Transcriptome Analysis of the Barley-*Rhynchosporium secalis* Interaction

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Leaf scald caused by the infection of *Rhynchosporium secalis*, is a worldwide crop disease resulting in significant loss of barley yield. In this study, a systematic sequencing of expressed sequence tags (ESTs) was chosen to obtain a global picture of the assembly of genes involved in pathogenesis. To identify a large number of plant ESTs, which are induced at different time points, an amplified fragment length polymorphism (AFLP) display of complementary DNA (cDNA) was utilized. Transcriptional changes of 140 ESTs were observed, of which 19 have no previously described function. Functional annotation of the transcripts revealed a variety of infection-induced host genes encoding classical pathogenesis-related (PR) or genes that play a role in the signal transduction pathway. The expression analyses by a semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) revealed that *Rar1* and *Rpg4* are defense inducible genes, and were consistent with the cDNA-AFLP data in their expression patterns. Hence, the here presented transcriptomic approach provides novel global catalogue of genes not currently represented in the EST databases.

Keywords : barley, interaction, leaf scald, semi-quantitative RT-PCR, transcriptome changes

Rhynchosporium secalis (Oudem.) J. J. Davis, the causal agent of scald, is an important global disease of barley (*Hordeum vulgare* L.), responsible for large economic losses in cool and humid areas of temperate zones (Wagner, 2008). Crop rotations, fungicides, or resistant cultivars may control it. The latter, however, depends critically on a clear perception of the interaction between host and pathogen, which in turn requires a well-characterized set of differential cultivars or differential isolates (Walters et al., 2012).

However, it is highly challenging to control this disease in barley due to a poor understanding of the mechanisms of plant resistance and since no highly resistant barley cultivar is yet available (Bjørnstad et al., 2002).

Significant advances have been achieved in understanding the molecular basis of plant-pathogen interactions, especially in specific race-cultivar interactions (Looseley, 2012; Wagner, 2008). However, using Amplified fragment length polymorphism (AFLP) display of complementary DNA (cDNA) technique that scrutiny can reveal altered expression of any gene that carries suitable restriction sites leads to an accurate way for understanding plant responses to pathogens (Baldwin et al., 1999; Wendy et al., 2000). The cDNA-AFLP approach, once established, is an efficient and economical method to display whole transcript profiles of single tissues, specific developmental stages or other inducible characters (Bachem et al., 1996).

Transcript profiles and spatial expression patterns of genes provide an important basis for functional analysis of unknown genes by correlating those patterns with biological process of interest (Vuylsteke et al., 2007). In addition, studying global gene expression coordinately expressed genes can help to discover the networks involved in the process (Al-Daoude and Jawhar, 2009).

Stages of *R. secalis* infection in barley are well characterized (Lehnackers and Knogge, 1990), and each stage is a potential recognition point with the possible release of pathogen or plant-derived signaling molecules. Significant variation occurs, however, at the early stages of infection. Linsell et al. (2011) reported that at early stages of infection, fungal attachment and germination are accompanied by the release of proteins, carbohydrates, lipids, glycoproteins, and peptides from the spores, and many of these molecules can trigger general host defense responses (Steiner-Lange et al., 2003). In addition, primary germ tubes are also capable of breaching host epidermal walls leading to the initiation of cytoplasmic aggregates in underlying host cells (Lehnackers and Knogge, 1990). Therefore, the initial induction of defense responses most likely occurs at the early stages of infection and is possibly the result of syner-

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gistic effects of recognition of multiple pathogen-derived molecules.

However, in spite of the fact that scald disease exerts a substantial impact on barley production in many regions of the world; limited information is available about the genetic background and regulation of interaction mechanisms in resistant scald barley genotypes during the early phase of infection. Therefore, the primary objective of this research was to monitor the global response of the assembly of barley genes differentially expressed during early interaction stages between the fungal pathogen *R. secalis* and the barley resistant genotype Banteng, via cDNA-AFLP method.

