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# Transcriptome Profile of Early Responsive Genes in Susceptible Barley during *Rhynchosporium secalis* Infection

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Scald caused by *Rhynchosporium secalis*, is an economically important disease found worldwide. In order to profile genes and pathways responding to *R. seclais* infection, leaf transcriptomes before and after fungus inoculation in susceptible barley were compared using cDNA-AFLP technique. Transcriptional changes of 144 expressed sequence tags (ESTs) were observed, of which 18 have no previously described function. Functional annotation of the transcripts revealed a wide range of pathways including cell wall fortification, cytoskeleton construction and metabolic processes at different time points. Furthermore, the results of RT-PCR analysis on candidate genes, ABC transporters and lycine-specific demethylase were consistent with the cDNA-AFLP data in their expression patterns. Taken together, our data suggest that susceptible barley reprograms metabolic and biological processes to initiate a suitable response *R. secalis* infection.

**Keywords:** barley, *Rhynchosporium secalis***,** defense response, RT-PCR, transcriptomics

## Introduction

Leaf scald disease, caused by *Rhynchosporium secalis* (Oud) Davis, is a major constraint to high grain yield and quality in many barley-growing regions of the world (Brown et al. 1996; Meles et al. 2004). Although crop rotations and fungicides can be effective in reducing scald severity, the most effective and environmentally sound means of control is through the use of resistant cultivars (Looseley 2012). However, it is highly challenging to control this disease in barley due to a poor understanding of the mechanisms of plant reaction and since no highly resistant barley cultivar is yet available (Bjørnstad et al. 2002).

In a histological study of scald infection, Xi et al. (2000) observed differences in the infection process between resistant and susceptible cultivars. In susceptible plants the disease can result in more dead and collapsed tissue and further uncontrolled spread leading to visible necrotic lesions (Yahyaoui 2003). In addition, even susceptible hosts are not fully accessible to *R. secalis* but express different degrees of background resistance that is mainly achieved by penetration defense (Looseley 2012).

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Significant advances have been achieved in understanding the molecular basis of plant–pathogen interactions, particularly in specific race-cultivar relations. The amplified fragment length polymorphism (AFLP) display of complementary DNA (cDNA) technique that when scrutinized can reveal altered expression of any gene that carries suitable restriction sites can lead 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, particular developmental stages or other inducible characters (Polesani et al. 2008). The initial product of genome expression is the transcriptome, a collection of RNA molecules derived from those protein-coding genes whose biological information is required by the cell at a particular time. 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).

Previous transcriptome study using cDNA (Al-Daoude et al. 2014) has attempted to understand interaction between a resistant barley genotype and scald, however, still, little is known about the genetic background and regulation of interaction mechanisms of susceptibility, which would greatly facilitate the development of new control strategies through the identification of pathogen and host factors required for disease progression. Moreover, 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, peptides and many of these molecules can trigger general host defense responses. Therefore, the aim of the present research was to better understand the interaction between the fungal pathogen *R. secalis* and the barley susceptible genotype WI2291, via the cDNA-AFLP method.

## Materials and Methods

### *Isolation of fungus*

The most virulent isolate (Rs46) to all barley genotypes available so far (Arabi et al. 2010) was used in this study. 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 ± 1 °C in the dark. Then, conidia were collected with 10 mL of sterile distilled water. The conidial suspension was adjusted to $0.5 \times 10^6$ conidia/mL using hemacytometer counts of conidia to provide estimates of the inoculum concentration. A surfactant (polyoxyethylene-20-sorbitan monolaurate) was added (100 µL/L) to the conidial suspension to facilitate dispersion of the inoculum over the leaf surfaces.

### *Host genotype and inoculation*

The universal susceptible cultivar from Australia cv. WI2291 was used in this study. 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 × 40 × 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 ± 1 °C (day) and 17 ± 1 °C (night) with a day length of 12 h and a relative humidity of 80–90%. Seedlings were irrigated with Knop's nutrient solution (1 g $NaNO_3$; 0.25 g $KNO_3$; 0.25 g $MgSO_4$ $7H_2O$; 0.25 g $KH_2PO_4$; and 10 mg $FeCl_3$ per 1000 ml of water). Inoculation tests of the Rs46 isolate was performed using the method described by Arabi et al. (2010). 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 (100–200 mg) for mRNA 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. mRNA controls were extracted from water-treated leaves incubated under the same conditions and at the same time points. mRNA was isolated using Trizol (Invitrogen) reagent and cleaned with RNeasy Mini Kit for RNA cleanup (Macherey-Nagel, MN, Germany) according to 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 (Al-Daoude et al. 2014). 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 (Dynal). Digestion with *Mse*I yielded fragments that were ligated to adapters for amplification (*BstYI*-Forw: 5'-CTC GTA GAC TGC GTA GT-3'; *BstYI*_Rev: 5'-GAT CAC TAC GCA GTC TAC-3'; *MseI*-Forw: 5'-GAC GAT GAG TCC TGA G-3'; *MseI*-Rev: 5'-TAC ATC AGG ACT CAT-3'). Pre-amplification was performed with an *Mse*I primer (Mse0: 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 (BstT0: 5'-GAC TGC GTA GTG ATC T-3'; BstC0: 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 pre-amplification, the mixture was diluted 100-fold 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 selective amplifications were as follows: 5 min denaturation at 94 °C, followed by 30 s denaturation at 94 °C, 30 s annealing at

