondary hyphae that penetrate additional distal epidermal cells (48 h post inoculation). In the case of the resistant *mlo* genotype, fungal sporelings do not achieve host cell penetration, while partially susceptible *mlo ror1* double mutants show an intermediate phenotype (host cell entry rate ca. 20 % at 48 h post inoculation; Freialdenhoven et al. 1996). After addition of dimethyl sulfoxide (DMSO, 50 µl/20 mg FW), 50–100 µg samples of barley leaves were homogenized with metal beads (diameter 4 mm) in a Mixer Mill MM 400 (Retsch, Haan, Germany) and centrifuged for 15 min at 20,000*g*. The supernatants were collected and subjected to analysis on an Acquity UPLC system (Waters, Milford, MA, USA) equipped with PDA eλ detector.

Results

Initial fine genetic mapping

The rice collinearity approach generated 21 new gene-based markers closely linked to the barley *Ror1* locus. For 13 genes, polymorphisms identified by amplicon sequencing (Table S4) were used to devise marker assays based on CAPS or direct-sequencing assays, and the other eight genes were mapped by RFLP analysis (Table S5). These markers, together with four others previously described (Collins et al. 2001) were scored on the panel of recombinants for the *Ror1* interval (Table S6; Fig. 1). With the exception of the *AK355835* gene, which was polymorphic only for the C69 (*mlo*-5 *ror1*-4) × Grannenlose Zweizeilige (*mlo*-11) cross, the A89 (*mlo*-5 *ror1*-2) × Malteria Heda (*mlo*-3) cross provided more recombination events and polymorphic sites. Hence, only the genetic map constructed using the latter cross is presented (Fig. 1). At this stage, there were no signs of break in collinearity between the mapped locations of the barley genes and the corresponding genes in the rice genome, except for *AK364468* (ortholog of Os05g03910) (Fig. 1).

Amplicon sequencing revealed a non-random distribution of polymorphic sites with respect to the demonstrated or predicted positions of genes along barley chromosome 1HL (see gene blocks I to III in Fig. 1; Table S5). Gene blocks I (seven genes; 17 polymorphisms) and III (six genes; eight polymorphisms) but not II, were polymorphic between Malteria Heda and the other two mapping parent backgrounds, whereas block II (three genes; three polymorphisms), but not block I and III, was polymorphic between the BCIngrid *mlo*-5 background and the other two parental backgrounds.

The *Ror1* locus was delimited to a 0.18 cM interval and was resolved from all mapped genes. One recombination event separated it from *AK355699* in the distal direction (from here named *Con*, encoding a conserved protein of unknown function), and four recombination events separated it from a group of eight genes in the proximal direction (Fig. 1; Table S6). From the latter group, *AK375542* (from here named *Pol*, encoding a RNA polymerase I

