

Transcriptome Profiling Reveals Distinct Gene Activations in Barley Responding to Scald and Spot blotch

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(Received 11 January 2018; Accepted 13 April 2018;
Communicated by V. Korzun)

Scald (*Rhynchosporium secalis*; *Rs*) and spot blotch (*Cochliobolus sativus*; *Cs*) are important diseases of barley (*Hordeum vulgare* L.) worldwide. Similar mechanisms and gene transcripts are assumed to be involved in the barley defense response since both these pathogens are necrotrophic fungi. In the current study, the transcriptome in leaves of the same barley genotype WI2291 inoculated with *Rs* and *Cs* was compared at different times postinoculation. Comparison of data for barley *Rs*- and *Cs*- inoculated plants with mock-inoculated plants revealed gene expression changes that included basal defense transcripts and transcripts specific to the establishment of a necrotrophic interaction with associated fungi. During barley–pathogen interaction pathway, WI2291 activated a higher number of genes and pathways in response to *Rs* infection than in response to *Cs* invasion. However, families of genes encoding pectin-degrading enzymes, secondary metabolism enzymes, transporters and peptidases are expanded to cover *Rs* and *Cs* at an early stage following inoculation. Our results demonstrate differences in the pathways and activated genes of barley cv. WI291 challenged by *Rs* and *Cs*, and that expression patterns of the same defense-associated genes were altered in adaptation to different pathogens. Our work provides new insights into the underlying mechanisms related to regulation of different pathways in response to fungal infection.

Keywords: barley, scald, spot blotch, transcriptome, gene expression

Introduction

Scald [*Rhynchosporium secalis* (Oudem.) J. J. Davis] and spot blotch [*Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex Dastur] of barley (*Hordeum vulgare* L.) are among the most devastating fungal diseases causing heavy crop losses worldwide (Kumar et al. 2002; Karimi and Khaledian 2016). They develop haustoria within the lumen of the host cell, which function to absorb nutrients (Kan 2006). Throughout infection, these fungi actively manipulate host cellular machinery that repress defenses and/or aid in disease progression (Lev et al. 2005; Sahu et al. 2016). Tremendous advance has been made in our understanding of pathosystems involving *Rs* and *Cs* pathogens. However, our understanding of the molecular mechanisms mediating barley infection by these two necrotrophic fungi is still limited.

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Transcriptome analysis is a common way for understanding responses of both the pathogens and the host during the infection process (Arabi et al. 2015; Al-Daoude et al. 2016). It is well known that signaling pathways are stimulated upon infection, being linked to specific rebuilding of the barley cell transcriptional event in response to the presence of the pathogens, therefore, transcript profiles and spatial expression patterns of genes can supply a substantial basis for functional analysis of unknown genes by correlating those patterns with biological process of interest (Mosquera et al. 2009; Wei et al. 2017). Moreover, plant cell reprogramming has been spotted in many pathosystems, including barley (Ghannam et al. 2016; Huang et al. 2016). A transcriptional profiling of barley genes during *Rs* and *Cs* infection revealed a complex cell reprogramming due to specific transcriptional and metabolic alterations generated by the pathogen (Jawhar et al., 2017; Al-Daoude et al. 2016). However, these studies suggested that transcripts for basal plant defense genes continued to increase in plenty during the infection process. However, this conclusion still remains to be completely confirmed for this pathosystem.

Screening for differentially expressed genes is a direct way to reveal the molecular basis of a biological system. The complementary DNA-amplified fragment length polymorphism (cDNA-AFLP) method has been effectively used to detect the altered expression of any gene that carries suitable restriction sites drives to a precise way for comprehension plant responses to pathogens (Sarosh and Meijer 2007; Xiao et al. 2016). In arbitrage with microarray technique and RNA sequencing, cDNA-AFLP costs less and does not seek sequence information (Reijans et al. 2003). When compared with subtractive hybridization, cDNA-AFLP is highly reproducible (De Paepe et al. 2004).