Fungal isolate. The Syrian pathotype *R. secalis* (Rs46) used in the study was the most virulent of 115 isolates collected in 1997 and 2006 from naturally infected barley in different regions of Syria, as described by Arabi et al. (2010). The fungal mycelia were transferred from a stock culture into Petri dishes containing lima bean agar (LBA) with 13 mg/l kanamycin sulphate and incubated for 2 weeks at $15 \pm 1^\circ\text{C}$ in the dark. Then, conidia were collected with 10 ml of sterile distilled water. The conidial suspension was adjusted to 0.5×10^6 conidia/ml using hemacytometer counts of conidia to provide estimates of the inoculum concentration. A surfactant (polyoxyethylene-20-sorbitan monolaurate) was added (100 $\mu\text{l/l}$) to the conidial suspension to facilitate dispersion of the inoculum over the leaf surfaces.

Plant material and experimental design. After an extensive screening for several years in the greenhouse and laboratory experiments, the German cv. Banteng was proved to be the most resistant genotype to all scald isolates available so far. Therefore, it was selected for the cDNA-AFLP analysis. Inoculation tests of Rs46 isolate was performed using the method described by Arabi et al. (2010). Seeds were surface-sterilized with 5% sodium hypochlorite solution for 5 min, washed three times in sterile distilled water, then planted in plastic flats ($60 \times 40 \times 8$ cm) filled with sterilized peatmoss, and arranged in a randomized complete block design with three replicates. Each experimental unit consisted of two rows of 18 seedlings. Flats were placed

in a growth chamber at $22 \pm 1^\circ\text{C}$ (day) and $17 \pm 1^\circ\text{C}$ (night) with a day length of 12 h and a relative humidity of 80–90%. Seedlings were irrigated with Knop's nutrient solution (1g NaNO_3 ; 0.25 g KNO_3 ; 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.25 g KH_2PO_4 ; and 10 mg FeCl_3 per 1000 ml of water).

Plant inoculation. Plants were inoculated at growth stage 13 (Zadoks et al., 1974) by uniformly spraying each flat with 25 ml of conidial suspension with a hand-held spray bottle. After inoculation, plants were maintained in the dark at 95–100% R.H. for the first 18 h. Non-inoculated control plants were sprayed with distilled water.

mRNA isolation. Leaf samples for RNA isolation were taken at different time points post inoculation (2, 3, 4 and 5 days) according to the developmental stages of the fungus during infection (Table 1). Leaves were collected at each time point from 20 individual plants, labeled and immediately frozen in liquid N_2 before they were stored at -80°C till needed. As controls, mRNA was extracted from water-treated leaves incubated under the same conditions and at the same time points. mRNA was extracted from samples (100–200 mg) with the Nucleotrap mRNA mini kit (Macherey-Nagel, MN, Germany) following the manufacturer's protocol.

cDNA-AFLP analysis. The cDNA-AFLP protocol was performed according to the method described by Breyne et al. (2002) with minor modifications which permit the visualization of one single cDNA fragment for each messenger originally present in the sample, thus reducing the redundancy of sequences obtained. Briefly, double-stranded cDNA was synthesized from 1 μg mRNA using the Superscript II reverse transcription kit (Invitrogen, UK) and a biotinylated oligo-dT primer (Roche). The cDNA was digested with *Bst*YI (restriction site RGATCY), and the 3' ends of the fragments were captured on streptavidin magnetic beads (Dyna). Digestion with *Mse*I yielded fragments that were ligated to adapters for amplification (*Bst*YI-Forw: 5'-CTC GTA GAC TGC GTA GT-3'; *Bst*YI_Rev: 5'-GAT CAC TAC GCA GTC TAC-3'; *Mse*I-Forw: 5'-GAC GAT GAG TCC TGA G-3'; *Mse*I-Rev: 5'-TAC ATC AGG ACT

Table 1. Sampling time-points according to the developmental stages of *R. secalis*

Sampling time point	Days after inoculation
Few small lesions mainly at the tip or in the edge of the leaf blade	2
A dark margin mainly on the edge of the leaf blade	3
Somewhat larger dark lesions on the edge of the leaf blade	4
Elongated dark lesions with initial chlorosis in the leaf blade	5

