Differentially Expressed Gene Transcripts in Wheat and Barley Leaves upon Leaf Spot Infection

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Abstract: A cDNA library was created on the basis of transcripts that were generated during the process of infection of wheat and barley with *Pyrenophora tritici-repentis* or *P. teres*. Due to the time course of infection assays, the leaves were collected at various intervals after inoculation until 100 h after inoculation. We compared the temporal development of the two pathogens, as well as the effect of varieties of the same crop species, and differences among isolates of a single pathogen. The appressoria and vesicular structures developed more rapidly in barley varieties inoculated by *P. teres* than in wheat varieties inoculated by *P. tritici-repentis*. The lower level of susceptibility of the wheat variety Globus and the barley variety Heris to *P. tritici-repentis* and *P. teres*, respectively, was apparent from 12 h after infection. Information on changes in expression profiles during pathogenesis and on the clones that could be functionally assigned to the known gene sequences is presented. Out of the 360 transcript derived fragments (TDFs) produced in the barley-*P. teres* pathosystem in our trials, 62% were specific to the plant-pathogen interaction. In the wheat-*P. tritici-repentis* pathosystem 291 TDFs occurred, 63% were specific to the plant-pathogen interaction.

Keywords: cDNA-AFLP; infection development; net blotch; *Pyrenophora tritici-repentis*; *Pyrenophora teres*; tan spot

Wheat tan spot and barley net blotch are cereal diseases of worldwide economic importance caused by ascomycetous fungi *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* /Died./ Schoem.) and *Pyrenophora teres* Drechs. (anamorph: *Drechslera teres* /Sacc./ Shoemaker). Both pathogens can cause severe yield losses due to a reduction of kernel number per ear, size of kernels, and the extent of photosynthetically active leaf area.

Typical net blotch symptoms on leaves (form *P. teres* f. *teres*) are distinctive dark-brown, longitudinal necrotic lesions that often form a net-like pattern. Another form (*Pyrenophora teres* f. *maculata*), known as the spot form, causes circular or elliptical brown lesions. Both forms induce surrounding chlorosis in the affected barley tissues. These distinct disease symptoms, the spot form and the net form, can be explained by differences in fungal growth (Lightfoot & Able 2010).

Typical tan spot (*P. tritici-repentis)* symptoms include tan-coloured necrotic lesions surrounding small infection sites. The pathogen induces tan necrosis and/or extensive chlorosis in its wheat host.

The induction of chlorosis and necrosis symptoms in both barley (net blotch) and wheat (tan spot) pathosystems is caused by mycotoxin activity. Toxins appear to act as pathogenicity factors (Orolaza *et al.* 1995; Ciuffetti *et al.* 2010). *P. tritici-repentis* ability to induce two distinct symptoms, tan necrosis and extensive chlorosis, on a set of differential wheat varieties is the basis of distinguishing eight *P. tritici-repentis* races (Lamari *et al.* 2003). Each race is differentiated by the expression of one toxin or a combination of *Ptr* ToxA, *Ptr*ToxB and *Ptr* ToxC toxins.

Plant disease resistance often plays a role in the infection process. After conidia germination, an elongating germ tube is formed. The appressorium is produced at the tip of the germ tube, followed by the formation of penetrating hyphae, vesicles, and infection hyphae. The host starts to respond to the invading pathogen. Papilla depositions, occasionally haloes or aggregation of cytoplasm can appear at the site of *P. tritici-repentis* or *P. teres* penetration (Keon & Hargreaves 1983; Dushnicky *et al.* 1998).

Endogenous levels of reactive oxygen intermediates, nitric oxide, salicylic acid, jasmonic acid, and ethylene are increased in the incompatible reaction. Plant disease resistance can be triggered by specific recognition of microbial effectors by plant nucleotide binding-leucine rich repeat (NB-LRRs) receptors, and series of defence responses are activated. These responses include the hypersensitive response, upregulation of phenylalanine ammonium lyase, deposition of cell wall reinforcing materials, and synthesis of antimicrobial compounds including pathogenesis related proteins and phytoalexins (Veronese *et al.* 2003; Jones & Dangl 2006; Collier & Moffett 2009; Bernoux *et al.* 2011).

A cDNA-AFLP (Amplified Fragment Length Polymorphism; Vos *et al.* 1995) technique is available for the characterization of genes involved in biochemical processes. It enables to isolate genes that are differentially expressed during a process of interest. It was employed to clone cDNA copies of transcripts that are upregulated during *P. teres* conidial germination (Dilger *et al.* 2003) to describe interactions between the fungal pathogen *C. sativus* and its resistant barley host (Al-Daoude & Jawhar 2009) and to identify differentially expressed genes in chickpea during root infection by *Fusarium oxysporum* (Nimbalkar *et al.* 2006). Adhikari *et al.* (2007) analysed the expression patterns in association with resistance response of wheat to *Mycosphaerella graminicola* using cDNA-AFLP. Zheng *et al.* (2009) used cDNA-AFLP in a study on genes expressed during compatible interactions between *Blumeria graminis* and wheat.