In this work, a transcriptional study of the barley *Rs* and *Cs* pathosystem during early stages was conducted. This is the first study to compare two necrotrophic stresses using a global expression profiling strategy in the same barley genetic background.

Materials and Methods

Plant material and experimental design

Under inoculation screening experiments in greenhouse and laboratory, the Australian cv. WI2291 proved to be the most susceptible genotype to all scald and spot blotch isolates available so far (Arabi and Jawhar 2004; Arabi et al. 2010). Therefore, it was selected for the cDNA-AFLP analysis. Barley seeds were 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 for each disease isolate consisted of two rows of 18 seedlings each. Flats were put 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%.

Inoculation

The most Syrian virulent *Rs*46 and *Cs*16 pathotypes (Arabi and Jawhar 2004; Arabi et al. 2010) were used in this study. The *Rs* fungal mycelia were transferred from a stock cul-

ture 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 0.5×10^6 conidia/ml using hemacytometer counts of conidia to provide estimates of the inoculum concentration. The *Cs* fungal mycelia were cultured on potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) with 13 mg/l kanamycin sulphate. The conidial suspension was adjusted to 2×10^4 conidia/mL using hemacytometer counts of conidia to provide estimates of the inoculum concentration. Plants were inoculated separately with scald and spot blotch at growth stage 13 (Zadoks et al. 1974) by uniformly spraying of the 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

Total RNA was extracted from samples of fungal-inoculated barley leaves (100–200 mg) at the specified time points post inoculation (24, 48 and 72 hpi) for each disease using the Nucleotrap mRNA mini kit (Macherey-Nagel, MN, Germany) following the manufacturer's protocol. As controls, mRNA was extracted from water-treated leaves, incubated under the same conditions and at the same time points. RNA was used for cDNA synthesis with the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions; the resulting cDNA was stored at -20 °C.

cDNA-AFLP analysis

The cDNA-AFLP protocol was performed according to the method described by Breyne et al. (2002) with minor modifications. 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 (*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 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 pre-amplification, 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 selective 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).

Data analysis

Data from each pathogen at each time point (three replications of each treatment) with transcript levels above background were analyzed by ANOVA with time and treatment effects. Transcripts were organized into groups based on their transcript accumulation profiles by a K-Means clustering analysis. Gene classification and categorization were performed using BLASTX hit (top hits with an e value greater than 10^{-10} were considered to have an unknown function).

Results

The reaction of barley plants is presented in Figure 1. In this study, cDNA libraries were constructed from barley leaves inoculated with *Rs* or *Cs* at different time points with three biological replicates. Comparison with mock-inoculated data revealed gene expression changes that included basal defense transcripts and transcripts specific to the establishment of a necrotrophic interaction connected with fungi. Differentially-expressed transcripts were visually scored relative to the first sampling time point which was arbitrarily attributed a zero value. An analysis of variance (ANOVA, $P < 0.0001$) was performed at each time point for each cultivar compared to its non-inoculated control. A total of 46 transcripts were detected in the barley WI2291 cultivar, 19 for *Rs* and 17 for *Cs* (Table 1).



Figure 1. Symptoms of spot blotch (a) and scald (b) diseases on barley cv. WI 2291 after 72 h post inoculation

Table 1. Defense-related gene classes transcripts, detected at $P < 0.0001$ in *R. secalis* (Rs) and *C. sativus* (Cs)-barley W12291 cultivar

	By pathogen		By hour after inoculation		
			24	48	72
All classes	R.s	27	8	10	9
	C.s	19	4	7	8
Defense-related	R.s	4	1	1	2
	C.s	1	0	1	0
Transport	R.s	3	2	1	0
	C.s	5	1	2	2
Regulatory	R.s	3	0	2	1
	C.s	5	1	2	2
Unknown	R.s	12	4	5	3
	C.s	7	2	2	3
Transcription	R.s	1	0	0	1
	C.s	1	0	0	1
Kinase	R.s	4	1	1	2
	C.s	0	0	0	0