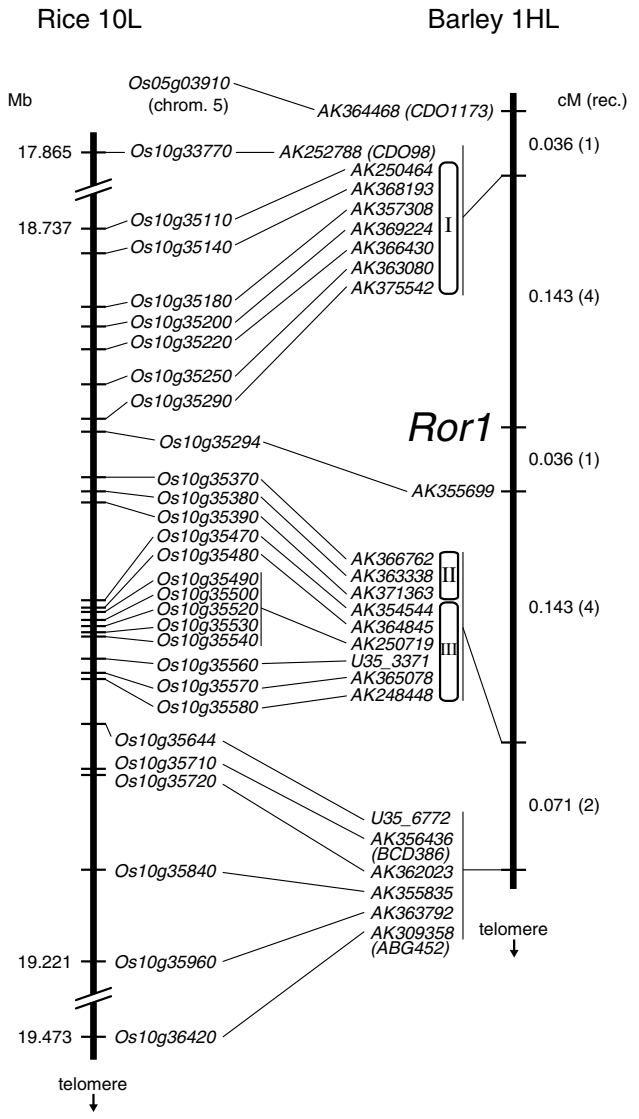

Fig. 1 A89 × Malteria Heda genetic map of the barley *Ror1* region on the long arm of chromosome 1H, aligned to the corresponding sequence interval on the long arm of rice chromosome 10. *Lines* connect orthologous genes. Positions (Mb) in the rice chromosome 10 sequence are shown to the left. Genetic distances (cM) and number of observed recombinants (out of 2,798 meioses) are shown to the right of the barley map. Roman numerals designate groups of co-segregating genes that show the same pattern of polymorphism across the three barley mapping parents (see Table S5). Names of corresponding RFLP probes previously mapped in grasses are indicated in *brackets*

subunit 2) was predicted to be the closest gene to *Ror1* based on the rice collinearity. In the sequenced rice and *Brachypodium distachyon* genomes, genes corresponding to *Con* and *Pol* are immediate neighbors, arranged head-to-head, and are separated by respective intervals of 2.5 and 4.7 kb (between the transcription start sites). These intervals contained no predicted genes. Screening the *Brachypodium sylvaticum* BAC library identified two clones (78G14 and 77I12), each containing orthologs of both *Con* and *Pol*. The region between the two genes was PCR-amplified from one of the clones and sequenced, revealing a 2.5 kb fragment containing no predicted gene(s). Therefore, at this stage a break-down in collinearity prevented the identification of a candidate gene for *Ror1* from the small-genome grasses *O. sativa*, *B. distachyon* or *B. sylvaticum*.

Chromosome walking with YACs

To establish a physical contig spanning the *Ror1* locus, a barley YAC library was screened by PCR for the presence of clones containing the closest predicted genes, *Con* and *Pol* (Table S1). Two YAC clones containing *Pol* (YHV82-B11 and YHV158-C12) and one containing *Con* (YHV87-A3) were isolated (Fig. 2a and b). Subsequently, the left (L) and right (R) ends of these YACs were recovered. The YAC end sequences were used to design primer pairs for re-screening of the YAC library to identify overlapping clones (Table S1). Where highly repetitive DNA was encountered at the YAC ends, amplified YAC end sequences were cleaved with restriction enzymes to identify unique repeat variants that could help identifying genuine overlapping clones (Table S1). When this approach was not successful, low copy sequences found in the partially assembled YAC insert sequences (see next section; File S1) were used to design primer pairs (Table S1) for re-screening the barley YAC library. The walking steps resulted in a contig of five YAC clones around *Pol* and three YAC clones around *Con* (Fig. 2b).

Sequencing of YAC clones

Genomic DNA from all YAC clones, with the exception of YHV415-F4, was isolated and paired-end sequenced via Illumina technology. Clone YHV415-F4 grew poorly, possibly due to instability of its YAC, and it was impossible to isolate its genomic DNA. For all sequenced YACs, except for clone YHV66-H11, 36 bp reads and an average of approximately 40 million reads per clone were achieved. In the case of clone YHV66-H11, 100 bp per read and ca. 15 million reads were obtained. To identify the barley genes present on the YACs, the short sequence reads were filtered and the non-yeast sequences were aligned to publicly accessible barley EST and cDNA sequences. In addition to *Con* and *Pol,* we identified ten more genes localizing on the YACs. BLAST searches using the IPK Gatersleben database (barley BLAST server, sorted chromosome arms, http://webblast.ipk-gatersleben.de/barley/) confirmed these genes were from barley chromosome 1HL. Non-overlap of the two YAC groups was supported by the absence of a gene common to both. Furthermore, clone YHV72-C11 was found to be chimeric, containing DNA sequences from barley