The aim of our study was to determine if the cDNA-AFLP method can be usefully applied to study the infection development and host reaction in wheat and barley leaves during leaf spot infection. Clones with similarity to gene products described earlier from various organisms were detected. The function of detected gene products and their possible role in plant defence are discussed. The temporal development of *P. tritici-repentis* and *P. teres* pathogens, as well as the effect of varieties of the same crop species, and differences among isolates of a single pathogen were examined under the microscope. This information could be applied for predicting genes participating in processes of resistance mechanism for further, more detailed studies.

MATERIAL AND METHODS

Infection of plant materials

The barley varieties Beate (susceptible), Heris (medium resistant) and Prestige (susceptible) and wheat varieties Caphorn (susceptible), Biscay (susceptible), Globus (medium resistant) and Glenlea (susceptible) were chosen to study the development and degree of infection by fungi of the genus *Pyrenophora* (Figure 1 and 2).

The plantlets were raised in greenhouse conditions at the temperature of 20°C during 10 days up to the second leaf stage. These plants were inoculated with a suspension containing conidia, mycelial fragments, and sterile water.

The inoculum was prepared from isolate H602 of *P. teres* f. *teres* (PTT) that was collected in Drnholec, Czech Republic during 1998 and from isolate H615 of *Pyrenophora teres* f. *maculata* (PTM) that was collected in Lužany during 2005. Both isolates were obtained by courtesy of Ing. Věra Minaříková, Agricultural Research Institute in Kroměříž, Ltd., Czech Republic. *P. tritici-repentis* isolate PTR-1 was collected in Přerov, Czech Republic in 2000. Isolate PTR-1 belongs to race 1 which is prevalent in the Czech Republic like in many countries of *P. tritici-repentis* occurrence.

The detergent Tween 20 was added to the suspension (1µl/100 ml). The inoculum contained 20 conidia in 5 µl of the suspension. Every pot, containing approximately 30 plants, was inoculated with 10 ml of the suspension. The inoculum was dispersed evenly. The inoculum preparation was based on the method of Evans *et al.* (1993) and Ali and Francl (2001). The inoculated plants were incubated for 24 h in the closely covered glass cylinders. Three different isolates of *P. teres* and *P. tritici-repentis* were used. Segments 2.5 cm long were taken from the middle part of the leaf blade of the second leaf from the infected plants in time intervals of 4, 23, 28, 47, 52, 71, 76, 94 and 100 h after inoculation (Table 1). Both infections