CAT-3'). Pre-amplification was performed with an *Mse*I primer (*Mse*0: 5'-GAT GAG TCC TGA GTA A-3'), combined with a *Bst*YI primer carrying either a T or a C at the 3' end (*Bst*T0: 5'-GAC TGC GTA GTG ATC T-3'; *Bst*C0: 5'-GAC TGC GTA GTG ATC C-3'). Pre-amplification PCR conditions were as follows: 5 min denaturation at 94°C and

then 30 s denaturation at 94°C, 60 s annealing at 56°C, 60 s extension at 72°C (25 cycles), followed by 5 min at 72°C. After preamplification, the mixture was diluted 100 folds and 4 µl was used for selective amplification with 14 primer combinations, carried out with two selective nucleotides on the *Mse*I primer. Touch-down PCR conditions for selec-

Table 2. Homologies of sequenced AFLP fragments (accession numbers in NCBI databases) to sequences in the databases at 2, 3, 4 and 5 days after inoculation of barley cv. Banteng by the *R. secalis* Rs46

Frag- ment no.	2d		3d		4d		5d		Accession no.	Length (bp)	Blast match	BlastX % Simi- score	Simi- larity
	I	II	I	II	I	II	I	II					
1	–	–	–	–	–	+	–	+	JZ714794	136	<i>Hordeum vulgare</i> subsp. vulgare mRNA cds, clone	2 e-29	98
3	–	–	–	–	–	+	–	+	JZ714795	168	<i>Hordeum vulgare</i> subsp. Vulgare cultivar Haruna Nijo HOX-1 gene	5 e-39	95
5	–	+	–	+	–	+	–	+	JZ714796	138	<i>Hordeum vulgare</i> subsp. Vulgeare Rpg4 gene complete sequence, RGA gene complete cds	7 e-30	95
9	–	–	–	–	–	+	–	+	JZ714797	134	<i>Hordeum vulgare</i> subsp. Vulgare LK2 transcriptional factor	94	
10	–	+	–	+	–	–	–	–	JZ714798	210	<i>Hordeum vulgare</i> Cepada capa Rym4 gene complete	7 e-51	77
11	–	+	–	+	–	+	–	+	JZ714799	143	<i>Hordeum vulgare</i> tonoplast intrinsic protein 1 (TIP1), intrinsic protein 2 (TIP2) and (Rar1) gene	2 e-31	96
35	–	–	–	+	–	+	–	–	JZ714800	143	<i>Hordeum vulgare</i> subsp. Vulgare mRNA for predicted protein	2 e-31	98
37	–	–	–	–	–	–	–	+	JZ714801	169	<i>Hordeum vulgare</i> subsp. Vulgare cultivar Haruna Nijo HOX-1 gene	5 e-39	95
49	–	–	–	+	–	+	–	+	JZ714802	152	<i>Hordeum vulgare</i> CBF9 (CBF9) gene complete sequence and retrotransposon	5 e-34	92
60	–	–	–	+	–	+	–	–	JZ714803	167	putative SCARECROW gene regulator (<i>Oryza sativa</i>)	2 e-12	71
64	–	+	–	+	–	–	–	–	JZ714804	144	<i>Oryza sativa Japonica</i> Group OsO5g0110400 mRNA	2 e-27	83
80	–	–	–	–	–	+	–	–	JZ714805	176	Retrotransposon protein, putative, Ty3-gypsy subclass (<i>Oryza sativa Japonica</i> Group)	6 e-13	65
85	–	–	–	–	–	+	–	+	JZ714806	177	Putative RIRE2 retrotransposon protein (<i>Oryza sativa Japonica</i> Group)	7 e-13	65
88	–	+	–	–	–	–	–	–	JZ714807	177	Putative gag-po precursor protein (<i>Oryza sativa japonica</i> group)	8 e-13	65
95	–	–	–	+	–	+	–	+	JZ714808	232	<i>Hordeum vulgare</i> Cepada capa Rym4 and MCT-1 genes complete	5 e-51	77
99	–	–	–	+	–	+	–	+	JZ714812	232	<i>Hordeum vulgare</i> subsp. Vulgare cultivar Haruna Nijo Rym4 and MCT-1 genes	5 e-51	77
100	–	–	–	+	–	+	–	–	JZ714809	230	<i>Hordeum vulgare</i> subso. vulgare LKs gene for putative short internodes family	2 e-50	77
130	–	+	–	+	–	+	–	+	JZ714810	80	<i>Hordeum vulgare</i> tonoplast intrinsic protien 1 (TIP1) tonoplast, intrinsic protein 2 (TIP2), Rar1 (Rar1) gene	6 e-33	97
140	–	–	–	+	–	+	–	+	JZ714811	77	<i>Hodeum vugare</i> subsp. VULGARE Rpg4 gene, complete sequence, RGA1 gene, complete cds, Rpg5 gene complete sequence, PP2C gene complete cds, and ADF3 gene complete sequence	3 e-31	96