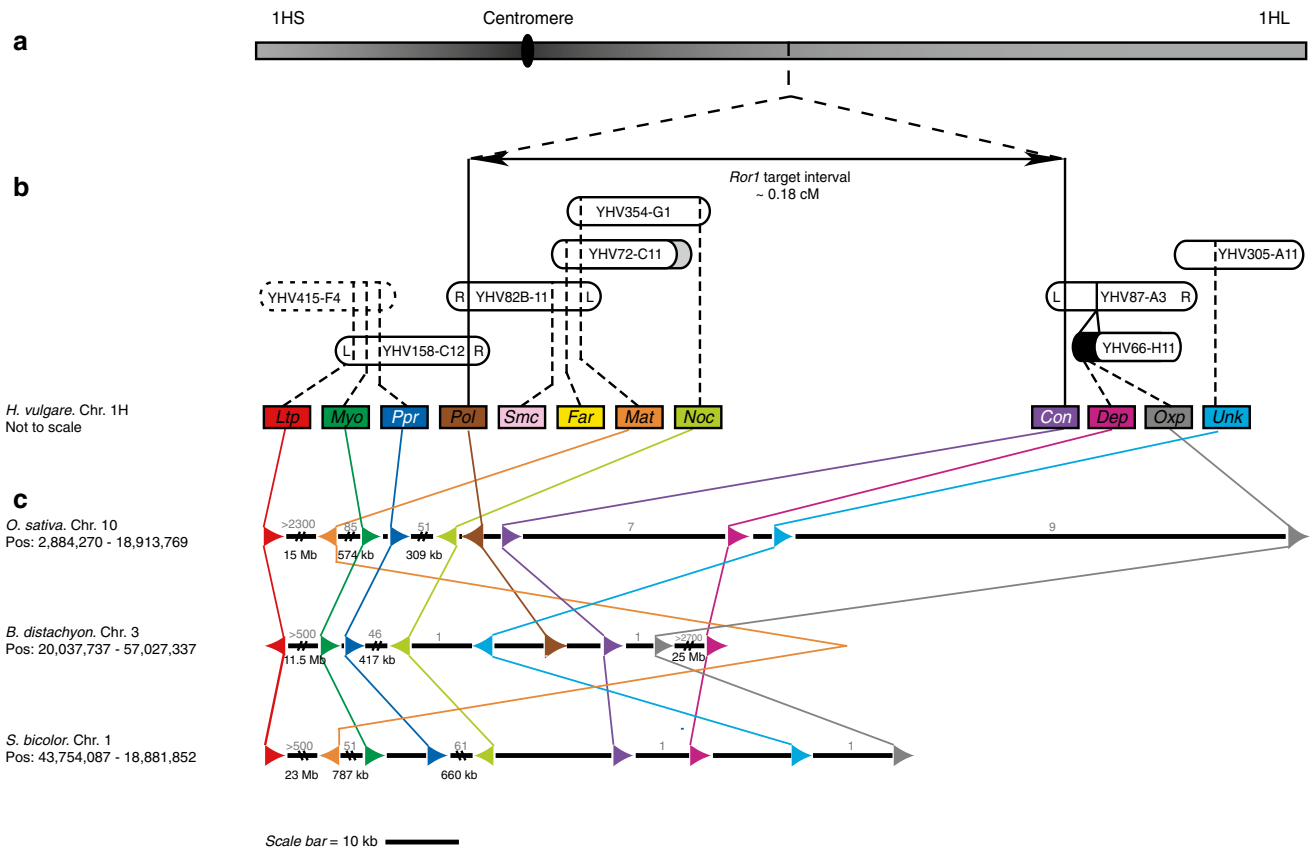

Fig. 2 Schematic representation of the genes in the *Ror1* region relative to the YAC contigs in chromosome 1H in *H. vulgare* and their rearrangements in the syntenic region of *O. sativa* chromosome 10, *B. distachyon* chromosome 3 and *S. bicolor* chromosome 1. **a** Approximate physical location of the *Ror1* locus on chromosome 1HL based on FISH analysis (see Fig. 3) 1HS, short arm of barley chromosome 1H; 1HL, long arm of barley chromosome 1H. **b** YAC contigs established around the predicted *Ror1* flanking genes, *Con* and *Pol* (indicated by *vertical solid lines*). The remaining genes contained in the sequenced YAC clones are indicated by *vertical dashed lines*. A putative chimeric region in clone YHV72-C11 is shown in *grey* and the presumptive deletion from clone YHV87-A3 present in clone YHV66-H11 is shown in *black*. The unstable YAC clone YHV415-F4 is drawn in *dashed lines*. The YAC clones are not displayed to scale. Genes present in the sequenced YAC clones are shown as *colored boxes*; note that the spaces between them do not represent physical distances. The orientation of the YAC clones around *Pol* relative to *Con* is arbitrary and not supported by experimental data. L, Left YAC end; R, Right YAC end. **c** Presence of candidate genes in the syntenic regions of three grass species. Orthologous genes are shown as *arrows* in the chromosomes of the three other monocot species. *Arrows* are drawn in the direction of gene transcription. Note that the orientation of genes *Ltp*/*Myo*/*Ppr* and *Smc*/*Far*/*Mat*/*Noc* in barley relative to *Pol* and the order of genes *Ltp*/*Myo*/*Ppr* is not resolved. Numbers of additional genes in each of the species intervals are shown in *grey*. Chr: chromosome; Pos: starting and ending position from the first to the last identified orthologous gene in each chromosome. All chromosomes have been drawn from the short arm to the long arm (left to right) except for chromosome 1 in *S. bicolor*, which has been inverted