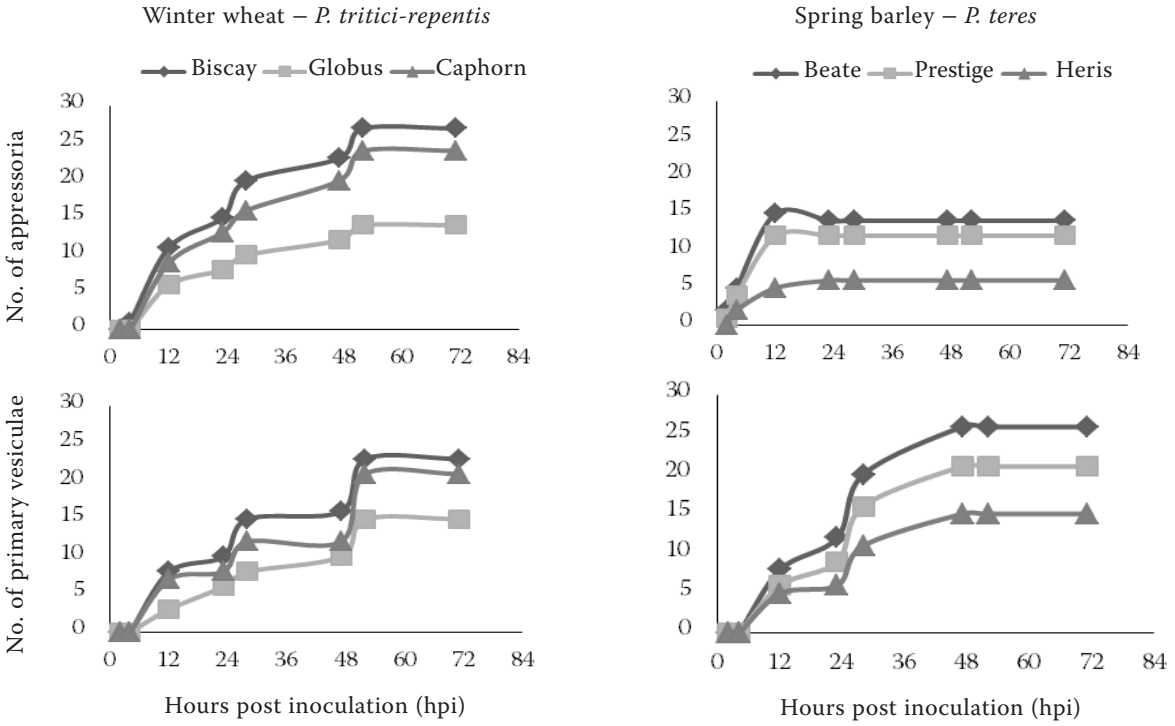

Figure 1. Development of fungal appressoria and vesicles on wheat leaves after inoculation by *Pyrenophora tritici-repentis* and on barley leaves after inoculation by *Pyrenophora teres* 0–72 hpi; the plants were inoculated with a suspension containing conidia, mycelial fragments and sterile water

and collecting of samples were conducted simultaneously in all involved varieties. One second leaf was always used for image analysis, and the other for molecular assay. Samples for molecular analysis were frozen in liquid nitrogen and kept at –80°C until use. Leaf segments of barley and wheat were analysed under a microscope (CX40, Olympus, Tokyo, Japan, 400× magnification) at the same time to determine the speed of formation of secondary infection structures, their amount, and the time when their development stopped. Staining with trypan blue was used for better differentiation of the structures.

RNA extraction and cDNA synthesis. Freshly harvested leaves before inoculation and 4, 23, 28, 47, 52, 71, 76, 94 and 100 h after inoculation were ground in liquid nitrogen into a powder using the mortar and pestle and suspended in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted according to the PureLink™ RNA Mini Kit protocol (Invitrogen, Carlsbad, CA, USA). The complete removal of DNA was controlled by PCR using the primers specific to 18S rRNA (Dilger *et al.* 2003) in 15-µl reaction volumes containing 200nM primers, dNTPs at 200µM each, 1U of Taq polymerase (Qiagen, Hilden, Germany) and 1× PCR buffer (Qiagen, Hilden, Germany) on a UNOII thermocycler (Biometra, Goettingen, Germany). The concentration of RNA samples was determined by spectrophotometry. Samples were diluted to the concentration of 300 ng/µl. cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol.

cDNA-AFLP analysis. Generation of cDNA-AFLP amplicons was conducted with each cDNA sample prepared as described earlier using the restriction enzyme combinations MseI/EcoRI and MseI/PstI. For visualization, EcoRI and PstI primers were labelled with fluorescent labels 6-fam, ned, joe or hex (Table 2). AFLP analyses were carried out according to the AFLP™ Plant Mapping Protocol (Applied Biosystems, Foster City, CA, USA). Products of selective amplification were separated by capillary electrophoresis in ABI PRISM 310 (Perkin-Elmer, Foster City, CA, USA). Rox500 (PN 401734, Applied Biosystems) was used as the internal size standard. Chromatograms were processed by the software GeneScan and Genotyper (Applied Biosystems). A binary matrix, reflecting the presence (1) or absence (0) of specific AFLP fragments, was generated for

Table 1. Samples used in the study

Host plant	Variety	Pathogen	Isolate of patogen	Sample	Hours after inoculation (hpi)
Wheat	Glenlea	–	–	3	0
		P. tritici-repentis	PTR-1	16	4
				21	23
				26	28
				31	47
				36	52
				41	71
				46	76
Barley	Beate	–	–	1	0
		P. teres	H602	4	4
				8	23
				12	28
				17	47
				22	52
				27	71
				32	76
				37	94
				42	100
			H615	5	4
				9	23
				13	28
				18	47
				23	52
				28	71
				33	76
				38	94
				43	100
	Prestige	–	–	2	0
		P. teres	H602	6	4
				10	23
				14	28
				19	47
				24	52
				29	71
				34	76
				39	94
				44	100
			H615	7	4
				11	23
				15	28
				20	47
				25	52
				30	71
				35	76
				40	94
				45	100

