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Expression Analysis and Physical Mapping of a cDNA Library of Fusarium Head Blight Infected Wheat Spikes

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Abstract

Resistance to Fusarium head blight (FHB) (caused mainly by *Fusarium graminearum* Schw.) is difficult to obtain because of the quantitative nature and the numerous types of resistance involved. cDNA expression arrays have become a rapidly growing area for the identification and characterization of genes involved in complex pathways. A cDNA library was made from wheat (*Triticum aestivum* L.) spikes of the variety 'Sumai 3', 24 h after inoculation with *F. graminearum.* Clones were sequenced and putative function assigned to each clone based on BLASTX alignments. Nylon membrane arrays were made from the 580 unigenes present in the library, of which 75 expressed clones were induced in the first 24 h after inoculation. Of these unigenes, 14 are involved in defense response, 9 in gene expression and regulation, 29 in other cell functions, and 24 are without a known function. The induced genes catalyze key steps in the formation of lignin, energy production, and production of phytoalexins suggesting that resistance in wheat to *Fusarium* is provided by a different pathway than that of the hypersensitive response. Two of the induced genes, BE585589 and BE585627, were physically mapped to regions of chromosomes 3AS and 6BL, respectively, known to contain major QTL for FHB resistance.

Abbreviations: DON, deoxynivalenol; EST, expressed sequence tag; FHB, Fusarium head blight; hai, hours after inoculation; OPR, 12-oxophytodienoate reductase.

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Fusarium head blight has become a significant pathogen of both wheat and barley (*Hordeum vulgare* L.) in the world, including the USA. In 1993, this disease caused US$826 million in crop losses in the USA. (McMullen et al., 1997). The resulting crop damage is due to shriveled grain, poor germination, and contamination with mycotoxins that are toxic to animals and humans (Bai and Shaner, 1994). Finding natural resistance in wheat germplasm has proven elusive. The present levels of resistance in wheat to FHB are quantitative and may be due to either hindering the initial infection or slowing of the spread of the infection (Schroeder and Christensen, 1963). Resistance to FHB is separated into five categories: Type I, resistance to initial infection; Type II, resistance to spread of infection; Type III, resistance to kernel infection; Type IV, tolerance; and Type V, resistance to the mycotoxin deoxynivalenol (DON) (Schroeder and Christensen, 1963; Wang and Miller, 1988; Mesterhazy, 1995). No complete resistance is available, but Type II resistance is common, mostly limited to varieties from China and South America, and may be controlled by up to five minor genes (Singh et al., 1995; Van Ginkel et al., 1996; Bai et al., 2000).

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Little is known about the early stages of infection. Fungal colonization of wheat spikes is limited to a window of 10 to 20 d, going from anthesis to the soft dough stage of seed development (Schroeder and Christensen, 1963). The germinated macroconidia of *F. graminearum* is evident on the surface of glumes 6 to 12 h after inoculation (hai), hyphae at 12 to 24 hai, and conidiophores at 48 to 76 hai (Pritsch et al., 2000). No direct penetration of the glumes is evident, but hyphae within the tissue are observed at 48 to 76 hai (Pritsch et al., 2000). Further investigation with the close relative *F. culmorum,* W.G. Smith demonstrate that at 48 hai hyphal networks are evident on the inner epidermal surfaces of spikelets, on hairs on the top of the ovary, or on the remaining anthers. However, penetration of the inner surface of the palea and lemma is only observed 48 hai (Kang and Buchenauer, 2000a.). In both of these cases there is no direct evidence for the mode of penetration.

Cytologically there is no evidence supporting a specific recognition by the host or specific point of entry into the host. There is molecular evidence, though, that the host recognizes an infection.

Type II resistance was initially believed to be based on an unknown physiological factor because *F. graminearum* was observed to grow faster in a susceptible than a resistant wheat cultivar (Schroeder and Christensen, 1963). Pritsch et al. (2000) found no differences in timing or development of symptoms on the glume between resistant and susceptible cultivars. However, cellular differences were evident. Lignin accumulates at a higher rate in resistant cultivar host cells. Deoxynivalenol accumulates more slowly in resistant lines as well (Kang and Buchenauer, 2000b). Cytologically there is no evidence supporting a specific recognition by the host or specific point of entry into the host. There is molecular evidence, though, that the host recognizes an infection. Pritsch et al. (2000) determined that transcripts for the defense response genes peroxidase, PR-1, PR-2, and PR-3 accumulate similarly in both resistant and susceptible cultivars. But, transcripts for PR-4 and PR-5 were significantly higher in resistant cultivars.

The advancement of automated sequence analysis and the use of randomly selected cDNA clones have advanced the understanding of genome expression in many organisms and tissues. End sequencing of cDNAs is sufficient to assign putative function to a given clone through amino acid similarity to other cloned genes. Expression arrays, made of large numbers of expressed sequence tags (ESTs), provide a global view of cell responses to defined stimuli. In wheat, genomics and gene expression were limited to ESTs because of its large genome size of 16.0×10^9 bp (Arumuganathan and Earle, 1991), and until recently, wheat was not well represented in the public databases. Quantitative traits that are difficult to follow in subsequent generations, such as resistance to FHB, would significantly benefit from EST development. The study of induction of genes during FHB infection has been limited to small libraries (Han et al., 2005; Kong et al., 2005) and only a few of the induced genes have been mapped. The objectives for this research were to partially sequence, characterize, and assign possible function to the clones of a cDNA library from wheat spikes infected with FHB (Li et al., 2001). Second, we set out to profile unigene expression during the first 24 h after inoculation in a resistant cultivar and determine if induced genes are associated with loci associated with resistance. Although the number of genes represented in the expression array is small in comparison to commercial microarrays, key defense response pathways were identified in this study.