chromosomes 1H, 5H and 7H, as confirmed by the closest BLAST matches (IPK Gatersleben database, barley BLAST server, http://webblast.ipk-gatersleben.de/barley/). In addition, clone YHV87-A3 has probably undergone a rearrangement leading to an internal deletion that includes part of the clone YHV66-H11 carrying genes *Dep* and *Oxp* (Fig. 2b). This conclusion is based on the combination of the following features: (1) presence of common contigs, obtained by partial assembly of short reads (File S1) in clones YHV87-A3 and YHV66-H11, (2) absence of the left and right YAC ends of YHV87-A3 on YHV66-H11, suggesting that clone YHV87-A3 extends beyond the ends of clone YHV66-H11, yet (3) presence of genes *Dep* and *Oxp* on clone YHV66-H11, but not on clone YHV87-A3.

Analysis of candidate genes from the YAC clones

The ten extra genes identified in the sequenced YAC clones (Fig. 2b; Table 1) were analyzed in more detail as *Ror1* candidate genes by mapping them on the panel of recombinants for the *Ror1* interval (Tables S2 and S7). The genes *Dep* (*AK366762*), *Oxp* (*AK354544*) and *Unk* (*AK363338*), were previously mapped telomeric to *Con* using the rice collinearity approach described above (Fig. 1 and Tables S4–S6). However, we found that in barley those genes are

Table 1 Genes present in the sequenced YAC clones and their orthologs in *O. sativa*, *B. distachyon* and *S. bicolor*

Gene ID	HarvEST ID	FLcDNA ID	Gene annotation[a]	Orthologous genes		
				Oryza sativa[a]	*Brachypodium distachyon*	*Sorghum bicolor*
Ltp	U35_540	AK372510	Protease inhibitor/seed storage/lipid transfer protein (LTP) family	LOC_Os10g05720	BRADI3G21070	SB01G026220
	U35_39431					
	U35_39189					
Myo	U35_19977	AK370653	Myosin-2 heavy chain family protein	LOC_Os10g34710	BRADI3G29440	SB01G018770
	U35_28313					
	U35_42973					
Ppr	U35_34977		Pentatricopeptide (PPR) repeat-containing protein	LOC_Os10g34720	BRADI3G29450	SB01G018760
Pol	U35_7164	AK375542	Protein DNA-directed RNA polymerase I subunit RPA2	LOC_Os10g35290	BRADI3G29917	SB04G001790
	U35_35390					
	U35_43139					
Smc	U35_6091	AK363441	Structural maintenance of chromosomes (SMC) N-terminal domain-containing protein	LOC_Os05g51790	BRADI2G14160	SB10G027780
Far	U35_26899		Far-red-impaired response 1 (Far1) transcription factor, *Triticum aestivum*	No orthologs	No orthologs	No orthologs
Mat	U35_2581		Meprin and TRAF homology (MATH) domain-containing protein	LOC_Os10g33830	No orthologs	SB01G019210
	U35_2582					SB09G026713
Noc	U35_25890		Nucleolar complex protein 2 (NOC2)	LOC_Os10g35280	BRADI3G29890	SB01G018260
Con	U35_5789	AK371545	Fiber protein Fb34	LOC_Os10g35294	BRADI3G29930	SB01G018250
		AK355699				
Dep	U35_14486	AK353904	Oxidoreductase, short chain dehydrogenase/reductase family domain-containing protein	LOC_Os10g35370	BRADI3G57380	SB01G018230
	U35_14487	AK359203				
	U35_14489	AK366762				
	U35_36325	AK355367				
Oxp	U35_4615	AK354544	Oxidoreductase protein	LOC_Os10g35470	BRADI3G29950	SB01G018200
Unk	U35_3986	AK363338	Protein of unknown function	LOC_Os10g35380	BRADI3G29910	SB01G018220
	U35_7604	AK362931				

[a] Gene annotation and gene identifier according to *Orzya sativa* at MSU6 (http://rice.plantbiology.msu.edu/) except for *Far*, whose annotation is derived from BLAST search

not arranged in the same order as in *O. sativa*, *B. distachyon* or *S. bicolor* (see comparative genomics below and Fig. 2c). Genes *Ltp*, *Myo*, *Mat* and *Noc* were all separated from *Ror1* by four recombination events and co-segregated with *Pol* (Tables S2 and S7). *Far*, *Ppr* and *Smc* could not be genetically mapped owing to an absence of polymorphism; however, *Far* and *Smc* could be physically ordered within the YAC contig around *Pol* (Fig. 2b). We deduced that *Far* and *Smc* were separated from *Ror1*, because they were physically flanked by *Ltp*/*Myo*/*Ppr* and *Noc*, which were recombined with *Ror1*. *Ppr* was excluded to represent *Ror1* on the basis of direct sequencing of amplicons derived from the *ror1* mutants. Therefore, by a combination of genetic and physical mapping (Tables S6 and S7; Fig. 2b), the ten additional genes identified via the YAC clones were shown to be distinct from *Ror1*.