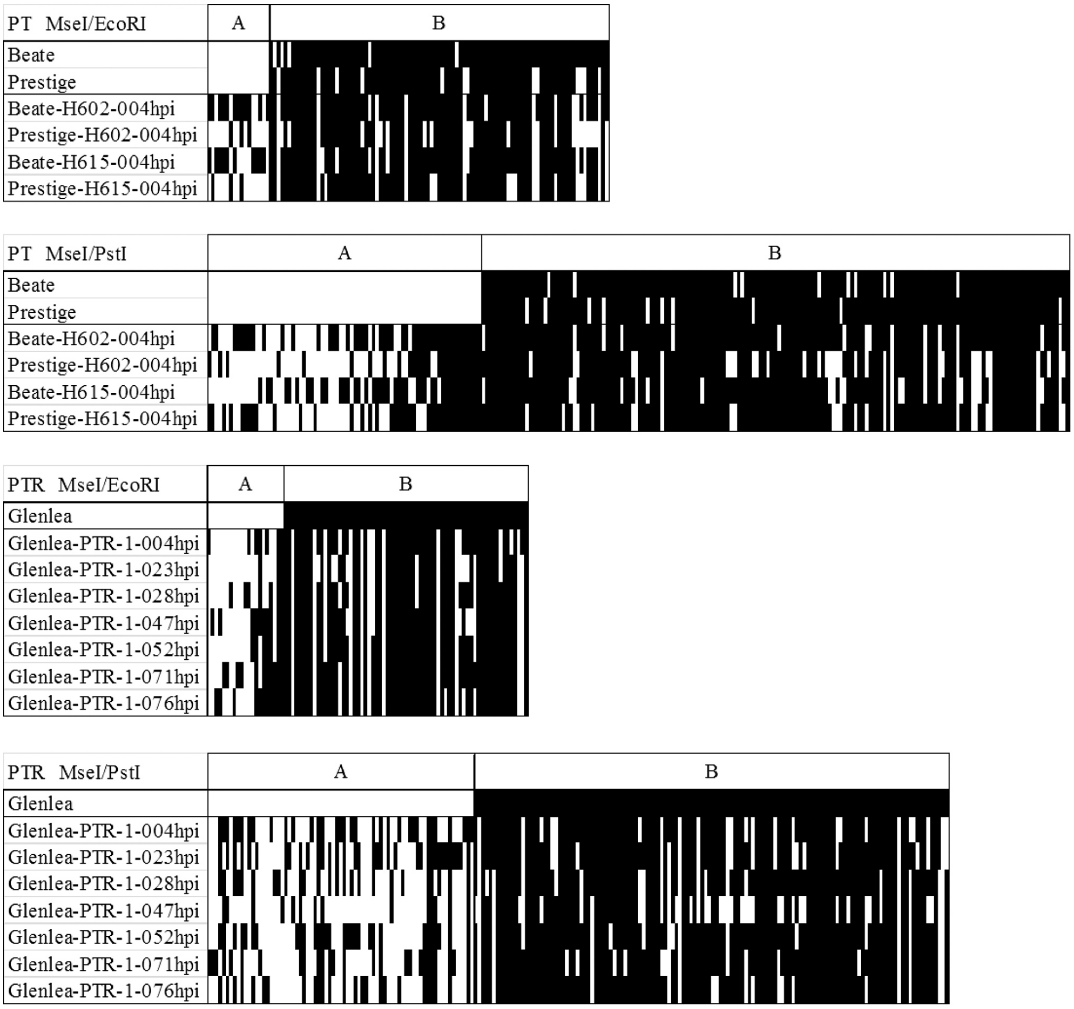

Figure 2. Early expressed profiles of the barley-*P. teres* pathosystem 4 h after inoculation (hpi) and profiles of the wheat-*P. tritici-repentis* pathosystem expressed 4–76 hpi: (A) differential cDNA AFLP bands unique to inoculated samples; (B) differential cDNA AFLP bands detected both in inoculated and uninoculated samples

each sample. A colormap was prepared in MS Excel software.

Sequence analysis. Selected amplicons were extracted from 2% agarose gel using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into the pCR®4-TOPO plasmid vector. The resulting ligation products were used to transform *Escherichia coli* TOP10 competent cells according to the manufacturer's protocol (TOPO® TA Cloning Kit, Invitrogen, Paisley, UK). Purification of plasmids was carried out using a High Pure Plasmid Isolation Kit (Roche, Mannheim, Germany). Inserts were sequenced using a Big Dye Terminator 3.1 Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and universal sequencing primers, either M13+ or M13–. Extension products were separated on an ABI PRISM 3130 sequencer (Applied Biosystems, Foster City, CA, USA). Sequence alignments were assembled using the T-Coffee software (Notredame *et al.* 2000) and blasted (Zhang *et al.* 2000) to find homologies within sequences already deposited into databases (http://blast.ncbi.nlm.nih.gov/). Hits were considered significant if the expected value (E-value) was less than 1.0E-5.

RESULTS AND DISCUSSION

Infection of plant materials

There was no apparent germination of conidia within two hours or less after the infection in all varieties and pathogens tested. Appressoria and the penetration pegs started to form within 4–6 h.

Host tissue penetration and formation of vesicles occurred twelve hours after inoculation. The maximum and no longer considerably changing number of appressoria was observed in the segments sampled 47 h after inoculation or later. Vesicular formation was variety specific (Figure 1). Similarly, Keon and Hargreaves (1983) described the germination of conidia, formation of appressorium and penetration of host epidermal cells in *P. teres* within 24 h after inoculation. According to Dushnicky *et al.* (1996) the penetration and infection stage of *P. tritici-repentis* generally occurs within the first 24 h both in susceptible and resistant varieties.