*Table 1*. Homologies of sequenced AFLP fragments (accession numbers in NCBI databases) to sequences in the databases at 2, 3, 4 and 5 days post inoculation of barley cv. WI2291 by the *R. secalis* Rs46

| Fragment no. | 2d | | 3d | | 4d | | 5d | | Accession no. | Length (bp) | Blast match | BlastX score | % similarity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | I | II | I | II | I | II | I | II | | | | | |
| 2 | – | + | – | | – | + | + | – | JZ820448 | 217 | Dasypus novemcinctus LIM domain kinas 2 | 9 | 98 |
| 6 | – | – | – | + | – | + | – | – | JZ820449 | 168 | *Hordeum vulgare* subsp. *vulgare* eIF4E gene locus, complete sequence | 2 e-36 | 88 |
| 9 | – | – | – | – | – | + | – | + | JZ820450 | 252 | *Hordeum vulgare* cultivar *Cepada capa* Rym4 gene | 3 e-37 | 81 |
| 10 | – | + | – | – | – | + | – | – | JZ820451 | 240 | *Hordeum vulgare* subsp. *vulgare* genes for putative iron-deficiency specific<br>4 protein and putative ethylene-responsive transcription factor | 2 e-32 | 93 |
| 12 | – | + | – | + | – | – | – | – | JZ820452 | 358 | Hypothetical protein OsJ_31040 [*Oryza sativa* Japonica group] | 6.4 | 52 |
| 22 | – | + | – | + | + | – | – | – | JZ820453 | 210 | ABC transporter substrate-binding protein [*Streptomyces* sp. NRRL F-5639] | 7.2 | 46 |
| 25 | + | – | + | + | – | + | – | + | JZ820454 | 197 | Lysine-specific demethylase 3B [*Aegilops tauschii*] | 0.028 | 91 |
| 38 | – | – | – | + | + | – | – | – | JZ820455 | 326 | Formin-like protein 7-like [*Setaria italica*] | 5.1 | 85 |
| 51 | – | + | – | + | – | – | + | – | JZ820456 | 335 | GTP-binding protein LepA [*Desulfovibrio alaskensis*] | 2 | 63 |
| 55 | – | – | – | – | – | + | + | + | JZ820457 | 243 | Putative disease resistance protein RGA4 [*Aegilops tauschii*] | 3 e-31 | 95 |
| 63 | – | – | + | + | – | + | – | + | JZ820458 | 270 | Calcineurin-like metallo-phosphoesterase super family protein, putative isoform 2 [*Theobroma cacao*] | 7. 1 | 47 |
| 82 | – | + | – | – | – | – | – | – | JZ820459 | 180 | *Hordeum vulgare* subsp. *vulgare* mRNA for predicted protein, complete cds, clone: NIASHv2106N12 | 0.002 | 78 |
| 84 | – | – | – | – | – | + | + | + | JZ820460 | 211 | *Hordeum vulgare* subsp. *vulgare* cultivar Haruna Nijo *Hox-1* gene | 1 e-09 | 78 |
| 92 | – | – | – | + | – | + | + | – | JZ820461 | 214 | *Hordeum vulgare* subsp. *vulgare* eIF4E gene locus | 7 e-05 | 80 |
| 110 | – | – | – | – | – | – | – | + | JZ820462 | 240 | *Hordeum vulgare* cleistogamy 1 gene | 3 e-81 | 75 |
| 114 | – | – | – | – | – | + | – | – | JZ820463 | 167 | *Triticum aestivum* cultivar Chinese Spring 4A DELLA protein (Rht-A) gene | 1 e-07 | 85 |
| 123 | – | + | – | + | – | – | – | – | JZ820464 | 215 | *Hordeum vulgare* subsp. *vulgare* mRNA for predicted protein | 5 e-26 | 88 |
| 130 | – | + | – | + | – | + | – | – | JZ820465 | 256 | *Vitis vinifera* contig VV78X259514.3, whole genome shotgun sequence | 5 .7 | 86 |

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

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). Selected cDNA-AFLP bands were cut from the 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, Perklin-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.