Materials and Methods

Sequencing and Analysis of cDNA Library

Construction of the cDNA library is described in Li et al. (2001). *Escherichia coli* cultures were grown in 1 mL of Terrific Broth (Sigma-Aldrich, St. Louis, MO) containing 100 mg L^{-1} of ampicillin for 20 h, at 250 rpm, and 37°C. Plasmids were isolated from colonies using a Qiagen Biorobot 3000 and Qiagen Turbo 96 isolation kits (Qiagen, Valencia, CA). Templates were 5′ single pass sequenced using T7 primers (IDT, Coralville, IA) and the BigDye terminator kit (Applied Biosystems, Foster City, CA). Sequenced clones containing a poly-A^+ tail were then resequenced using SP6 (IDT). Sequence reactions were precipitated with 60% (v/v) final concentration of isopropanol and washed with 70% ethanol (v/v). After being air dried, the pellets were resuspended in highly deionized formamide (Applied Biosystems). Sequence analysis was performed on an ABI 3700 DNA Analyzer (Applied Biosystems). Sequencing data was analyzed for quality and overlap using *phred*, *phrap*, and *consed* (Ewing et al., 1998; Ewing and Green, 1998; Gordon et al., 1998). Sequences were deposited in the public EST database dbEST with the accession numbers BE585476–BE586199.

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Amplification of cDNA Inserts

The pGEM-T vector contained the SP6 and T7 priming sites flanking the insert. The 580 singletons were used for polymerase chain reaction (PCR). PCR reactions of 50 µL contained 10 to 20 ng of plasmid, 20 pmol of each primer, 0.25 m*M* of each nucleotide (New England Biolabs), 1× *Taq* polymerase buffer (Sigma-Aldrich), 4 µ*M* $MgCl_2$, and one unit of *Taq* polymerase (Sigma-Aldrich). The reaction was performed as follows: 92°C for 1 min; 35 cycles of 92°C for 1 min, 60°C for 2 min, and 72°C for 2 min; 72°C for 10 min. After PCR, the products were precipitated by adding isopropanol to a final concentration of 60% (v/v) then washed with 70% ethanol. The pellets were allowed to air dry and dissolved in 10 m*M* Tris-HCl pH 8.5 at a final concentration of 100 ng μL^{-1}.

Reverse Northern Analysis

cDNAs were arrayed onto Hybond N nylon membranes (Amersham Pharmacia, Piscataway, NJ) using a 96-pin arrayer (V&P Scientific, San Diego, CA, Part Number VP408). Each cDNA clone was double spotted onto a single membrane and two copies of the membrane were made. After the blots were air dried overnight, they were treated for 5 min with denaturing solution (1.5 *M* NaCl, 0.5 *M* NaOH), 5 min with neutralization solution (1.5 *M* NaCl, 0.5 *M* Tris HCl pH 8.0), washed with 2× SSC (Sambrook et al., 1989), and then air dried again before hybridization. Each blot was probed four times using probes made from cDNA.

Reverse transcription reactions contained 200 ng of mRNA from spikes 0 and 24 h following inoculation, 0.5 µg of oligo dT, 1× first strand buffer (Gibco-BRL), 1 m*M* dNTPs, 0.01 *M* DTT, and 1 U of SuperScript RT (Gibco-BRL, Carlsbad, CA). cDNA probes were labeled with antifluorescein-AP by random priming 1/5 of the RT reaction (NEN, Wellesley, MA). Blots were hybridized overnight at 65°C and washed with 2× SSC 0.5% SDS for 15 min at 65°C and 0.2× SSC 0.1% SDS for 15 min at 65°C. Detection using antifluorescein-AP conjugate and nucleic acid chemiluminescence reagents was done according to NEN protocol.

Blots were exposed to autoradiography film for 30 min. Films were analyzed using Gel Expert software (NucleoTech, San Mateo, CA). Intensity values for a 60S ribosomal subunit were used as a control, while the pGEM-T vector was used as a negative control. Means of the square root of intensities within a blot were used to determine the ratio of expression of 24 hai versus 0 hai. Ratios of the controls were used as the divisor to normalize the ratio of expression. Herwig et al. (2001) have shown that membrane arrays can determine ratios of 1:1.5 accurately with adequate replication. Therefore, an induction ratio of 1.5 was used as a cutoff.

Bin Mapping

ESTs were assigned to chromosomal bins by either Southern hybridization or by BLASTN alignment to sequences of ESTs mapped to chromosomal bins (http://wheat.pw.usda.gov/, verified 16 June 2006). Southern hybridization procedures and the Chinese Spring wheat chromosomal deletion stocks that were used are described in Qi et al. (2004).

Results

Sequence Analysis of the Sumai 3 cDNA Library

The construction of the cDNA library used in this work was previously described in Li et al. (2001). However, details about the quality of the library were not available. A total of 864 colonies were arrayed into 96 well plates. One 96-well plate was randomly selected for determination of insert size and percentage of plasmids with insert. Amplified products demonstrated that inserts ranged from 800 bp to 2.4 kbp, with an average size of 1.47 kbp. Also, 94 of the 96 colonies contained inserts. These results indicate that the library is of good quality and the total of number sequenceable clones was 799 (92%).

Using default settings, raw sequence data from the ABI 3700 were analyzed by the base calling program, *phred* (Ewing et al., 1998; Ewing and Green, 1998), which assigns quality scores to each callable base. The average length of sequence called by *phred* was 721 bases, with an average quality score of 32.1 per base. FASTA files were created and vector sequence was trimmed using cross_match with settings of minmatch 12 and minscore 20. After trimming, the average lengths of the reads were 621 bases. Cleaned ESTs were then subjected to a batch BLAST (Altschul et al., 1990) search using BLASTX with the database option of “nr” which searches all public protein databases associated with GenBank. *Phrap* (Ewing et al., 1998; Ewing and Green, 1998) analysis was used to identify reads containing overlaps and identical sequences. *Phrap* identified 475 clones having no matching sequences and 324 clones with at least one matching sequence within the group of 799. The 324 clones present in two or more copies could be combined into 105 contiguous unique unigenes with the number of overlapping members ranging from 2 to 24 per contig. Several of the contigs aligned with the same sequence (i.e., glyceraldehyde 3-phosphate dehydrogenase). However, *phrap* analysis still placed the reads in different contigs. Rubisco was represented 10 times, UDP-glucose gucosyltransferase, eight times; and *S*-adenosylmethionine decarboxylase, six times. One representative was selected from each contig and added to the unigene set totaling 580.