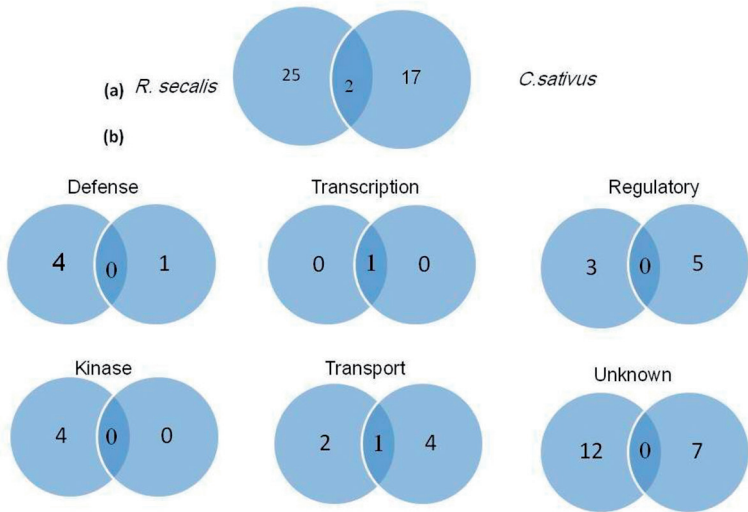


Figure 2. Venn diagrams of significant differentially accumulated transcripts in barley W12291 cultivar inoculated with *R. secalis* and *C. sativus* components of the diagrams are indicated by the left and right circles, respectively, of each Venn diagram. a. The number of transcripts exhibiting increased accumulation in W12291 during at least one examined time point after inoculation with *R. secalis* or *C. sativus*. b. Significant differentially accumulated transcripts in W12291 were separated for each pathogen by gene classification. The number of unique and common transcripts within each classification is indicated

Table 2. Significant differentially accumulated transcripts common between *R. secalis* (Rs) and *C. sativus* (Cs) detected at $P < 0.0001$

Accession no.	Gene category and annotation	Hour after inoculation		
		24	48	72
JZ820458	Calcineurin-like metallo-phosphoesterase superfamily protein,		R.s	R.s
JZ820448	Dasypus novemcinctus LIM domain kinase 2		R.s	R.s
BAA06731	<i>Nicotiana tabacum</i> NPK2, serine/Threonine protein Kinases,	C.s	C.s	C.s
EAZ37001	<i>Oryza sativa</i> hypothetical protein		C.s	C.s
EAY83985	<i>Oryza sativa</i> hypothetical protein		C.s	
AJ278817	partial mRNA for putative cysteine protease (pCYSPORT gene)			C.s
AJ290421	<i>Hordeum vulgare</i> mRNA for BAX inhibitor 1(pBI-1 gene)		C.s	C.s
EAZ10281	<i>Oryza sativa</i> hypothetical protein	C.s		C.s
AAU9536	<i>Solanum phureja</i> osmotin-like protein	C.s		C.s
AAG16625	<i>Solanum cryoprotective</i> osmotin-like protein			C.s
JZ820453	ABC transporter substrate-binding protein	R.s	R.s	
EAZ23888	<i>Oryza sativa</i> hypothetical protein			C.s
JZ820451	protein and putative ethylene-responsive transcription factor	R.s		
EAY86781	<i>Oryza sativa</i> hypothetical protein	C.s	C.s	
BAC43449	<i>Arabidopsis thaliana</i> putative disease resistance protection RPP8		C.s	
JZ820463	Triticum aestivum 4A DELLA protein (Rht-A) gene			S
JZ714808	<i>Hordeum vulgare</i> Cepada capa Rym4 gene complete.		R	R/S
JZ820456	GTP-binding protein LepA [<i>Desulfovibrio alaskensis</i>]	R.s	R.s	
JZ820457	Putative disease resistance protein RGA4			R.s
JZ714795	Vulgare cultivar Haruna Nijo HOX-1 gene.			R.s
AAA63149	Myosin heavy chain homolog		C.s	C.s
BAB44079	<i>Oryza sativa</i> putative NBS-LRR type resistance protein			C.s
JZ820452	hypothetical protein OsJ_31040	R.s	R.s/C.s	C.s
JZ820461	<i>Hordeum vulgare</i> subsp. vulgare eIF4E gene locus		R.s/C.s	R.s/C.s
JZ820464	<i>Hordeum vulgare</i> subsp. vulgare mRNA for predicted protein	R.s	R.s	
JZ820465	<i>Vitis vinifera</i> contig VV78X259514.3	R.s	R.s	R.s
JZ820454	Lysine-specific demethylase 3B		R.s	R.s
JZ820455	formin-like protein 7-like [<i>Setaria italica</i>]		R.s	