In barley varieties, a higher incidence of appressoria followed by the secondary mycelium formation was registered in several observations in the Beate variety. The Prestige variety showed a lower occurrence of appressoria in comparison with Beate. The difference between both varieties within 47 hpi made about 10% of the incidence of infection structures on the examined leaf segment area: 14 appressoria and 26 vesicles in Beate, 12 appressoria and 21 vesicles in Prestige. The appressoria, vesicles, and secondary mycelium formed in both varieties at the same time. We observed fewer appressorium, vesicular, and secondary mycelium structures 28 h after the inoculation in the Heris variety than in Beate and Prestige. There were 6 appressoria and 11 vesicles in Heris, 14 appressoria and 20 vesicles in Beate and 12 appressoria and 16 vesicles in Prestige on the examined leaf segment area.

In wheat varieties, the highest proportions of appressoria and vesicular structures in 52 h post inoculation were produced in the Biscay variety, 27 appressoria and 23 vesicles on the examined leaf segment area. At the same time point, 18% less of these structures were present in the Caphorn variety, 24 appressoria and 21 vesicles. In addition, the Globus variety showed 14 appressoria and 15 vesicles at the same time point, which means 45% less of appressoria and vesicles than in Biscay.

Growth rate studies on the hosts revealed that the invasion of barley varieties by *P. teres* was faster than that by *P. tritici-repentis*. *P. tritici-repentis* produced appressoria on the wheat varieties later, after 12 h. However, the influence of the particular isolates was not important for the rate and percent of infection. The image analysis revealed no correlation between the symptom development and the time after inoculation.

Many factors are involved in the manifestation of symptoms induced by the toxins varying not only chemically but also in the mechanism of their activity. In addition, the phenotypic observation of the disease is influenced by the plant ability to recover impairment. When the formation of an infection peg does not lead to penetration, as may happen due to the host response, the fungus attempts to penetrate again. Papillae formation may stop the entry of infection hyphae into some epidermal cells, but not into all of them, even in resistant varieties (Dushnicky *et al.* 1996). In all these processes, multiple parameters may display a substantive influence, for example even a small variation in the leaf state and in light, moisture, temperature or other conditions.

cDNA-AFLP analysis

The restriction endonuclease combinations MseI/EcoRI and MseI/PstI were used for cDNA-AFLP analysis. In total, 39 primer combinations (21 for MseI/EcoRI enzyme combination, and 18 for MseI/PstI enzyme combination) were obtained for 46 cDNA samples.

The MseI primer was extended with seven combinations of three selective nucleotides, whereas the extensions of EcoRI and PstI primers had two nucleotides. Produced fragments were in the size range of 80–500 bp.

In the barley-*Pyrenophora teres* pathosystem, 62% of the total 360 transcript-derived fragments (TDFs) were supposed to be specifically expressed only during the plant-pathogen interaction (Table 2). MseI/EcoRI primer combinations applied in the barley-*P. teres* pathosystem produced 123 transcript-derived fragments (TDFs). Out of 123 fragments, 89 (72%) were supposed to be specific to the host-pathogen interaction. The MseI/PstI primer combination provided 237 TDFs in the barley-*P. teres* pathosystem, out of which 133 (56%) were supposed to be specific to the host-pathogen interaction.

In the wheat-*P. tritici-repentis* pathosystem, 291 TDFs were identified, out of which 183 fragments (63%) were supposed to be specific to plant-pathogen interaction. The MseI/EcoRI primer combination used in the wheat-*P. tritici-repentis* pathosystem rendered 88 TDFs, with 49 fragments (56%) involved in the host-pathogen interaction. In the wheat-*P. tritici-repentis* pathosystem, 203 TDFs were given by MseI/PstI combinations, with 134 fragments (66%) involved in the host-pathogen interaction. The number of obtained

Table 2. List of selective primers used for cDNA AFLP analysis, their range of detection and the numbers of TDFs detected in plant-pathogen interactions