Table 1. Library unigenes associated with pathogen response, the number of cDNAs in GenBank found in *Fusarium*-infected wheat libraries, and the expression ratios (24:0 h).

GenBank no.	BLASTX alignment	*P* value	No. of ESTs†	Ratio
Induced				
BE585508	12-oxophytodienoate reductase OPR2	6×10^{-98}	19	1.6
BE585604	carbonic anhydrase, chloroplast precursor	1×10^{-113}	4	1.5
BE585504	cytoplasmatic aconitate hydratase	1×10^{-116}	2	1.5
BE586153	putative dioscorin class A precursor	8×10^{-65}	5	3
BE586125	glutathione transferase (*Triticum aestivum* L.)	1×10^{-116}	14	2.1
BE586141	glycolipid transfer protein–like (*Oryza sativa* L.)	7×10^{-94}	1	2.2
BE585700	putative lipase (*O. sativa* [japonica])	5×10^{-71}	4	1.6
BE585849	lipase class 3 family protein (Arabidopsis)	4×10^{-61}	1	1.5
BE586181	pathogenesis related protein-1	2×10^{-20}	4	1.5
BE585502	immediate-early salicylic acid–induced glucosyltransferase	6×10^{-28}	10	2.5
BE586107	putative betanidin-5-*O*-glucosyltransferase	1×10^{-44}	10	1.9
BE585478	acyl-CoA thioesterase family protein	2×10^{-62}	5	1.5
BE585541	cinnamoyl CoA reductase 2 (*Solanum tuberosum* L.)	7×10^{-31}	4	1.8
BE586095	*trans*-cinnamate 4-monooxygenase	8×10^{-58}	2	1.9
Not Induced				
BE585872	12-oxophytodienoate reductase OPR2	1×10^{-95}	19	1.2
BE585988	12-oxophytodienoate reductase OPR2	5×10^{-96}	20	1
BE585640	BAX inhibitor 1 (*Hordeum vulgare* L.)	7×10^{-74}	2	1.3
BE586190	caffeic acid *O*-methyltransferase	3×10^{-79}	4	1
BE585697	chalcone synthase (*T. aestivum*)	3×10^{-35}	10	1
BE585793	chitinase II precursor (*Triticum*)	1×10^{-114}	7	1.2
BE585656	putative glucan synthase (*O. sativa*)	1×10^{-125}	1	0.6
BE585704	glucan endo-1,3-ß-D-glucosidase	3×10^{-65}	7	1
BE585769	glucan endo-1,3-ß-glucosidase precursor	2×10^{-12}	1	1
BE585868	putative glutathione *S*-transferase	9×10^{-96}	4	1
BE585907	glutathione *S*-transferase 1	8×10^{-99}	9	0.7
BE585647	glutathione *S*-transferase 1	2×10^{-90}	9	1.1
BE585942	glutathione transferase F4 (*T. aestivum*)	1×10^{-101}	5	0.7
BE585540	glutathione *S*-transferase GST 24 (*Zea mays* L.)	7×10^{-33}	2	0.7
BE586158	glyoxalase I (*O. sativa*)	1×10^{-112}	9	1.3
BE585829	glyoxalase I (*O. sativa*)	2×10^{-22}	4	0.9
BE585798	Cw-21 peptide, nonspecific lipid transfer protein	2×10^{-35}	4	1.1
BE585909	oxalate oxidase 2 precursor (Germin)	1×10^{-99}	2	0.6
BE585499	peroxidase 6 (*T. monococcum* L.)	4×10^{-95}	9	1.2
BE585838	putative peroxidase (*O. sativa*)	1×10^{-60}	5	0.8
BE585557	TPA: class III peroxidase 124 precursor	3×10^{-52}	4	0.8
BE586060	peroxidase 1 (*T. monococcum*)	8×10^{-84}	9	1.1
BE585760	phenylalanine ammonia-lyase	1×10^{-127}	6	1
BE586193	small Ran-related GTP-binding protein	1×10^{-114}	5	1.4
BE585853	superoxide dismutase [Cu–Zn] 4a	2×10^{-69}	7	0.9
BE585855	prolyl 4-hydroxylase (*Dianthus caryophyllus* L.)	7×10^{-64}	2	1.1
BE586124	putative cinnamyl-alcohol dehydrogenase	2×10^{-17}	1	1.2

† Expressed sequence tag (EST) number based on number BLASTN alignments to ESTs from other *Fusarium*-inoculated wheat libraries.

Categorized Clones

Unigenes were grouped into three major categories: (i) clones aligned with sequences of known function with probabilities of 1×10^{-5} or better; (ii) clones aligned with sequence of unknown or hypothetical function with probabilities of 1×10^{-5} or better; and (iii) clones with weak or no alignment with sequences in the database. Eighteen categories were identified (Fig. 1). There were 118 unigenes (Fig. 1) that had significant alignment to sequences in the database, but no functions were assigned to them. Of these, 76 aligned to unknown proteins from *Arabidopsis*, five from rice