(+): presence and (–): absence of fragment; I (un-inoculated controls), II (post inoculation)

tive amplifications were as follows: 5 min denaturation at 94°C, followed by 30 s denaturation at 94°C, 30 s annealing at 65°C, 60 s extension at 72°C (13 cycles, scale down of 0.7°C per cycle); 30 s denaturation at 94°C, 30 s annealing at 56°C, 60 s extension at 72°C (23 cycles) and 5 min at 72°C. Selective amplification products were separated on a 6% polyacrylamide gel in a Sequi-Gen GT Sequencing Cell (38 × 50 cm) (Bio-Rad, USA) running for 2.5 h at 105 W and 50°C, and silver stained (Silver Sequence kit, Promega, Cat. Q4132).

Sequence characterization of amplified cDNA fragments. Bands of interest were excised from gels with a surgical blade and eluted in 100 µl of sterile distilled water. An aliquot of 5 µl was used as a template for reamplification using non-labeled primers identical to those employed for selective AFLP amplification. PCR products were purified with MultiScreen PCR µ96 plates (Millipore) and sequenced directly (BMR Genomics). Prior to sequencing, PCR products were purified with QIAgen gel extraction kit according to the manufacturer's recommendations. Sequencing was carried out on a Genetic Analyzer (ABI 310, Perkin-elmer, Applied Biosystems, USA). Each sequence was identified by homology search using the Basic Local Alignment Search Tool (BLAST) program (Altschul et al., 1997) against the GenBank no redundant public sequence database using an *E*-value (BLASTX expectation values [*E*] of $<10^{-5}$) to database entries with assigned identities.

Expression analysis of known specific genes. A portion of seedling leaves was used for semi-quantitative RT-PCR analysis to validate the expression patterns detected by cDNA-AFLP. Gene specific oligonucleotides were designed from the conserved regions of plant defense related genes using sequences available in the NCBI Genbank database (<http://www.ncbi.nlm.nih.gov>); *Rar1F*: CAGGC-GCCAGTAAAGAATGC; *Rar1R*: TGCTTGGAACCTTCTACCGTT. *Rpg4F*: GCGTCGTCCATATTGTACAC; *Rpg4R*: TTGATCCGTTCTGTCTTTGG. *EF1aF*: CCTGTATGGTTGTGACCTTTGG; *EF1aR*: GGGCTTG-GTGGGAATCATCTTC. RT-PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 20 to 25 cycles of 94°C for 1 min denaturation, 55°C for 1 min annealing and 72°C for 1 min extension. Final extension was carried out at 72°C for 7 min. The annealing temperature was varied according to the sequence of the oligonucleotide used. PCR products were visualized on 1.5% agarose gel by electrophoresis in TAE buffer system. Template used for this analysis was the cDNA from unin-

oculated and inoculated with *R. secalis* 2, 3, 4 and 5 days post inoculation.