Sign	MseI	EcoRI (fam, joe, ned)	Signal range	TDFs in barley-*P. teres* pathosystem/TDFs	TDFs in wheat-*P. tritici-repentis* pathosystem/TDFs
				involved in host-pathogen interaction	
A	CAC	TG	119–475	11/7	2/1
		AG	107–437	9/8	9/5
		AT	129–395	5/2	6/2
B	CAG	TC	85–393	9/7	6/3
		TA	81–115	2/0	3/2
		AT	107–293	5/4	6/5
C	CAA	TC	101–269	6/4	2/0
		AA	89–485	12/8	5/1
		AT	97–319	4/3	3/1
D	CTA	TG	111–257	3/1	1/0
		TA	–	0/0	0/0
		TT	–	0/0	0/0
E	CTG	TC	185–449	2/2	1/0
		AA	123–365	7/7	2/1
		AT	87–325	4/4	3/0
F	CTT	TG	111–461	11/7	5/4
		AG	97–475	11/9	11/8
		TT	–	0/0	0/0
G	CTA	TC	145–415	3/2	9/9
		AA	111–423	8/4	6/1
		AT	99–497	11/10	8/6
Total				123/89	88/49
		PstI (fam, hex, ned)		involved in penetration	
K	CAC	GAT	91–471	12/6	8/6
		GCA	97–443	14/10	23/17
		GA	93–443	16/7	18/10
L	CAC	GC	133–443	9/5	7/5
		GT	95–317	6/2	2/0
		GG	103–429	18/9	16/8
M	CTG	GAT	139–453	7/3	4/1
		GCA	99–387	16/10	13/11
		GA	97–453	13/4	7/5
N	CTG	GC	97–409	14/5	6/4
		GT	97–413	15/8	13/8
		GG	81–389	22/17	26/22
O	CAA	GAT	135–417	7/5	3/3
		GCA	91–389	14/9	14/10
		GA	97–335	17/10	11/3
P	CAA	GC	97–391	14/7	7/3
		GT	95–321	11/7	12/11
		GG	81–295	12/9	13/7
Total				237/133	203/134

TDFs was comparable with that reported in previous studies (Al-Daoude & Jawhar 2009; Zheng *et al.* 2009). The results of cDNA AFLP analysis were summarized in colormaps showing the expression profile of both pathosystems (Figure 2). The varieties Prestige and Beate differed significantly in their expression profiles in 4 hpi. A slower course of the infection process for some combinations of varieties and isolates is a possible explanation. We must admit that the detected fragments do not reflect the infection process, but rather some other factors such as microenvironmental differences in the greenhouse. Frequent coincidence of the presence of fungal and plant bands can indicate mere presence or development of mycelium in the plant.

In the barley-*P. teres* pathosystem, the infected plants of barley varieties Beate and Prestige were examined with primer combinations MseI/EcoRI 4 hpi. Fragments present only in the uninfected variety compared to the infected variety can correspond with differentially expressed genes involved in the plant defence. It is possible that some processes could already be displayed in some varieties, but delayed in the others, during the early stage of infection (4 hpi). Thus the infected variety Beate showed seven TDFs possibly connected with the plant defence, and two such TDFs were shown in Prestige. Another band specific to the plant infected with isolate H602, CAG/TC-111 bp, was observed. Fragment CAG/TC-111 bp distinguished *P. teres* isolates H602 (PTT) and H615 (PTM). Fragment CAG/TC-261 bp distinguished the varieties Prestige and Beate regardless whether they were infected or not.

Another primer combination, MseI/PstI, distinguished *P. teres* isolates H602 and H615 by means of fragments CAA/GG 195 bp, CAC/GCA 111 bp. Since the fragments are in the region where also fungal fragments are present, this unequal band reproduction could be caused by slower development of one of the fungal isolates. The MseI/PstI primer combination did not distinguish the varieties Prestige and Beate regardless whether they were infected or not. We noticed possible downregulation while searching for transcripts specific to the plant response to fungal infection. The fragment was present in some infected varieties, while in the uninfected varieties it was always absent.

The wheat-*P. tritici-repentis* pathosystem was examined with MseI/EcoRI primer combinations. Ten fragments vanished after inoculation of var. Glenlea with *P. tritici-repentis*. In one case, a fragment was detected only after inoculation. It was probably linked to a gene expressed by the pathogen. In addition, specific fragments were present in the region of the fungus expression only for definite time periods after inoculation.

MseI/PstI primer combinations in the wheat-*P. tritici-repentis* pathosystem revealed three cases of a strong fungal band at the same time point when infecting var. Glenlea. It implies specificity to the plant infection, and more precisely for the presence of the mycelium. The primer combinations resulted in the amplification of 405 differentially amplified PCR fragments.

Sequence analysis

Thirty-six differential cDNA-AFLP fragments with the strongest signal were chosen for a sequence analysis. The results of the sequence comparisons are summarised in Table 3. No significant hits were detected for seven clones. Though their function is unknown, they still may represent novel transcripts associated with defence response (Nimbalkar *et al.* 2006). No clones were recognized as fungal genes based on their homology to known fungal gene sequences. Generally, there are few fungal gene sequences, namely of the genus *Pyrenophora,* present in the databases.

Seven clones showed homology to a hypothetical protein of unknown function. Five of them showed similarity to a sequence known from *Hordeum vulgare* (Matsumoto *et al.* 2011), the other two to a sequence from *Sorghum bicolor*. Both *Sorghum bicolor* clones showed similarity to the gene known as Sb01g031570 (Paterson *et al.* 2009).