Physical mapping of *Ror1* and its flanking genes by FISH

We investigated the physical distance between *Ror1*-flanking genes, *Con* and *Pol,* and the order of these and *Myo* and *Unk* by performing FISH of suitable probes on barley metaphase chromosomes. Gene-specific PCR products were pooled to generate FISH probes with a length between 2.6 and 3.3 kb (Table S3). The pHv-1112 repeat-specific probe (Kato 2011) was used to identify the long arm of chromosome 1H. Multi-color FISH of the gene-specific probes in combination with the chromosome 1HL-specific probe revealed in repeated experiments for all genic signals an interstitial position at roughly a third of the way down the long arm of chromosome 1H from the centromere (Fig. 3; depicted also in Fig. 2a). The identified location of all tested genes in the same

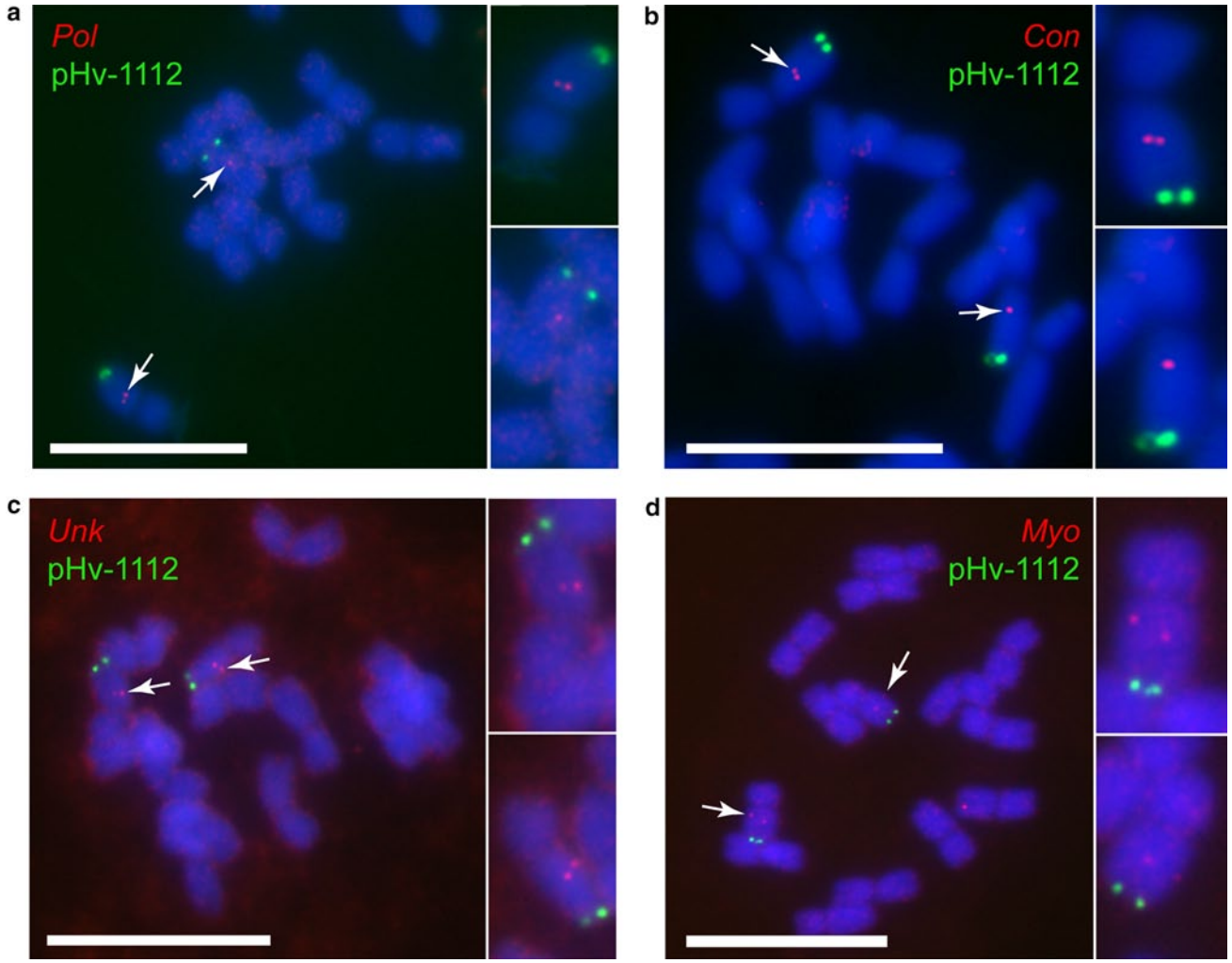

Fig. 3 Micrographs of multicolor FISH on barley metaphase chromosomes of cv. Ingrid. Shown in *green* is the signal of the chromosome 1HL-specific probe pHv-1112 (Kato 2011); shown in *red* and indicated by *arrows* are the signals for the genes of interest: **a** Probe for *Pol*. **b** Probe for *Con*. **c** Probe for *Unk*. **d** Probe for *Myo*. The *insets* show a magnification of the chromosomes with the FISH signals (in part rotated to suit the Figure format). Blue fluorescence, DNA staining by DAPI (4′,6-diamidino-2-phenylindole). *Scale bar* 20 μm (color figure online)

chromosomal region (Fig. 3) provided additional evidence that the proposed YAC contigs around the *Ror1* locus (Fig. 2b) were likely to be correct with respect to the presence of the suggested genes in the isolated YAC clones. Subsequently, we used multi-color FISH with three combinations: (1) *Con* (red) + *Pol* (green) + pHv-1112 (far red); (2) *Con* (red) + *Unk* (green) + pHv-1112 (far red) and (3) *Pol* (green) + *Myo* (red) + pHV-1112 (far red). The probes labeled in red yielded a clear signal; however, the signal of the probes labeled in green was not as strong (Fig. S1). FISH analysis with probes derived from *Con* and *Pol* showed non-overlapping signals on both sister chromatids (Fig. S1a), suggesting a more than one megabase-sized distance (Ma et al. 2010) between these two *Ror1*-flanking genes. However, the order of the genes *Unk* and *Myo* with respect to *Con* and *Pol* could not be resolved (Fig. S1b).

Comparative genomics between various monocots species