In this study, a systematic sequencing of expressed sequence tags (ESTs) was chosen to document the early resistant barley response to *R. secalis* infection (Looseley, 2012). It demonstrated differential gene expression in barley leaves during the early phase of infection, before any visible symptoms are apparent in the tissues (Table 2). cDNA-AFLP analysis carried out on mRNA samples of infected leaves at different points of time, and on water-sprayed leaves (healthy control), as described by Breyne et al. (2002). Different sampling points were chosen to cover early barley responses to scald which leads within 5 days to a visible hypersensitive cell death on a resistant genotype. For each of the 8 primer combinations, 20–35 transcript derived fragments (TDFs) were visualized as bands, 50–700 bp in size, representing approximately 140 transcripts overall. Only 19 transcripts showed a differential expression pattern with cDNA-AFLP results (accession numbers in NCBI databases are given in Table 2). To determine the reproducibility of these profiles, the experiments were repeated using additional samples of a biological replicate.

Based on the assumption that disease resistance involves the early recognition of the invading pathogen and the activation of defense response genes, the cDNA-AFLP patterns of resistant plants were screened for expressed fragments which occur 2, 3, 4 and 5 days after fungal attack (Fig. 1). The time points for taking samples for RNA isolation in the first experiments were chosen for capturing mRNA samples to wrap up barley response to *R. secalis* invasion, taking into account the findings of Xi et al. (2000) on the production of barley scald symptoms. Differentially-expressed transcripts were visually scored relative to the first sampling time point which was arbitrarily attributed a zero value.

BlastX score to sequences on the database were presented in this study (Table 2). The majority of genes were observed to be related to either metabolism or cellular defense. Among these, fragments 3 and 5 with high similarities (high BlastX scores) to barley pathogenically-induced genes including *HOX1* (JZ714795) and the *Rpg4* (JZ714796) appeared two days post inoculation. Moreover, fragment 140 showed strong similarities to the barley *Rpg5* gene (JZ714811). The *Rpg4* and *Rpg5* genes are known from barley to be involved in resistance against *Puccinia graminis* (Wang et al., 2013). They are located on the long arm of chromosome 5H where Looseley (2012) reported a QTL in connection with barley-*R. secalis* symptom expression. Furthermore, over expression of particular *PR-5* proteins delays or halts the onset of disease symptoms of

Fig. 1. A portion of a silver stained AFLP gel using *EcoRI*+ACG/*MseI*+CAT primer combination. I: Controls, plants sprayed with water. II: Treatments, plants infected with *R. secalis*. 2d, 3d, 4d, and 5d are sampling time points in days (d). M: 20bp DNA ladder.

fungal pathogens in rice (Datta et al., 1999). Additionally, *Rym4* (JZ714808) appeared at 2 days of inoculation, which is the recessive bymovirus resistance locus in barley corresponding to the eukaryotic translation initiation factor 4E gene (Kanyuka et al., 2005). The percents of similarity are given in Table 2.

The study also identified genes believed to be involved in the signal transduction pathway. Several genes encoding for transcription factors placed in this category appeared 3 days post inoculation, including fragments that showed homology with *LK2* (JZ714810), *SCARECROW* (JZ714803), and *RIRE2* (JZ714806) (Table 2).

To validate the expression patterns of some R genes detected by the cDNA-AFLP, RT-PCR on candidate genes *Rar1* and *Rpg4* with significantly changed expression pattern before and after the infection was performed. The oligonucleotides designed from the sequence information used for RT-PCR in inoculated and non-inoculated samples could reveal consistently reproducible and differential am-

Fig. 2. (A) Expression profile of *Rar1* and *Rpg4* genes at the different time points in days (d) following *R. secalis* infection in the resistant barley cv. Banteng determined by semi-quantitative RT-PCR. *EF1α* was used as the control gene. (B) Changes of band genes intensity via time points in days (d).