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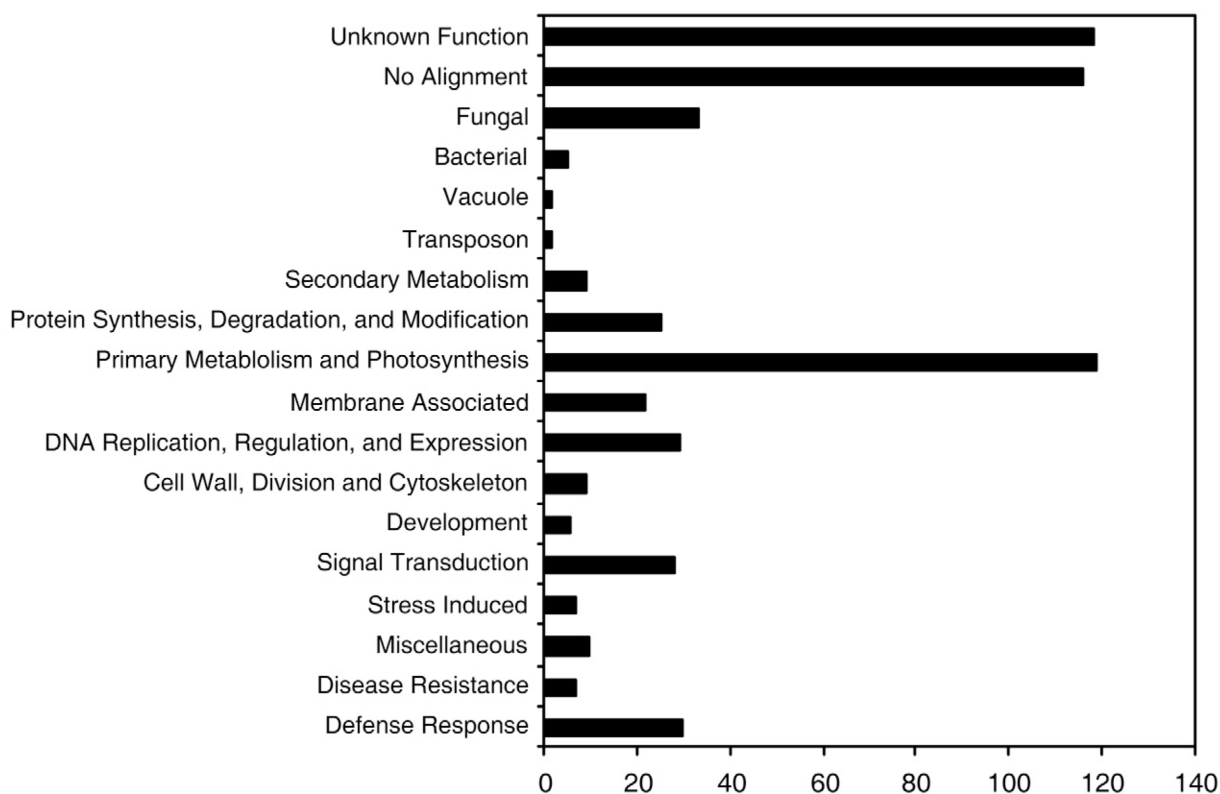

Fig. 1. Categorized singletons based on BLASTX alignments.

(*Oryza sativa* L.), and the remainder from various other organisms. The probability scores ranged from 1×10^{-5} to 1×10^{-94} with the majority of the values around 1×10^{-50}. Also in the unigene set were 116 (Fig. 1) with no similarities to sequences in the database or the alignment was weak, including 19 in which BLASTX returned no alignment results.

Putative function could be assigned to 346 unigenes based on significant alignment to sequences in the database and categorized according to function (Fig. 1). Of these, 122 unigenes were assigned function in primary metabolism and photosynthesis. The most common sequence in this group and in the library was glyceraldehyde 3-phosphate dehydrogenase with 41 clones in the library. Within the primary metabolism and photosynthesis category are several assignments involved in sucrose and starch biosynthesis. There were few representatives of other major housekeeping genes such as ribosomal proteins, photosynthesis proteins, and ubiquitin. Two unigenes aligned with transposition proteins indicating active transposons within the tissue sampled.

Of particular interest to this study were genes associated with resistance and plant response to pathogen infection. The 41 unigenes identified are listed in Table 1 and were categorized according to previous reports of induction during biotic stresses. On the basis of the total library, including clone redundancy, this category represented 77 (9.6%) of the 799 sequenceable clones. The number of similar sequences found in this and other *Fusarium*-inoculated wheat spike libraries are listed in Tables 1, 2, and 3 (Anderson et al. [unpublished data, 2000] and Ouellet et al. [unpublished data, 2002] deposited at www.ncbi.nlm.nih.gov/entrez, verified 16 June 2006). There were seven abiotic stress-induced singletons present. The signal transduction category contained 28 singletons of which several were serine/threonine kinases. Three copies of a 14–3-3 protein A homolog were present as well as chaperonins and a GTPase-activating protein. Also present were seven unigenes identified to have similarities to cloned resistance genes.

Reverse Northern Expression Analysis

There were 80 wheat unigenes induced (Tables 1, 2, and 3) in Sumai 3, 24 hai. The induced genes were categorized according to inferred function from their alignment. Fourteen of the previously identified defense response genes from the library (Table 1) were induced 24 hai at a ratio of ≥1.5. A homolog of PR-1 was induced at a ratio of 1.5 and provided validation to the macroarray technique. *trans*-Cinnamate 4-monooxygenase and cinnamoyl-CoA reductase were induced and are key steps in the formation of lignin. However, phenylalanine ammonia-lyase, had no change in expression. The enzyme that is key to jasmonic acid formation, 12-oxo-phytodienoate reductase (OPR), was induced. Other

Table 2. Induced unigenes with putative assigned function based on alignments.