Ten out of the twelve genes present in the YAC contigs defined a syntenic region on chromosome 10, 3 and 1 of *O. sativa*, *B. distachyon* and *S. bicolor*, respectively. The core syntenic intervals in *O. sativa*, *B. distachyon* and *S. bicolor* were delimited as physical units of ~1040, ~492 and ~1540 kb, harboring 161, 55 and 122 putative genes, respectively (Fig. 2c). This comparison excludes *Ltp* and *Dep* orthologs, located >10 Mb away from the core syntenic intervals in one or more of the species. Breaks in the gene collinearity included a lack of an ortholog of *Mat*

in *B. distachyon,* an absence of an ortholog of *Far* in any of the three model species, the position of the *S. bicolor Pol* ortholog on chromosome 4, and the location of *Smc* orthologs on chromosome 5, 2 and 10 in *O. sativa*, *B. distachyon* and *S. bicolor*, respectively (Table 1). Within the core syntenic intervals, the conservation was also perturbed by several other gene directionality, gene order and insertion/deletion differences (Fig. 2c).

Utilization of the barley draft genome assembly

Examination of the draft assembly of the barley genome recently released by the International Barley Sequencing Consortium (2012) largely validated our contigs and maps in the *Ror1* region (Table S8). Of the twelve genes in the YAC contigs, *Ltp*, *Myo*, *Ppr*, *Pol*, *Mat*, *Dep*, *Oxp* and *Unk* were identified by the anchoring strategies AC1, AC2 or AC3 and assigned a specific position on chromosome 1H (given in cM/bp; Table S8). The identified contigs contained 95 additional high confidence (HC) barley genes. Gene *Smc* was part of the non-high or low confidence genes and *Far* did not show a clear hit (Table S8), possibly because it may represent part of a retrotransposon erroneously assigned as a protein-coding gene. In addition, *Con* and *Noc* together with 18 other HC barley genes found by synteny ("genomic stratification") with *Bd* are located in relative position 345720720721 on chromosome 1H; unfortunately, this "relative position" is not informative regarding their proximity to the remaining genes found in the YAC contigs. Use of the barley draft assembly therefore allowed identification of 103 genes in the barley *Ror1* region. At first sight *PR17c*, a gene located at the same fingerprint contig position as *Ltp*, *Myo*, *Ppr*, *Pol* and *Mat* caught our attention because of its recently demonstrated function in penetration resistance against barley-powdery mildew (Zhang et al. 2012). The coding sequence of the gene was sequenced from the eight *ror1* mutants but no mutations were found in it, suggesting it was not *Ror1*.