We inferred the function of differentially expressed genes of 22 clones from their similarities to the sequences in databases. The sequences from clones J26, J7 and L4c show homology to amino-acid N-acetyltransferase from *Oryza sativa*, an enzyme supposed to catalyse the transfer of an acetyl group from acetyl-CoA to a primary amine of acceptor molecules. The sequence from clone A4b shows homology to peroxin Pex 14 from *Zea mays* (Alexandrov *et al.* 2009), a protein involved in the biogenesis and maintenance of peroxisomes, organelles that rapidly assemble, multiply and degrade in response to metabolic needs. Clone O16 shows homology to *T. aestivum* translational initiation factor 4 (Allen *et al.* 1992), which takes part in the gene expression during protein biosynthesis. Clone L5 shows homology to *Zea mays*

Table 3. Similarities between the nucleotide and deduced amino acid sequences of the differentially expressed clones and sequences in databases; in total, 36 TDFs with the strongest signal were chosen for cloning and sequencing

Clone	Length (bp)	Maximum similarity		
		Gene product	Accession No.	E-value
A16	285	Rye 26S rRNA 3' end and 18S rRNA, 5' end	M37231.1	3E-33
A4	285	Rye 26S rRNA 3' end and 18S rRNA, 5' end	M37231.1	4E-87
A4b	480	*Zea mays* peroxin Pex14	NM_001155897.1	5E-51
A4d	800	*Chasmanthium latifolium* 16S ribosomal RNA (rrn16) gene	HM363119.1	0.0
A7	285	Rye 26S rRNA 3' end and 18S rRNA, 5' end	M37231.1	5E-80
B5	800	*Triticum aestivum* (L.) partial chloroplast 16S rRNA gene	AJ239003.1	0.0
C1	200	unknown		
I16	175	nucellin-like aspartic protease-like (*Oryza sativa* Japonica Group)	BAD19623.1	2E-06
		pepsin A (*Zea mays)*	NP_001151415.1	5E-05
I26	175	*Sorghum bicolor* hypothetical protein, mRNA	XM_002467601.1	5E-66
I4	175	*Chasmanthium latifolium* 16S ribosomal RNA (rrn16) gene	HM363119.1	5E-158
I6	175	*Hordeum vulgare* subsp. *vulgare* mRNA for predicted protein	AK363881.1	3E-09
I5	150	*Hordeum vulgare* subsp. *vulgare* mRNA for predicted protein	AK358266.1	2E-29
I6	150	*Sorghum bicolor* hypothetical protein, mRNA	XM_002467601.1	3E-69
J26	500	putative amino-acid *N*-acetyltransferase (*Oryza sativa* Japonica Group)	AAR87160.1	3E-26
J7	250	putative amino-acid *N*-acetyltransferase (*Oryza sativa* Japonica Group)	AAR87160.1	5E-22
L4	200	PP2C (*Hordeum vulgare* subsp. *vulgare)*	ABO32801.1	3E-10
L4b	250	unknown		
L5	250	*Zea mays* chlorophyll a/b binding protein 8	NM_001155126.1	1E-16
L7	250	unknown		
L4c	380	putative amino-acid *N*-acetyltransferase (*Oryza sativa* Japonica Group)	AAR87160.1	3E-26
L4d	400	*Hordeum vulgare* subsp. *vulgare* mRNA for predicted protein	AK369867.1	6E-20
L6	400	*Fragaria* × *ananassa* mRNA for β-galactosidase (*beta-gal2* gene)	AJ278704.1	1E-14
		Hordeum vulgare subsp. *vulgare* PP2C (PP2C) mRNA	EF446603.1	3E-10
E4	111	*Brachypodium pinnatum* ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit (*rbcL*) gene	AY632361.1	2E-16
E1	250	*Thamnosma trifoliata* partial *rbcL* gene for ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	FN552686.1	1E-05
E5	250	unknown		
E5b	400	ATP binding protein, putative (*Ricinus communis)*	XP_002511570.1	1E-40
		putative methyltransferase (*Solanum tuberosum)*	ABO93008.1	3E-40
		dehydration-responsive family protein (*Arabidopsis thaliana)*	NP_192782.1	1E-38