GenBank no.	BLASTX alignment	*P* value	No. of ESTs†	Ratio
Cell cycle				
BE585583	small GTP-binding protein RAB5B (*Oryza sativa*)	3×10^{-54}	1	2.3
Development				
BE586111	Argonaute protein (*Arabidopsis thaliana*)	1×10^{-103}	2	1.8
DNA replication, regulation, and expression				
BE585571	26S proteasome regulatory particle non-ATPase	1×10^{-81}	6	1.8
BE585600	putative zinc finger protein (*O. sativa* [japonica])	1×10^{-48}	2	1.6
BE585643	putative nuclear WD protein (*Solanum bulbocastanum* Dunal)	1×10^{-80}	1	1.5
BE585854	elongation factor 1-α (EF-1-α)	1×10^{-133}	6	1.5
BE585900	STAM binding protein–like protein (*O. sativa*)	5×10^{-95}	2	1.9
BE585934	26S proteasome regulatory subunit S5A	8×10^{-82}	1	1.9
BE585938	putative ATP synthetase α chain (*O. sativa*)	3×10^{-77}	3	2
BE585994	eukaryotic translation initiation factor 6	1×10^{-48}	8	1.8
BE586142	PRLI-interacting factor L-like (*O. sativa*)	4×10^{-64}	1	2
Membrane associated				
BE585535	PDR3 ABC transporter (*O. sativa* [japonica])	2×10^{-95}	2	1.5
BE585561	putative ribosomal protein (*O. sativa* [japonica])	5×10^{-5}	1	2.3
BE585614	putative microtubule-associated protein	1×10^{-73}	2	2.2
BE586152	transporter-related–like (*O. sativa* [japonica])	6×10^{-89}	1	2.3
BE586160	putative aldose 1-epimerase (*O. sativa* [japonica])	5×10^{-57}	1	2.1
Signal transduction				
BE585615	ankyrin-like protein (*O. sativa* [japonica])	1×10^{-110}	1	1.9
BE585845	putative Ras-GTPase activating protein SH3 domain	9×10^{-72}	3	1.5
BE585891	copper chaperone (CCH)-related protein-like	2×10^{-14}	1	1.8
BE586006	14–3-3–like protein A (14–3-3A)	1×10^{-110}	4	1.8
BE586099	MAP kinase homolog (*Triticum aestivum*)	4×10^{-97}	1	1.6
BE586143	putative In2.1 protein (*T. aestivum*)	1×10^{-117}	4	1.8
Primary metabolism and photosynthesis				
BE585588	chlorophyll a/b-binding protein II precursor–rice	1×10^{-113}	5	3
BE585589	glyceraldehyde 3-phosphate dehydrogenase	5×10^{-98}	23	1.9
BE585627	aspartate aminotransferase, cytoplasmic	1×10^{-115}	3	2.3
BE585631	*S*-adenosylmethionine decarboxylase (*T. turgidum*)	1×10^{-103}	11	1.7
BE585657	SLT1 protein (*Nicotiana tabacum* L.)	3×10^{-60}	1	2.1
BE585710	putative selenium-binding protein (*O. sativa*)	1×10^{-98}	7	1.5
BE585717	glutamate dehydrogenase (*Zea mays*)	1×10^{-108}	2	1.6
BE585936	putative ubiquitin carboxylase	2×10^{-4}	5	1.6
BE585952	possible apospory-associated protein c	1×10^{-53}	2	6.1
BE585970	isocitrate dehydrogenase (NAD+) (Nicotiana)	2×10^{-53}	5	1.9
BE585971	urease accessory protein UreD [*Glycine max* (L.) Merr.]	3×10^{-41}	1	1.8
BE586018	putative pollen specific protein SF21 (*O. sativa*)	8×10^{-38}	1	1.5
BE586173	phosphate/phosphoenolpyruvate translocator protein	1×10^{-117}	1	2
BE586178	dentin sialophosphoprotein precursor-like	1×10^{-68}	1	1.5
BE586183	putative anthranilate *N*-benzoyltransferase	1×10^{-65}	2	1.6
Secondary metabolism				
BE585827	cytochrome p450 51 (CYPL1) (p450-l1a1)	5×10^{-21}	3	1.9

† Expressed sequence tag (EST) number based on number BLASTN alignments to ESTs from other *Fusarium*-inoculated wheat libraries.

unigenes similar to pathogen response genes that were induced included a cytoplasmic acconitate hydratase, dioscorin class A precursor, glutathione *S*-transferase, glycolipid transfer protein, and peroxisomal acyl-CoA thiosesterase.

Infection of wheat spikelets also induced numerous genes involved in other cell pathways (Table 2). *Argonaute*, rab5B, and cytochrome P450 CYPL1 are involved in development, cell cycle, and secondary metabolism, respectively. Gene expression pathway

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Table 3. Induced unigenes with no assigned function, as well as unigenes with weak or no alignments to database sequences. These represent uncharacterized genes from wheat.

GenBank no.	BLASTX alignment	*P* value	No. of ESTs†	Ratio
BE585483	unknown protein (*Oryza sativa*)	6×10^{-95}	3	2.4
BE585563	short-chain dehydrogenase/reductase protein–like	8×10^{-46}	1	2.7
BE585575	unknown protein (*O. sativa* [japonica])	5×10^{-57}	1	1.7
BE585593	putative protein (*Arabidopsis thaliana*)	2×10^{-5}	4	1.6
BE585642	similar to ATP-dependent helicase	2.5	1	1.7
BE585651	putative stress-related–like protein interactor	9×10^{-91}	3	1.5
BE585655	hypothetical protein (*Picea mariana* Mill.)	3×10^{-35}	1	2.1
BE585803	none		1	1.8
BE585835	unknown protein (*O. sativa* [japonica])	8×10^{-17}	1	1.5
BE585864	hypothetical protein (*O. sativa*)	6×10^{-23}	5	1.8
BE585973	none		4	1.7
BE585997	270 kDa ankyrin G isoform (Rattus)	0.61	1	1.6
BE586005	OSJNBb0021A09.5 (*O. sativa* [japonica])	4×10^{-50}	1	2.7
BE586028	none		1	1.9
BE586034	hypothetical protein (*O. sativa* [japonica])	8×10^{-19}	1	2
BE586035	none		2	1.5
BE586093	unknown protein (*O. sativa* [japonica])	7×10^{-75}	3	1.6
BE586096	unknown protein (*A. thaliana*)	1×10^{-5}	2	1.8
BE586108	tubulointerstitial nephritis	0.33	1	1.8
BE586109	P0436D06.15 (*O. sativa* [japonica])	5×10^{-18}	2	2
BE586110	none		2	2.1
BE586129	hypothetical protein (*O. sativa* [japonica])	2×10^{-59}	1	1.6
BE586137	none		1	2.3
BE586174	unknown protein (*O. sativa* [japonica])	1×10^{-12}	1	3.5

† Expressed sequence tag (EST) number based on number BLASTN alignments to ESTs from other *Fusarium*-inoculated wheat libraries.

genes included Zn-finger proteins, elongation factors, translation initiation factors, and regulatory complex subunits. Five different membrane associated proteins as well as six proteins involved in signal transduction were also induced. Primary metabolism included numerous amino acid modifying enzymes. Present were induced homologs of pyruvate kinase and phosphate/phosphoenolpyruvate, which are involved in energy production in the Krebs cycle. Twenty-four induced genes have no known function (Table 3).