Secondary metabolite analysis

To identify potentially novel *Bgh*-inducible barley metabolites and to examine if accumulation of any of these was dependent on *Ror1*, we performed ultra-high performance liquid chromatography—photodiode array detection (UPLC-PDA) analysis of extracts from *Bgh*-inoculated leaves of cv. Ingrid, BCIngrid *mlo*-5, A89, C69 and C88 (*mlo*-5 *ror1*) and A44 *(mlo*-5 *ror2*) lines, at 24 and 48 h after *Bgh* inoculation. This revealed four compounds, which accumulated to much higher levels in the inoculated leaves relative to the non-inoculated controls (Fig. S2). Based on the UV-spectra obtained for the respective chromatogram peaks two were likely to be hordatine-related, while the remaining two likely represented other classes of secondary metabolites (Fig. S3; Stoessl and Unwin 1970; von Röpenack et al. 1998). Although one compound (RT 10.9) accumulated to higher levels compared to Ingrid wild type and the *mlo*-5 mutant in both the *mlo*-5 *ror1* and *mlo*-5 *ror2* double mutants tested, none of the four detected *Bgh*-induced compounds showed specifically altered levels in the tested *mlo*-5 *ror1* mutants as compared to the other lines (Fig. S2).

Discussion

Our previous *Ror1* mapping (Collins et al. 2001) and the fine-mapping performed in this study (Fig. 1 and Tables S4–S6) revealed an uneven distribution of polymorphisms, manifested as long-range sequence haplotype structures in this region of barley chromosome 1H. Two gene blocks showed no sequence polymorphism between BCIngrid *mlo*-5 (background of the C69 and A89 *ror1* mutants) and Grannenlose Zweizeilige, making the C69 × Grannenlose Zweizeilige cross useful for mapping only 14 out of the 25 genes mapped using the A89 × Malteria Heda cross. There was another potential block of sequence identity between Malteria Heda and Grannenlose Zweizeilige, defined by only three polymorphisms in three genes, although this had no practical consequence, as these two genotypes were not crossed for mapping. These sequence identities are surprising, given that the three parental genotypes were not known to be related. BCIngrid *mlo*-5 is derived from the Swedish cultivars Ingrid and Carlsberg II (Freialdenhoven et al. 1996), and Grannenlose Zwiezeilige is an Ethiopian landrace (Jørgensen 1976). Ethiopian and European barley gene pools are quite distinct (Bjørnstad et al. 1997; Orabi et al. 2007). Malteria Heda is an Argentinian cultivar from 1943 with no recorded Ethiopian parentage (Flavio Capettini, personal communication). This experience highlights the benefit of trialling multiple crosses in a positional cloning project, regardless of any perceived lack of relatedness between parents. Furthermore, this suggests these barley haplotype structures are ancient. While many of the genes in the same haplotype block co-segregated in the experimental populations, suggesting a role for suppressed recombination in the preservation of haplotype blocks through time, multiple haplotype switches were observed in some intervals showing no recombination (e.g. region between *U35_6772* and *AK363792*), indicating that recombination is not the sole driver of haplotype structure, and/or that recombination distribution is cross-dependent.

Barley genes mapping close to the centromeres may be intractable to positional cloning due to the recombination suppression that is typical of the pericentromeric and centromeric regions of barley chromosomes. These regions of

suppressed recombination can encompass as much as half of the physical length of each chromosome (The International Barley Genome Consortium 2012; Künzel et al. 2000; Mayer et al. 2011). *Ror1* was genetically mapped within 0.5 cM of the centromere (Collins et al. 2001) and was physically located by FISH to roughly a third of the way down the long arm of chromosome 1H from the centromere (Fig. 3). This region of 1H exhibits an average genetic/physical ratio of up to 47 Mb/cM (Künzel et al. 2000), giving an expected size of up to 8.5 Mb for the 0.18 cM *Ror1* interval. Consistent with these facts, the two YAC contigs did not overlap, despite considerable extension to both sides of *Con* and *Pol*, and the *Pol* and *Con* probes showed separate hybridization signals by FISH (Fig. S1), suggesting a greater than a megabase-sized distance between the two genes (Ma et al. 2010).

While the initial fine mapping revealed almost no breaks in collinearity between rice and barley, genes from the YACs uncovered multiple short and long-range breaks in gene order conservation between barley and the model grass genomes (Collins et al. 2001; Figs. 1, 2). Perhaps this reflects bias towards the type of genes mapped. For the initial fine mapping, genes that were shown by sequence database searches to be single-copy in rice and barley were preferentially chosen for marker generation, while evaluation of all genes identified from the YAC contigs was attempted, regardless of copy status. A clear statement in this respect is difficult for barley owing to the yet incomplete genome information; however, in rice 9 out of the 12 genes identified on the YAC contigs have more than one copy.

