Accumulation of Transcripts Abundance after Barley Inoculation with Cochliobolus sativus

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Spot blotch caused by the hemibiotrophic pathogen Cochliobolus sativus has been the major yield-reducing factor for barley production during the last decade. Monitoring transcriptional reorganization triggered in response to this fungus is an essential first step for the functional analysis of genes involved in the process. To characterize the defense responses initiated by barley resistant and susceptible cultivars, a survey of transcript abundance at early time points of C. sativus inoculation was conducted. A notable number of transcripts exhibiting significant differential accumulations in the resistant and susceptible cultivars were detected compared to the non-inoculated controls. At the p-value of 0.0001, transcripts were divided into three general categories; defense, regulatory and unknown function, and the resistant cultivar had the greatest number of common transcripts at different time points. Quantities of differentially accumulated gene transcripts in both cultivars were identified at 24 h post infection, the approximate time when the pathogen changes trophic lifestyles. The unique and common accumulated transcripts might be of considerable interest for enhancing effective resistance to C. sativus.

Keywords: Cochliobolus sativus, Hordeum vulgare, spot blotch, transcripts accumulation

Spot blotch (SB) caused by the fungus Cochliobolus sativus (Ito & Kurib.) Drechs. ex Dast. [anamorph: Bipolaris sorokiniana (Sacc. in Sorok.) Shoem.] is a serious foliar disease, particularly important in warm and moist areas during barley (Hordeum vulgare L.) growing season (Kumar et al., 2002). Effective control of SB can be achieved by the introduction of resistant cultivars as an important component of integrated disease management (Ghazvini and Tekauz, 2008). Hence, the search for parental stocks possessing an adequate level of resistance to C. sativus is essential.

Pathogenesis includes germination of conidia on the leaf surface and formation of an appressorium at the tip of the germ tube that supports direct penetration through the host cuticle (Kumar et al., 2001; 2002). The apparently biotrophic growth phase is primarily confined to a single epidermal cell invaded by infection hyphae, whereas the necrotrophic growth phase starts upon invasion of the mesophyll tissue followed by host cell death, which appears to be a consequence of toxin secretion (Apoga et al., 2002).

Few studies have been published on the cellular and molecular factors contributing to resistance to C. sativus (Schäfer et al., 2004). The impact of host-generated hydrogen peroxide ($H_2O_2$) on early infection stages of C. sativus has been analyzed by subcellular $H_2O_2$ detection in tissues infiltrated with diaminobenzidine. Intriguingly, $H_2O_2$ accumulates strongly in association with an unsuccessful fungal attack in the epidermis as well as in association with successful fungal growth in the barley mesophyll. However, still, little is known about the genetic background and regulation of interaction mechanisms (Al-Daoude et al., 2013).

Understanding the molecular basis of plant-pathogen interactions would greatly facilitate the development of new control strategies and the identification of pathogen and host factors required for disease progression (Kumar et al., 2002). However, using amplified fragment length polymorphism (AFLP) display of complementary DNA (cDNA) technique can reveal altered expression of any gene that carries suitable restriction sites leads to an accurate way for understanding plant responses to pathogens (Al-Daoude and Jawhar, 2009; Baldwin et al., 1999; Wendy et al., 2000). The cDNA-AFLP approach, once established, is efficient to display whole transcript profiles of single tissues, particular developmental stages or other inducible characters (Bachem et al., 1996). In this study, we used the cDNA-AFLP technique to identify transcripts that accumu-
lated in barley resistant and susceptible cultivars infected with the pathogen *C. sativus* at different early time points.