However, when transcripts were divided into one of six general categories (defense, regulatory, transport, metabolism and unknown function), the unknown group had the greatest number of common transcripts (Table 1). The comparison of the functional annotation of the main detected differential transcripts proposes that transcript detection method reveals functional groups with different preference (Fig. 2; Table 2). However, the only common transcripts detected at the same time point for *R*s and *C*s were eIF4E proteins (JZ820461), which is a fundamental translation initiation factor in eukaryotic cells that recruits the small ribosomal subunit(40S) to the mRNA cap structure, an event which is considered the first step in cap-dependent translation initiation (Malys and McCarthy 2011).

Discussion

Comparing the transcriptomes post *R*s and *C*s inoculation, most gene expression patterns displayed opposing patterns in the two treatments (Table 1), especially at the same time point. Those genes stimulated by *R*s infection were usually repressed in the same genotype by *C*s infection, and vice versa. For example, this pattern appeared by NBS-LRR like protein (BAB44079) that was animated after 72 h for *C*s only, which is one of the most large gene families in plants implicated in signaling, and they are regulated either as isolated genes or as linked clusters of different sizes that are thought to simplify rapid *R*-gene evolution (Christie et al. 2015). Also, ABC transporter (JZ820453) was up regulated 24 and 48h and down regulated 72 post inoculation by *R*s. This might be due to the fact that this group of transporters was sensitive to osmotic shock due to the loss of a substrate-binding protein from the periplasm (hence the term periplasmic binding protein-dependent transport systems) after *R*s infection of the susceptible barley genotype which was not obtained in the resistant one as documented in previous work (Al-Daoude et al. 2016). Additionally, Hox1 gene was up regulated 72 days post infection only in the inoculated plants suggesting a function in the early stages of defense against *R*s attack (Table 2).

Although both *R*s and *C*s are fungi, the number of genes induced by *R*s infection was higher than the number induced by *C*s infection at different time points of barley inoculation. This result indicated that there were different functional roles in response to different necrotrophic pathogens. Therefore, additional studies are required to distinguish additional low-abundance, basic, hydrophobic or membrane-bound proteins connected with susceptibility to *R*s and *C*s. However, analyses of transcription within single inoculated cells, as described by Gjetting et al. (2007), might be a useful method to further deep investigation for barley infection with *R*s or *C*s to know where and when the transcripts are differentially accumulated.

In the present study, we performed triplicate deep transcriptome surveys in leaves of the same barley cultivar ‘WI2291’ inoculated with *R*s and *C*s. The data has demonstrated the use of distinct sets of genes by barley in its response to *R*s or *C*s, and that a disease-susceptible barley cultivar activates different defense mechanisms during infection point times, as well as, that expression patterns of the same defense-associated genes were changed in adaptation to different pathogens. Moreover, transcripts of the infection-in-

duced barley genes were more abundant in *Rs*-infected leaves than in *Cs*-infected leaves at an early stage following inoculation, especially of genes in plant–pathogen interaction pathways. The results can provide a robust platform for further reconnaissance of disease-resistance on barley–fungus interactions.

Acknowledgements

We thank the Director General of AECS and the Head of Molecular Biology and Biotechnology Department for their much appreciated help throughout the period of this research.

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