Fungal Genes within the Library

It was expected that some of the ESTs would originate from *Fusarium* because of the manner in which the plants were inoculated and RNA isolated. Fourteen unigenes aligned with fungal sequences in the databases from *Aspergillus*, *Neurospora*, yeast, *Magnaporthe*, and *Rhizopus*. To further determine how many of the cDNAs were from *Fusarium*, we probed the cDNA library with chemiluminescent labeled DNA from the *F. graminearum* strain GZ3639 (provided by Dr. John Leslie, Department of Plant Pathology, Kansas State University). Fifty of the 799 clones in the library hybridized with the probe including the 14 clones with alignments to fungal sequences, thus supporting the computer alignments that these were of fungal origin. Several of the fungal unigenes were common housekeeping genes of ubiquitin, heat shock proteins, and ribosomal proteins. Most of the genes did not have an assigned function and represent uncharacterized *Fusarium* genes. *Fusarium* unigenes were included on the array membranes with wheat to evaluate induction of fungal genes during infection. Three of the 50 unigenes were found to be induced (Table 4). Alignments of the induced genes putatively assigned the functions in gene expression and protein assembly.

Table 4. Fungal unigenes induced during the first 24 h of infection of wheat spikes.

GenBank no.	BLASTX alignment	*P* value	Ratio
BE585542	hypothetical protein FG02337.1 (*Gibberella zeae* Schwein)	7×10^{-94}	1.5
BE586097	hypothetical protein FG09931.1 (*G. zeae*)	5×10^{-60}	1.6
BE585661	glycoside hydrolase 45 (*G. zeae*)	1×10^{-100}	3.5

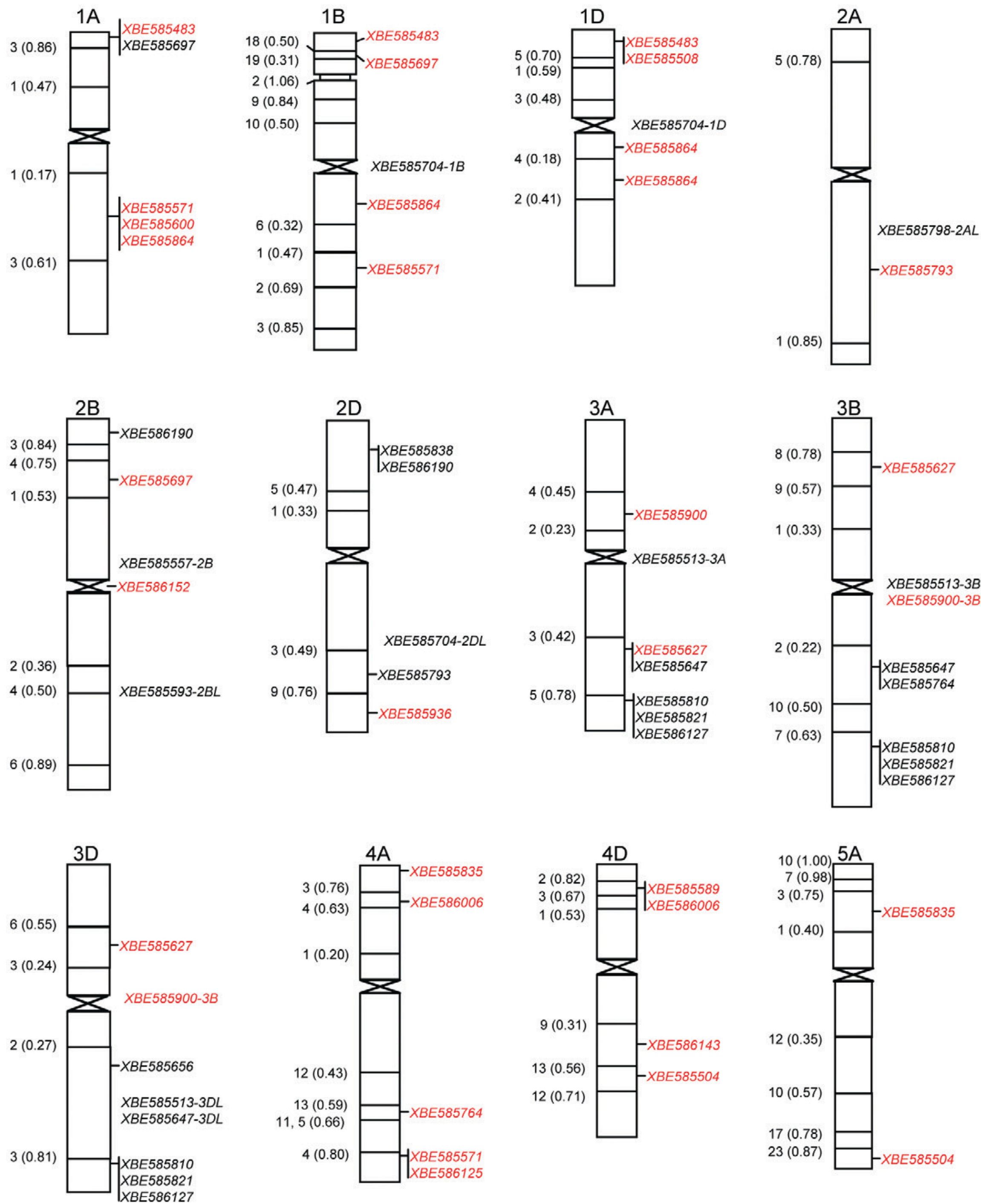

Fig. 2. Physical location of ESTs that are induced 24 h after inoculation. Also included are expressed clones that are involved with pathogen response, based on BLASTX alignments, but not induced.