**Plant materials.** After an extensive screening for over ten years in the greenhouse and laboratory experiments, the German cultivar Banteng was proved to be the most resistant genotype to all SB isolates available so far (Arabi and Jawhar, 2004), therefore, it was used in this study. A universal susceptible control (cv. WI 2291) from Australia was also included in the experiments. Seeds were planted in plastic flats (60 × 40 × 8 cm) filled with sterilized peat-moss and arranged in a randomized complete block design with three replicates. Each experimental unit consisted of 10 seedlings. Flats were placed in a growth chamber at temperatures 21 ± 1°C (day) and 17 ± 1°C (night) with a day length of 12 h and 80–90% relative humidity. Seedlings were irrigated by Knop nutrient solution (1 g NaNO₃; 0.25 g KNO₃; 0.25 g MgSO₄·7H₂O; 0.25 g KH₂PO₄; and 10 mg FeCl₃ per 1000 ml water) (Arabi and Jawhar, 2004).

**Inoculum preparation.** The virulent pathotype *C. sativus* (P4) used in the study was the most virulent of 117 isolates collected in 1998 and 2004 from naturally infected barley in different regions of Syria, as described by Arabi and Jawhar (2004). The fungal mycelia were transferred from a stock culture into Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) with 13 mg/l kanamycin sulphate and incubated for 10 days at 21 ± 1°C in the dark. Then, conidia were collected with 10 ml of sterile distilled water. The conidial suspension was adjusted to 2 × 10⁶ 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.

**Inoculation.** Infections were initiated by spraying the third and fourth barley leaves with a conidial suspension, and leaves covered for one night with plastic bags to increase humidity and the plants were kept in the same greenhouse at 20°C with a 16 h photoperiod. Disease ratings were determined on a 0–9 scale according to Fetch and Steffenson (1999).

**Material collection.** Leaf samples for RNA isolation were taken at different time points post inoculation (4, 24 and 48 hpi) 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 N₂ before they were stored at −80°C till needed. The remaining unsampled plants were moved to the greenhouse for disease assessment. Control plants of ‘Banteng’ and ‘WI2291’ were assessed only for disease phenotypes. 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 for plant tissues.

cDNA-AFLP analysis. The cDNA-AFLP protocol was performed according to the method described by Breyne et al. (2002) with minor modifications which permits the visualization of one single cDNA fragment for each messenger originally present in the sample, thus reducing the redundancy of sequences obtained (Al-Daoude and Jawhar, 2009). 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 *MseI* 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'; *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 *MseI* 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 (*Bst*0: 5'-GAC TGC GTA GTG ATC T-3'; *Bst*C0: 5'-GAC TGC GTA GTG ATC C-3'). Pre-amplification PCR conditions

|        | 4 hpi |        | 24 hpi |        | 48 hpi |        |
|--------|-------|--------|--------|--------|--------|--------|
|        |       |        |        |        |        |        |
| R      | 19    | 14     | 5      | 21     | 9      | 12     | 22     | 6      | 16     | 62     | 29     | 33     |
| S      | 8     | 4      | 4      | 7      | 5      | 2      | 11     | 6      | 5      | 26     | 15     | 11     |

Table 1. Totals of significant differentially accumulated transcripts by *C. sativus* and hours after inoculation, detected at *P* < 0.0001

|        | T | I | D |        | T | I | D |        | T | I | D |
|--------|---|---|---|--------|---|---|---|--------|---|---|---|
| R      | 19| 14| 5 |        | 21| 9 | 12|        | 22| 6 | 16|
| S      | 8 | 4 | 4 |        | 7 | 5 | 2 |        | 11| 6 | 5 |

Total (T), increased (I), or decreased (D) levels of transcript accumulation compared to the non-inoculated control. R (resistant) and S (susceptible).
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 MseI 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). PCR products were purified with MultiScreen PCR μ96 plates (Millipore) and sequenced directly (BMR Genomics). PCR products were purified with QIAgene gel extraction kit according to the manufacturer’s recommendations.

Data analysis. Data from each cultivar and its control 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 three 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).