*Validation of differentially accumulating transcripts*

Semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) was deployed to further support and validates the differentially accumulating barley transcripts. Two transcripts (fragment 22; ABC transporter and fragment 25; lysine-specific demethylase) were selected for this confirmation. 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); *ABC*F:ATGAACTTCTGG GGTGGGTT;*ABC*R:ACCCAAATTCAACGCAACGA.LysineF:;AGTGGATTGGTTT GCTCCCT;LysineR:CGTACCAATTCACGCACTGA.*EF1*aF(KJ943360):CCTGGTAT GGTTGTGACCTTTGG;*EF1*aR (KJ943365): GGGCTTGGTGGGAATCATCTTC. RT-PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 25 to 30 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. Annealing temperatures were varied according to the sequence of the oligonucleotide used. PCR products were visualized on a 1.5% agarose gel by electrophoresis in TAE buffer system. Template used for this analysis was the cDNA from uninoculated and inoculated with *R. secalis* 2, 3, 4 and 5 days post inoculation. Band intensity in selected genes was calculated in comparison to *EF1*a which was considered zero.

## Results

For each of the 14 primer combinations (see above), 25–29 ESTs were visualized as clear bands, 50–760 bp in size, representing approximately 144 transcripts overall, of which 18 have no previously described function (accession numbers in NCBI database are given in

Table 1). To ensure reproducibility, experiments were repeated using additional samples of a biological replicate. Based on the assumption that disease infection involves an early recognition of the invading pathogen, the cDNA-AFLP patterns of susceptible plants were screened for newly expressed fragments which occur 2, 3, 4 and 5 days following fungal attack.

BlastX score to sequences on the database and the percentage similarity are presented in Table 1. The majority of genes were observed to be related to either metabolism or cellular defense. Genes related to protein metabolism were also prevalently repressed in our experiment. Among these, fragments 110, 55, 51 and 63 with high similarities (high BlastX scores) to barley metabolism induced genes including. Cleistogamy (JZ820462), calcineurin like metallo-phosphoesterase (JZ820458), GTP-binding proteins (JZ820456) and putative disease protein RGA4 (JZ820457). Moreover, fragment 84 with high similarity (high BlastX scores) to barley pathogenically-induced gene *HOX1* (JZ820460) was

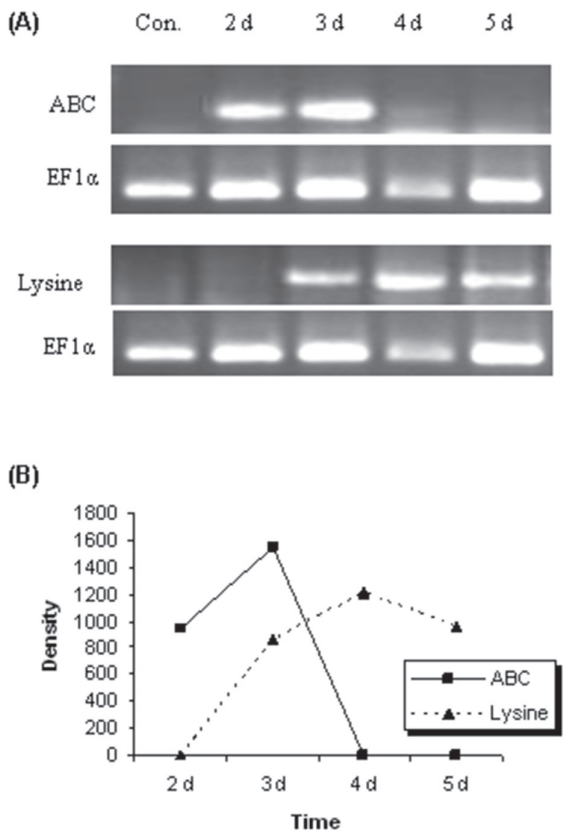


*Figure 1*. (A) Semiquantitative RT-PCR profile of ABC and lysine at different time points following the interaction of *R. secalis* with the susceptible barley cultivar WI 2291. *EF*a was used as a control. (B) Observed changes in band intensity over chosen time points. Band intensity in selected genes was calculated in comparison to *EF*a which was considered zero

identified after 4 and 5 days post inoculation. Additionally, a putative iron-deficiency specific gene (JZ820451) was up-regulated two days post inoculation (fragment 10; 93% similarity). Another differentially-expressed gene appeared after 3 days post inoculation was a formin-like protein (JZ820455). Formins (A fragment 38; JZ820450) was also identified 3 days post infection.