Physical Mapping

To determine whether the induced genes were associated with QTL for FHB resistance, Southern hybridizations and *in silico* mapping were used to map clones associated with defense response. A total of 88 loci were identified (Fig. 2). Of the induced genes, 46 loci were identified and two could only be assigned to either the arm or whole chromosome. Twenty-eight loci were identified for clones associated with pathogen response, but were not induced in the first 24 h of infection. Thirty-five of the loci were located in the terminal deletions of the respective

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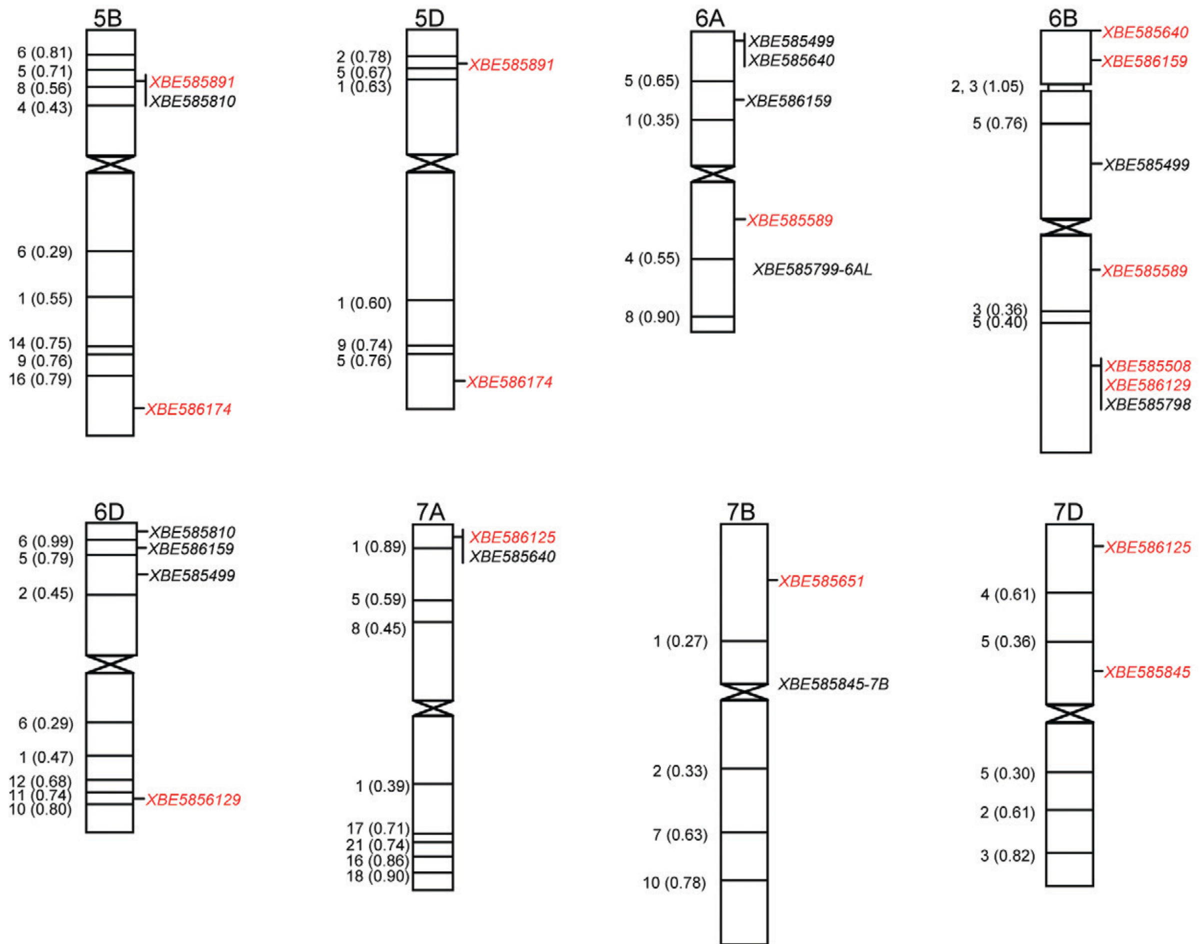

Fig. 2. Continued.

chromosome. No loci were found on chromosome 4B or the short arm of chromosome 2A. Group 7 chromosomes had the fewest loci.

Discussion

The molecular events during the early stages of infection by *Fusarium* are poorly understood. It is believed that *Fusarium* infects on or inside flowering spikelets at a conducive temperature (Pugh et al., 1933). Pritsch et al. (2000) determined that fungal spores sprayed onto the spikelets will germinate and enter through the open stomata. Conidia germinated on the glumes 6 to 12 hai and penetration occurred by 72 hai. However, penetration of the epidermis, other than through the stomata, was difficult to find. The difficulty in identifying the time of penetration was further shown by Kang and Buchenauer (1999, 2000a, 2000b). The plant appears to recognize infection, even though penetration is not apparent until 72 hai. PR-1, PR-2 (β-1,3 glucanase), PR-3 (chitinase), PR-4, and PR-5 began to accumulate as early as 6 to 12 h after inoculation, but no difference is seen between susceptible and resistant cultivars (Pritsch et al., 2000). Previously, two chitinase and two β-1,3 glucanase clones were isolated from this library using rice chitinase Ia and barley β-1,3 glucanase cDNAs as probes (Li et al., 2001). These genes accumulate more rapidly in the resistant parent, Sumai 3, than in the susceptible lines, however, the expression was highest between 24 and 48 hai for both. Another resistant line, Ning7840, was shown to have an increase in a homolog of actin depolymerizing factor and a gene of unknown function within 24 h of infection, while homologs of a cytochrome p450, an *S*-adenosylmethionine decarboxylase precursor, and two other unknown proteins had sharp increases at 72 h post inoculation (Kong et al., 2005). In our work, the chitinase II homolog (BE585793) had a ratio of 1.2 indicating some induction, however, significant induction would not have been seen until 48 hai (Li et al., 2001). The PR-1 homolog (BE586181) as well as a putative lipid transfer protein (BE586141) were induced within 24 h. It is interesting that no significant changes in expression were seen in the hydrogen peroxide/free radical–associated enzymes suggesting that senescence begins after the first 24 hai. Timing of

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tissue harvest can also explain the lack of induction of the other PR type genes that were in the library.