Table 3 to be continued

Clone	Length (bp)	Maximum similarity		
		Gene product	Accession No.	E-value
G5	500	*Lolium temulentum* 25S ribosomal RNA	EU328537.1	4E-31
M16	450	unknown		
M26	450	unknown		
M7	450	Putative retroelement (*Oryza sativa* Japonica Group)	AAN04214.1	2E-15
		putative non-LTR retroelement reverse transcriptase (*Oryza sativa* Japonica Group)	AAT01370.1	1E-14
		retrotransposon protein, putative, unclassified (*Oryza sativa* Japonica Group)	ABA97428.1	2E-14
N16	450	putative sexual differentiation process protein isp4 (*Oryza sativa* Japonica Group)	BAD67732.1	4E-18
		Oligopeptide transporter, putative (*Ricinus communis)*	XP_002518892.1	2E-13
O16	300	Wheat (clone p80k-34) initiation factor isozyme 4F p82 subunit	M95747.1	2E-91
O4	300	unknown		
O6	300	G10-like protein (*Zea mays)*	NP_001148940.1	2E-08
N5	450	*Hordeum vulgare* subsp. *vulgare* mRNA for predicted protein	AK354303.1	3E-81
N4	450	*Hordeum vulgare* subsp. *vulgare* mRNA for predicted protein	AK354591.1	6E-125

chlorophyll a/b-binding protein (Alexandrov *et al.* 2009) that has its role in a system for balancing the excitation energy between photosystems I and II in the light-harvesting process. The sequence of clone E5b shows homology to methyltransferase from *Solanum tuberosum* (Ballvora *et al.* 2007), an enzyme catalysing various metabolic functions through DNA methylation. The same clone showed homology also to dehydration-responsive family protein from *Arabidopsis lyrata* that acts mainly during cell dehydration and stress response and to ATP binding protein from *Ricinus communis.* Clone E4 and E1 showed homology to ribulose-1,5-bisphosphate carboxylase/oxygenase, an enzyme involved in the Calvin cycle, which catalyses the first major step of carbon fixation. Clone B5 showed homology to 16S rRNA from *T. aestivum* (Kovacs *et al.* 2000) as well as the clones A4d and I4 from *Chasmanthium latifolium* (Yankaitis *et al.* 2010). The sequences indicated plastid-encoded rRNA. Clones A16, A4, A7 and clone G5 showed similarity to eukaryotic ribosomal RNA, clones A16, A4, A7 from rye to 26S and 18S rRNA (Appels *et al.* 1986), clone G5 from *Lolium temulentum* to 25S rRNA (Dombrowski & Martin 2009). Clone L4 and L6 showed homology to *H. vulgare* HvPP2C, phosphatase 2C protein (Shen *et al.* 2001; Thiv *et al.* 2011). Clone L6 also showed homology to *Fragaria* × *ananassa* mRNA for β-galactosidase (Trainotti *et al.* 2001) catalysing the hydrolysis of β-galactosides into monosaccharides. Clone I16 was homologous with a sequence of nucellin-like, aspartic protease-like protein, hypothesized to play a role in the programmed cell death of the nucellus as aspartic protease. Another homology of the same sequence was found to pepsin (Alexandrov *et al.* 2009). Clone M7 showed homology to a putative retroelement from *Oryza sativa*. Clone N16 showed similarity to putative sexual differentiation process protein from *Oryza sativa* as well as to putative oligopeptide transporter from *Ricinus communis*. Clone O6 showed homology to G10-like protein from *Zea mays*, a highly conserved protein of unknown function present in a wide range of eukaryotic species (Alexandrov *et al.* 2009).

Similar transcripts have also been detected in other studies. Adhikari *et al.* (2007) identified genes with increased expression during the resistance response of wheat to *Mycosphaerella graminicola*. Novel genes not previously implicated in defence responses, but known from wheat plants treated with abiotic stressors such as drought were

detected. Similarly, homology to a dehydration-responsive family protein was found in our study. Al-Daoude and Jawhar (2009) randomly isolated sequences of several disease stress-responsive genes including phosphatases. Two clones from our study also showed homology to phosphatase, namely PP2C (protein phosphatase 2C). The expression of ribulose-1,5-bisphosphate carboxylase/oxygenase was induced in response to wheat infection with powdery mildew (Zheng *et al.* 2009). Retroelements appeared to be differentially expressed during the chickpea reaction to *Fusarium oxysporum* infection (Nimbalkar *et al.* 2006). Although plant retrotransposons have been found to be activated by abiotic and biotic stresses, their presence may be a mere evidence of the amplification of abundant RNA fragments by cDNA-AFLP method while some minor specific involved gene transcripts could be missed (Reijans *et al.* 2003).

We have described several differentially expressed transcript derived fragments (TDFs) in this study. Some of them showed homology to sequences of genes that may be involved in resistance mechanisms. The differentially expressed TDFs can provide information on changes in expression during pathogenesis including classical pathogenesis-related (PR) genes that play a role in the signal transduction pathway. Our cDNA-AFLP approach may allow the identification of the gene function in defence mechanisms. However, many sequences identified by cDNA-AFLP have homology to genes that may be part of the plant's general response to stress rather than having a specific role in defence. The characterisation of genes that play a role in biochemical processes during host-pathogen interactions could be useful for development of new methods for detection of resistant and susceptible lines in research programs. Thus the knowledge of changes induced in expression during pathogenesis can contribute to effective pest management.

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