The disease symptoms (presence of solid, dark necrotic lesions) were typically observed in infected plants with severity values being consistently higher in the universal susceptible cv. WI2291 at 48 hpi. The resistant cv. Banteng exhibited an infection type mode of 1 with a range of 1–3, whereas, WI2291 gave a susceptible infection type mode of 8 as expected (data not shown).

cDNA-AFLP analysis was carried out on mRNA samples of infected leaves to survey transcript accumulation in barley resistant and susceptible cultivars at three time points after C. sativus inoculation (4, 24 and 48 hpi) (Fig. 1), and on water-sprayed leaves (healthy control), as described (Breyne et al., 2002). Three different times were chosen to cover early barley responses to SB which leads within 48 hpi to a visible hypersensitive cell death on a susceptible genotype by considering the observations of Wisniewska et al. (1998) with barley susceptibility to C. sativus. 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 62 unique transcripts were detected in resistance cultivar, 29 of which exhibited increased accumulation. However, the number of identified transcripts slowly decreased across time points (Table 1). The susceptible cultivar ‘WI2291’ yielded a lesser number
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of unique transcripts with significant differential accumulation (26 total; Table 1). Of the 26 transcripts, 15 exhibited increased accumulation.

At the p-value of 0.0001, there were nine transcripts identified for at least one time point in both cultivars (Fig. 2a). When transcripts were divided into one of three general categories (defense, regulatory and unknown function), the unknown group had the greatest number of common transcripts (Fig. 2b; Table 2). Some transcripts were found in common at the same time point, such as AAK93796, a NBS-LRR like protein, and BAA06731, serine/threonine protein kinase (NPK2), in both cultivars at 24 hpi, while the majority accumulated at different time points between the cultivars.

In addition to identifying accumulated transcripts between the two cultivars, we identified transcripts that specifically responded to C. sativus in the resistant cultivar ‘Banteng’. Due to their well-known function in response to pathogens, we focused on the transcripts categorized as having some role in the defense response. The defense-related classifications of transcripts were unique in the resistant cultivar (Table 2). Transcript, AJ290420, pRac1 gene was identified only in the resistant cultivar. This gene is well-known to play a significant role in barley resistance to powdery mildew disease (Huckelhoven et al., 2001). Similarly, AB089942, Tad1, that encodes a thionin (TAD1), seems to be involved in resistance against pathogens during cold weather and a high expression level of 1-hordothionin from endogenous leaves of Arabidopsis thaliana resulted in increasing resistance against F. oxysporum (Hughes et al., 2000). Not surprisingly, the PR proteins were found in the resistant one. These unique defense sets show different patterns of expression. For example, AAZ94267, PR5, is detected at later time points in resistant cultivar (24 and 48 hpi) (Table 2). Moreover, while the number of detected genes is essentially consistent across all time points for both cultivars, the dramatic increase at 24 hpi coincide with the shift from biotrophism to necrotrophism within the pathogen (Kumar et al., 2002).

Transcripts of only one oxidative stress-related gene, AJ251717, putative NADPH oxidase (pNAox gene), was detected in the resistant cultivar after 24 hpi (Table 3). This gene is believed to be one of the earliest defense responses upon pathogen challenge (Lamb and Dixon, 1997). The observed pattern of reactive oxygen species (ROS) producers at the earlier stages of infection and reducers towards the later stages is supported by previous research that hypersensitive reaction as a resistance mechanism functions well against biotrophs and poorly against necrotrophs (Huckelhoven et al., 1999; Govrin and Levine, 2000). The pattern suggests that ‘Banteng’ targets C. sativus with ROSs to stop pathogen infection. Indeed, this supports previous

Table 2. Significant differentially accumulated transcripts common between resistant (R) and susceptible (S)cultivars detected at $P < 0.0001$