plification profiles. RT-PCR revealed a dramatic increase of *Rar1* gene 2 days following the *R. secalis* inoculation, reaching its highest level of expression at day five of the infection (Fig. 2). *Rar1* encodes an intracellular Zn^{+2} binding protein (Shirasu et al., 1999), and subsequent analyses have shown that *Rar1* is required for several R gene-mediated resistance responses in monocotyledonous and dicotyledonous plant species against different pathogen classes (Shirasu and Schulze-Lefert, 2003). Barley *Rar1* interacts with *Sgt1* (for suppressor of G-two allele of *skp1*) in the yeast two-hybrid assay and in planta. *Sgt1* is another intracellular protein engaged by a subset of NB-LRR R proteins (Azevedo et al., 2002). Genetically, some R genes require *Rar1* or *Sgt1*, whereas others require both or none (reviewed in Shirasu and Schulze-Lefert, 2003). In our study, *Rar1* did not require *Sgt1* during *R. secalis* interaction (Table 2).

Rpg4 gene was down-regulated in inoculated barley

plants as compared to its expression in healthy plants, suggesting its role in the first stages of defense against *R. secalis* attack (Fig. 2). This is concordant with the information available from barley stem rust resistance wherein, *Rpg4* have been shown to respond rapidly to signals after pathogen attack (Druka et al., 2000). However, it is well known that the *rpg4* gene by itself is not sufficient to confer resistance, perhaps due to failure to detect the fungus (Brueggeman et al., 2009).

The transcriptome analysis allowed the discovery of 19 ESTs having no previously described function or sequence identity in barley-*R. secalis* interaction, as a significant proportion of TDFs are not currently represented in barley EST databases. Database searches with the randomly isolated sequences identified a number of classical pathogenesis-related (PR) or genes that play a role in the signal transduction pathway. However, these results provide a vast amount of information that can guide hypothesis-driven research to elucidate the molecular mechanisms involved in transcriptional regulation and disease signaling networks in barley.

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References

- Al-Daoude, A. and Jawhar, M. 2009. Transcriptional changes in barley-*Cochliobolus sativus* interaction. *Austral. Plant Pathol.* 38:1–5.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl. Acids Res.* 25:3389–3402.
- Arabi, M. I. E., Al-Shehadah, E. and Jawhar, M. 2010. Pathogenic groups identified among isolates of *Rhynchosporium secalis*. *Plant Pathol. J.* 26:260–263.
- Azevedo, C., Sadanandom, A., Kitagawa, K., Freialdenhoven, A., Shirasu, K. and Schulze-Lefert, P. 2002. The RAR1 interactor SGT1, an essential component of R gene-triggered disease resistance. *Science* 295:2073–2076.
- Bachem, C. W. B., van der Hoeven, R. S., de Bruijn, S. M., Vreugdenhil, D., Zabeau, M. and Visser, R. G. F. 1996. Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. *Plant J.* 9:745–753.
- Baldwin, D., Crane, V. and Rice, D. 1999. A Comparison of gel – based, nylon filter and microarray techniques to detect differential RNA expression in plants. *Curr. Opin. Plant Biol.* 2:96–103.
- Bjørnstad, A., Patil, V., Tekauz, A., Marøøy, A. G., Skinnes, H., Jensen, A., Magnus, H., and MacKey, J. 2002. Resistance to scald (*Rhynchosporium secalis*) in barley (*Hordeum vulgare*) studied by near-isogenic lines: I. Markers and differential isolates. *Phytopathology* 92:710–720.
- Breyne, P., Dreesen, R. and Vandepoele, K. 2002. Transcriptome analysis during cell division in plants. *Proc. Natl. Acad. Sci. USA.* 99:14825–14830.
- Brueggeman, R., Steffenson, B. J. and Kleinhofs, A. 2009. The *rpg4/Rpg5* stem rust resistance locus in barley; resistance genes and cytoskeleton dynamics. *Cell Cycle* 8:977–981.
- Datta, K., Velazhahan, R., Oliva, N., Ona, I., Mew, T., Khush, G. S., Muthukrishna, S. and Datta, S. K. 1999. Over-expression of the cloned rice thaumatin-like protein (PR-5) gene in transgenic rice plants enhances environmental friendly resistance to *Rhizoctonia solani* causing sheath blight disease. *Theor. Appl. Genet.* 98:1138–1145.
- Druka, A., Kudrna, D., Han, F., Kilian, A., Steffenson, B., Frisch, D., Tomkins, J., Wing, R. and Kleinhofs, A. 2000. Physical mapping of barley stem rust resistance gene *rpg4*. *Mol. Gen. Genet.* 264:283–290.
- Kanyuka, K., Druka, A., Caldwell, D. G., Tymon, A., McCallum, Waugh, R. and Adams, M. J. 2005. Evidence that the recessive bymovirus resistance locus Rym4 in barley corresponding to the eukaryotic translation initiation factor 4E gene. *Mol. Plant Pathol.* 6:449–458.
- Lehnackers, H. and Knogge, W. 1990. Cytological studies on the infection of barley cultivars with known resistance genotypes by *Rhynchosporium secalis*. *Can. J. Bot.* 68:1953–1961.
- Linsell, K. J., Keiper, F. J., Forgan, A. and Oldach, K. H. 2011. New insights into the infection process of *Rhynchosporium secalis* in barley using GFP. *Fungal Genet. Biol.* 48:124–131.
- Looseley, M. E. 2012. Genetic basis of control *Rhynchosporium secalis* infection and symptom expression in barley. *Euphytica* 184:47–56.
- Shirasu, K., Lahaye, T., Tan, M. W., Zhou, F. S., Azevedo, C. and Schulze-Lefert, P. 1999. A novel class of eukaryotic zinc-binding proteins is required for disease resistance signaling in barley and development in *Caenorhabditis elegans*. *Cell* 99:355–366.
- Shirasu, K. and Schulze-Lefert, P. 2003. Complex formation, promiscuity and multi-functionality: Protein interactions in disease-resistance pathways. *Trends Plant Sci.* 8:252–258.
- Steiner-Lange, S., Fischer, A., Boettcher, A., Rouhara, I., Liedgens, H., Schmelzer, E. and Knogge, W. 2003. Differential defense reactions in leaf tissues of barley in response to infection by *Rhynchosporium secalis* and to treatment with a fungal avirulence gene product. *Mol. Plant-Microbe Interact.* 16:893–902.
- Vuylsteke, M., Peleman, J. D. and van Eijk, M. J. 2007. AFLP-based transcript profiling (cDNA-AFLP) for genome-wide expression analysis. *Nat. Prot.* 2:1399–1413.