Considering the definition of collinear genes recently proposed by Wicker and co-workers (2010), where "the gene has to be found in a syntenic chromosomal region and four out of its eight closest neighboring genes also have their closest homologs in the same location and order in the other species", we can state that none of the genes in the vicinity of the *Ror1* locus in barley is collinear compared to the other tested grass genomes. Erosion of collinearity increases with phylogenetic distance and mainly due to "gene movement" (Wicker et al. 2010). Chromosome translocations, gene duplications, double-strand break repair and transposable elements can all cause gene movement (Wicker et al. 2010, 2011). An example to highlight is *Ltp*, which is separated from the core syntenic regions in *O. sativa*, *B. distachyon* and *S. bicolor* by ~10–25 Mb (Fig. 2b). Additional instances comprise gene pairs *Myo-Mat* and *Ppr-Noc* that are just a few genes apart from each other in the barley genome, but separated by more than 40 genes each in two of the other grass genomes (Fig. 2c). However, there were also some examples of conserved gene proximity: *Myo*/*Ppr* (in all four grasses), *Dep*/*Unk* (in *O. sativa* and *S. bicolor*), and *Pol*/*Con* (in *O. sativa* and *B. distachyon*). Despite overall gross synteny, small rearrangements of gene content, order and orientation are common among grasses, even between closely related species such as wheat and barley or maize and sorghum (Bennetzen and Ramakrishna 2002). There are few detailed case reports about particular barley loci at which interruptions in microcollinearity in comparison to rice have been observed (Brueggeman et al. 2002; Brunner et al. 2003; Kilian et al. 1997; Taketa et al. 2011). Nevertheless, it seems as if generally breaks in microsynteny are particularly frequent in retrotransposon-rich pericentromeric regions of grass chromosomes (Bowers et al. 2005; Li et al. 2013), which is consistent with our findings of perturbed gene order in the *Ror1* region.

Our experience with *Ror1* is in stark contrast to the cloning of *Ror2*, which concluded rapidly due to the presence of an ortholog of the target gene at the corresponding position in the syntenic region of the rice genome (Collins et al. 2003). The failure to identify *Ror1* in comparison to the sequenced *O. sativa*, *B. distachyon* and *S. bicolor* genomes implies either that a corresponding gene has moved since the evolutionary divergence of barley from these species, or that such a gene is absent from these other genomes. For both scenarios there is precedence in barley. For example, the *Rar1* gene is located on barley chromosome 2H, which is syntenic with chromosomes 4 and 7 in *O. sativa*, but its ortholog is present on *O. sativa* chromosome 2 (Lahaye et al. 1998; Shirasu et al. 1999). The *Vrs1* gene is also located on barley chromosome 2H. Its neighboring genes are collinear with *O. sativa* chromosome 4, but *Vrs1* is an insertion from *O. sativa* chromosome 7 (Pourkheirandish et al. 2007). As a final example, *Rpg1*, located on barley chromosome 7H, does not have an ortholog in the rice genome (Brueggeman et al. 2002).

There is reason to suspect that a *Ror1* gene function exists in other grasses and might have been conserved since the monocot and dicot split. *B. distachyon* expresses penetration-based resistance against barley and wheat powdery mildews (*B. graminis* f. sp. *hordei* and *tritici*) (Draper et al. 2001), resembling *Ror1*-dependent resistance responses against wheat powdery mildew in barley (Trujillo et al. 2004). *Ror1* has been shown in barley to contribute to penetration resistance against *M. grisea*, which is a serious pathogen of rice (Jarosch et al. 1999, 2005). Furthermore the *Ror2*/*PEN1* and *Mlo* genes are conserved across barley and the dicot Arabidopsis (Collins et al. 2003; Consonni et al. 2006). However, phylogenetic sequence analyses of the Arabidopsis *PEN2* gene, which like *PEN1* helps to restrict entry by non-adapted pathogens, suggests this gene represents an evolutionarily recent innovation of the Brassicaceae lineage including Arabidopsis (Bednarek et al. 2011; Consonni et al. 2006; Xu et al. 2004). Therefore, the occurrence of *Ror1* as a gene conserved across one or

several grass lineages (or even in dicots), or as a gene specific to barley, both seem possible.

In analogy to the suspected role of the Arabidopsis PEN2 myrosinase in generating a lineage-specific toxic secondary metabolite crucial for *mlo2* resistance (Consonni et al. 2010) we considered the possibility that the accumulation of a secondary metabolite during *Bgh* pathogenesis could be dependent on *Ror1* function in barley. Although we found no metabolite whose production was dependent on *Ror1*, we identified two pathogen induced, likely hordatine-unrelated, compounds that are potentially worthy of study in relation to the barley-powdery mildew interaction (Figs. S2 and S3).

The recent release of the barley draft genome assembly, delivered by The International Barley Genome Consortium (2012), provides a new resource with which to pursue the *Ror1* gene. As a start, genes in the vicinity of *Ror1* that were identified in the assemblies in the current study could be tested as *Ror1* candidates by mutant sequencing, mapping on the panel of recombinants, and consideration of predicted biochemical functions.

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