DNA-PCR using the primer pairs for the two gene transcripts (fragment 22; ABC transporter and fragment 25; lysine-specific demethylase) resulted in no product, indicating that there was no contamination of DNA in the samples (data not shown). RT-PCR results are consistent with the data derived from the cDNA-AFLP approach. RT-PCR revealed a dramatic increase of ABC gene 2 days following the *R. secalis* inoculation, reaching its highest level of expression at day three of the infection (Fig. 1).

## Discussion

In this study, transcript accumulation in susceptible barley inoculated with *R. secalis* at different points of time was examined to characterized barley response to the pathogen invasion, taking into account the findings of Xi et al. (2000) on the production of barley scald symptoms. Results demonstrated that during *R. secalis* infection, low level defense responses were activated in susceptible plants, which is in line with those reported in barley (Lipka and Panstruga 2005; Fung et al. 2008).

In this experiment, an ABC transporter, which mediates the uptake of nutrients by microorganisms, was up-regulated 2 days and down-regulated 4 days post inoculation. Many of these ABC play important roles in eliminating waste products or toxins from the cell such as LmrA protein of *Lactococcus lactis* (Higgins 1995; van Veen et al. 2000), which could be activated during the first stage of *R. seclais* infection in the studied susceptible genotype. Lycine-specific demethylase was upregulated in infected leaves, reflecting the occurrence of important DNA methylation during scald infection, which is associated with transcriptional repression of transposable elements and protein-coding genes. However, expression of genes involved in protection against cell damage was not detected, indicating that there may be a higher degradation of cellular components in the barley susceptible genotype (Greenberg et al. 2013). This may contribute in turn, amino acid residues on histone proteins at early stage of *R. secalis* infection.

On the other hand, cleistogamy gene which has a role in flowering stage was up regulated 5 days post infection. This is can be attributed at least partially to the progress of stress caused by *R. secalis* infection on susceptible barley which might lead plants to activate this kind of genes for their reproduction. This is in agreement with the results of Kubo et al. (2010) a bout the basic role of this gene towards fungus.

Kinase (JZ820448) is also involved in intracellular and intercellular signaling and appeared 4 days post inoculation (Zhang and Klessig 2001). This suggested a general repression of protein synthesis and turnover. However, a gene involved in mRNA (JZ820464) for predicted protein was induced, in agreement with previous findings (Al-Daoude et al. 2014). Taken together, our data suggest that barley switches metabolic and biological processes to initiate the observed response to *R. secalis* colonization.

*Hox1* gene was up-regulated 4 and 5 days post infection only in the inoculated plants suggesting a role in the early stages of defense against *R. secalis* attack. This could be supported by our previous findings which indicate that *Hox1* responds rapidly to signals after the pathogen infection in a resistant barley cultivar (Al-Daoude et al. 2014). In addition, GTP-binding proteins were detected in non-inoculated plants. The proteins are often implicated in regulation of plant cell architecture, secondary wall formation, meristem signaling, and defense against pathogens (Liu et al. 2014). In the case of *R. secalis*, the roles of these proteins were not activated on susceptible plants or in resistant plants as previously reported (Al-Daoude et al. 2014). Additionally, formins (fragment 38; JZ820450) was identified 3 days post infection and could play a role in cytoskeleton, plasma membrane and cell-wall linkages related to defense responses (Cushman 2001). Interestingly, *Rym4* (JZ820450) appeared at 2 days of inoculation, which is the recessive by movirus resistance locus in barley corresponding to the eukaryotic translation initiation factor 4E gene (Kanyuka et al. 2005).

The expression of putative iron-deficiency gene was both up-regulated and down-regulated during *R. secalis* infection. It is well known that, Fe is essential for various cellular processes, as it serves as a cofactor for a range of plant enzymes, including cytochromes, catalase, peroxidase isozymes, ferredoxin, and isozymes of superoxide dismutase (Marschner 1995). It was therefore expected that the expression of these genes would be down regulated by disease infection, since the fungal infection reduces absorption of minerals and influences photosynthesis process.

In conclusion, this study provided a global profile of gene expression changes in susceptible barley during *R. secalis* attack, elucidating some novel gene regulations not currently represented in EST databases. The differentially expressed genes and putative signaling pathways generated in the present study revealed that the defense system of susceptible barley may be more complex than previously believed. The findings of this study will hopefully accelerate research on barley resistance to *R. secalis* and contribute to a better understanding of the barley defense response to plant pathogens. Further studies are required to identify additional low-abundance, basic, hydrophobic or membrane-bound proteins associated with susceptibility to *R. secalis*.

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