- Wagner, C. 2008. Quantitative resistance of barley against scald: a candidate gene approach In: Molina-Cano J. L. (ed.), Christou P. (ed.), Graner A. (ed.), Hammer K. (ed.), Jouve N. (ed.), Keller B. (ed.), Lasa J.M. (ed.), Powell W. (ed.), Royo C. (ed.), Shewry P. (ed.), Stanca A.M. (ed.). *Cereal science and technology for feeding ten billion people: genomics era and beyond*. Zaragoza :CIHEAM / IRTA, p. 175–177 (Options Méditerranéennes: Série A. Séminaires Méditerranéens; n. 81).
- Wang, X., Richards, J. and Gross, T. 2013. The Rpg4 –mediated resistance to wheat stem rust (*Puccinia graminis*) in barley (*Hordeum vulgare*) requires Rpg5, a second NBS-LRR gene, and an actin depolymerization factor. *Mol. Plant-Microbe Interact.* 26:407–418.
- Walters, D. R., Avrova, A. and Bingham, I. J. 2012. Control of foliar diseases in barley: towards an integrated approach. *Eur. J. Plant Pathol.* 133:33–37.
- Wendy, E. D., Rowland, O., Piedras, P., Kim, E. H. and Jonathan, D. G. J. 2000. cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. *Plant Cell* 12:963–977.
- Xi, K., Burnett, P. A., Tewari, J. P., Chen, M. H., Turkington, T. K. and Helm, J. H. 2000. Histopathological study of barley cultivars resistant and susceptible to *Rhynchosporium secalis*. *Phytopathology* 90:94–102.
- Zadoks, J. C., Chang, T. T. and Konzak, C. F. 1974. A decimal code for the growth stages of cereals. *Weed Res.* 14:415–421.