| Gene category and annotation | Hour after inoculation |
|-----------------------------|-----------------------|
|                            | 4  | 24 | 48 |
| DQ285022 1 Serine-threonine protein kinase (RK1) | R  | R / S | R / S |
| AB284130 1 OsMKP1 mRNA for MAP kinase phosphatase | R  | R  | R  |
| AJ276225 1 Protein 5 (pr-5 gene) antimicrobial protein | R  | R  | R  |
| AJ290420 1 GTP-binding protein (pRac1gene) | R  | R  | R  |
| AJ251717 1 Putative NADPH oxidase (pNAox gene). | R  | R  | R  |
| AAS07338 1 Antifungal zeaamin-like protein | R  | R  | R  |
| AAZ94267 1 PR5 | R  | R  | R  |
| AB089942 1 Tad1 mRNA for defense | R  | R  | R  |
| AAP80281 1 Protein Hod3 | R  | R  | R  |
| AAK93796 1 NBS-LRR like protein | R  | R / S | S  |
| BAA06731 1 NPK2, serine/Threonine protein | S  | S  | R  |
| AJ278817 1 Protease (pCYSSPORT gene) | S  | S  | R  |
| BAC43449 1 Protection RPP8 | S  | R / S | R / S |
| AAA63149 2 Myosin heavy chain homolog | S  | S  | S  |
| AJ290421 2 BAX inhibitor 1(pBI-1 gene) | S  | S  | S  |
| AAS9536 2 Osmotin-like protein | S  | S  | S  |
| AY685113 2 Transcription factor AP2D2 m. | R  | R  | R  |
| EAY73848 3 Unknown | R / S | R / S | R / S |
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Table 3. Defense-related gene classes containing significant differentially accumulated transcripts, detected at \( P < 0.0001 \) in resistant (R) and susceptible (S) barley cultivars

| Class                  | Unique | Common | By cultivar | By hour after inoculation |
|------------------------|--------|--------|-------------|--------------------------|
|                        |        |        |             | 4 | 24 | 48 |  
| All classes            | 30     | 9      | R           | 24 | 4  | 9  |
|                        |        |        | S           | 15 | 2  | 4  |
| Common classes         |        |        |             |    |    |    |
| Oxidative burst-related|        |        |             |    |    |    |
| General                | 1      | 0      | R           | 1  | 0  | 1  |
|                        | 0      | 0      | S           | 0  | 0  | 0  |
| PR protein             |        |        |             |    |    |    |
| General                | 2      | 0      | R           | 2  | 0  | 1  |
|                        | 0      | 0      | S           | 0  | 0  | 0  |
| R genes                | 4      | 1      | R           | 5  | 1  | 2  |
|                        | 0      | 1      | S           | 1  | 0  | 0  |
| MAPK related           | 2      | 0      | R           | 2  | 1  | 1  |
|                        | 2      | 0      | S           | 2  | 0  | 1  |
| Transcription          | 1      | 0      | R           | 1  | 0  | 0  |
|                        | 0      | 1      | S           | 1  | 0  | 0  |
| Protease inhibitor     | 0      | 0      | R           | 0  | 0  | 0  |
|                        | 1      | 0      | S           | 1  | 0  | 0  |
| Antifungal             | 1      | 0      | R           | 1  | 0  | 0  |
|                        | 0      | 0      | S           | 1  | 0  | 0  |
| Transport              | 0      | 0      | R           | 0  | 0  | 0  |
|                        | 2      | 0      | S           | 2  | 0  | 1  |
| PR protein             | 2      | 0      | R           | 2  | 0  | 1  |
|                        | 0      | 0      | S           | 0  | 0  | 0  |
| Defense-related        | 2      | 0      | R           | 2  | 0  | 1  |
|                        | 0      | 0      | S           | 0  | 0  | 0  |
| Unknown                | 4      | 3      | R           | 7  | 2  | 2  |
|                        | 5      | 3      | S           | 8  | 2  | 3  |

This report has demonstrated the use of distinct sets of genes by the resistant cultivar ‘Banteng’ in its response to \( C. sativus \). In particular, the type of oxidative stress-related transcripts and the timing of their accumulation appear to be important components distinguishing successful defense against \( C. sativus \) of its initial trophic lifestyle. The targeting of particular oxidative stress-related proteins or those proteins of other classes unique to a trophic lifestyle for use in breeding programs should be carefully evaluated to ensure broad usage. The few defense classes, namely PR proteins, might be of most interest for enhancing resistance to a wider range of \( C. sativus \). Since, over expression of particular \( PR-5 \) proteins delays or halts the onset of disease symptoms of fungal pathogens in rice (Datta et al., 1999). However, although further quantitative validation using Real-Time PCR (qPCR) is needed, 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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