In later stages of infection, a Type II resistant plant begins to build up layers of lignin and electron dense material along the cell wall in contact with the invading hyphae (Kang and Buchenauer, 2000b.).

In later stages of infection, a Type II resistant plant begins to build up layers of lignin and electron dense material along the cell wall in contact with the invading hyphae.

Expression analysis of the unigene set identified two genes in the pathway of lignin formation. The first, *trans*-cinnamate 4-monooxygenase (BE586095) is a cytochrome P450 that catalyzes the oxidative reaction of *trans*-cinnamic acid to *p*-coumaric acid (Ro et al., 2001). Second, cinnamoyl CoA-reductase 2 (BE585541) changes cinnamoyl CoA esters to cinnamyl aldehydes (reviewed by Vance et al., 1980). The induction of these two genes indicates that the plant is preparing for infection and would support the microscopy findings of Kang and Buchenauer (2000a, 2000b). The level of phenylalanine ammonia-lyase expression does not change in the first 24 hai, which has also been seen in cotton during infection by *Verticillium dahlia* Kleb (Cui et al., 2000). Phenylalanine ammonia-lyase provides substrates for several phenyl-propanoid pathways and a change in expression is unlikely.

There are no previous data to support the involvement of plant phytohormones abscisic acid, salicylic acid, and jasmonic acid in plant response to *Fusarium* infection. The homologs of an immediate early salicylic acid–induced glucosyltransferase (BE585502) and putative betadidin-5-*O*-glucosyltransferase (BE586107) were induced significantly suggesting production of salicylic acid. UDP-glucose glucosyltransferase has been found to be induced on wounding in tubers (Moehs et al., 1997) and involved in conjugating salicylic acid during pathogen attack (Lee and Raskin, 1999). Induction of a 12-oxophytodienoate reductase (OPR2) homolog in wheat suggests involvement of jasmonic acid. Formation of jasmonic acid from its precursor 12-oxophytodienoic acid is driven by OPR. *opr3* mutants in *Arabidopsis* are male sterile, and sterility can be restored by exogenous application of jasmonic acid (Stintzi and Browse, 2000). Application of abscisic acid and environmental stress have been shown to up-regulate PDR-like ABC transporters (BE585535; Smart and Fleming, 1996). These transporters are also involved in the efflux of cytotoxic compounds across a membrane and may be involved in detoxification of DON in the cells.

As infection starts, the cells may require an increased level of energy production as evident by induction of proteins gyceraldehyde 3-phoshate dehydrogenase, pyruvate kinase, and phosphate/phosphoenolpyruvate. Carbonic anhydrase precursor (BE585604) is induced, and in plants carbonic anhydrase facilitates the interchange of CO_2 and HCO_3^-, plays a key role in fixation of photosynthesis, and is affected by changes in the cytoplasmic pH (Hou et al., 1999; Hatch and Burnell, 1990).

Infection appears to be influencing transcription and the controlling mechanisms. Induction of an *Argonaute*-like protein (BE586111) suggests post-transcriptional control as *Argonaute* proteins have been shown to be involved in the RNA-induced silencing complex in *Drosophila* (Hammond et al., 2001). Induction of 14–3-3 proteins, along with induction of Zn finger–like proteins, also suggests that transcription is being controlled. 14–3-3 proteins (BE586006) have been shown to regulate the intracellular localization of bZIP transcriptional activators (Igarashi et al., 2001).

Wheat EST sequences in the public database contain several libraries from *Fusarium*-infected wheat spikes. Several of the pathogen response genes and clones with assigned function have numerous copies in the database and indicate high level of expression during infection. The low number of unknown genes indicate rare genes. Clones with high homology to *Arabidopsis* and rice proteins of unknown function present opportunities to study gene function. Mutations and complementation in *Arabidopsis* and rice will be useful methods of assigning a function to the unknown wheat genes found in the library.

Resistance to FHB is difficult to breed for in wheat because of the quantitative nature and the numerous types of resistance. For instance, one locus QTL analysis revealed seven QTL for Type I resistance (initial infection) on chromosome arms 2DS, 3AS, 3BS, 3BC, 4DL, 5AS and 6BS and four QTL for Type II (fungal spread) on chromosome arms 2DS, 3BS, 6BL, and 7BL (Yang et al., 2005). One QTL has been extensively mapped. *Qfhs.ndsu.3BS*, the major QTL for Type II resistance has been localized in deletion bin 3BS8 0.78–0.87 (Liu and Anderson, 2003a). Using rice chromosome 1S–wheat 3BS synteny, 27 wheat ESTs have been localized to this region, but expression analysis was not determined (Liu and Anderson, 2003b).

Expressed clones have been associated with FHB resistance QTL. Two clones from a 'Frontana' library are bin mapped to regions of QTL for Type II resistance on chromosomes 2AL, 3BL, and 3DL (Han et al., 2005). None of the expressed clones in our work share sequence homology with any of the clones mapped in previous reports. However, we determined that *XBE585627*(3BS) and *XBE585589*(6BL) mapped in the same deletion bins as major QTL on 3BS and 6BL (Yang et al., 2005). Close association of an expressed clone and a locus provides a strong tool in identifying the genes involved with FHB resistance QTL.

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