Plant Mol Biol Rep (2013) 31:925–935
DOI 10.1007/s11105-013-0567-2

ORIGINAL PAPER

Different Gene Expressions of Resistant and Susceptible Maize Inbreds in Response to *Fusarium verticillioides* Infection

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Published online: 31 January 2013

Abstract Ear rot, caused by *Fusarium verticillioides* (FV), is a destructive disease of maize as it reduces grain yield and increases risks of mycotoxin production, thus endangering livestock. To identify genes differentially expressed during FV infection, four cDNA libraries were constructed for suppression subtractive hybridization using RNA isolated from bracts of an FV-resistant inbred maize line, Bt-1, as well as an FV-susceptible maize inbred line, Ye478. A total of 145 clones were obtained following reverse dot-blot hybridization, and these were sequenced from these libraries. Similarity analysis revealed that of these genes, 93 were unique, including 68 of known function, 24 of unknown function, and a single novel gene. Most genes of known function were predominantly involved in plant defense such as cell defense, transcription regulation, signal transduction, and metabolism. Expression profiles of eight representative genes, using semiquantitative reverse transcription-polymerase chain reaction, confirmed that differential gene regulation during FV infection was involved. These findings suggested that these differentially expressed genes might be involved in FV defense responses in maize.

Keywords Ear rot · *Fusarium verticillioides* · Dot-blot hybridization · SSH · *Zea mays*

Introduction

Maize ear rot is one of the prevalent diseases in the world and mostly caused by several fungal pathogens, such as *Fusarium verticillioides* (FV) (Sacc.) Nirenberg (synonym *Fusarium moniliforme* Sheldon; teleomorph, *Gibberella moniliformis*) and *Fusarium proliferatum* (Matsushima) Nirenberg (teleomorph, *Gibberella intermedia*). *Fusarium* ear rot, predominantly caused by syn. *F. verticillioides*, is among the most destructive diseases because of quality losses and quantity reduction in maize grain (Robertson-Hoyt et al. 2006; Reid et al. 1999). The symptom of the disease usually consists of a white or light pink mold on individual kernels, groups of kernels, or physically injured kernels (Munkvold 2003). Especially, a high incidence of *Fusarium* ear rot occurs in the moist and humid regions of Southwest China, as well as other regions with similar longitude in other countries (Wen et al. 2002; Ali et al. 2005). In addition, the ingestion of FV-infected grain can cause severe adverse effects in both humans and livestock due to the production of diverse and potent mycotoxins (Vigier et al. 2001). The *Fusarium* ear rot has been commonly observed worldwide for its destructiveness in maize, one of the important crops for food in Asia (Weidenborner 2001). Although application of fungicide provides some level of disease control, ear rot outbreaks can be common and severe when climatic conditions are favorable for the pathogen (Chungu et al. 1996). It has been proven that the most efficient way to control plant diseases is to build up strong resistance in new inbred lines. However, few genes associated with maize ear rot resistance against FV have been identified.

After specific recognition of a pathogen, a variety of active defense mechanisms are known in plants to protect them during microbial pathogens infection, including generation of reactive oxygen species at the site of infection, cell wall thickening, and production of antimicrobial compounds and enzyme inhibitors (Heath 2000; Glazebrook 2005). Genes encoding pathogenesis-related (PR) proteins

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are a primary target during the early response to pathogen attack and are considered as a signature of the hypersensitive responses (HR) (Van Loon and Gerritsen 1989). Signal molecules, such as ethylene (ET), salicylic acid, and jasmonic acid, play an important role in the defense signalling networks (Pieterse and Van Loon 1999; Dong 1998). A dynamic exchange of signals and metabolites occurs between the pathogen and the host cell, and a central role for the nonexpressor of PR genes1 protein has been highlighted, providing more important information on basal defense (Kinkema et al. 2000; Fung et al. 2008). In recent years, considerable progresses have been made in understanding the resistant system on maize ear rot, including isolation of disease resistance genes, characterization of defense responses, and elucidation of signal transduction leading to activation of defense responses (Casacuberta et al. 1991, 1992; Cordero et al. 1992, 1994a, b; Multani et al. 1998; Murillo et al. 2001; Yuan et al. 2012). In response to FV infection, the pathogenesis-related-like proteins, chitinases, β-1, 3-glucanases, and calcium-dependent protein kinase were overproduced in maize (Multani et al. 1998). Moreover, disease-resistant genes Hm1 and guanylyl cyclase-like protein (ZmGC1) were isolated closely involving in maize ear rot (Johal and Briggs 1992; Yuan et al. 2008). It is widely accepted that plant disease resistances are controlled by multigenes or quantitative trait loci (QTL) (Young 1996). Some of QTLs for *Fusarium* disease resistance have been identified with molecular markers, which can be used for marker-assisted selection in maize breeding (Ali et al. 2005; Robertson-Hoyt et al. 2006; Ding et al. 2008). In our previous studies, a total of ten resistant QTLs were mapped on chromosomes 1, 2, 3, 4, 6, 7, and 9 by using maize inbred lines R15 (resistant) and Ye478 (susceptible) for FV ear rot (Zhang et al. 2006). Although mapping of many QTLs has advanced our knowledge regarding the genetic mechanisms of disease resistance, the molecular processes and gene regulation of the defense system relevant to maize ear rot remain poorly understood.

Here, we report on investigation of differentially expressed genes associated with *Fusarium* ear rot in two unique genetic backgrounds inbred lines, Bt-1 and Ye478, respectively. Bt-1 was derived from the tropical germplasm with high resistance to *Fusarium* ear rot and excellent agronomic characters. Ye478 is an elite Chinese inbred with high susceptibility to *Fusarium* ear rot after many years of screening with FV inoculation in southwestern China. In addition, Bt-1 has better agronomic characters than Ye478, including outer waxy cuticle and drought tolerance. However, Ye478 has better combining ability than Bt-1 (Zheng et al. 2010; Chen et al. 2002; Li et al. 2011). To better understand the differential defense processes in maize ear rot upon FV infection, biochemical and physiological enzyme activities were analyzed simultaneously for both inbred lines. Then, the differentially expressed genes responding to FV infection in maize were identified by suppression subtractive hybridization (SSH). A total of 93 single-gene fragments were isolated in response to FV infection from the libraries. Finally, expression profiles of representative defense-related genes associated with maize ear rot were indeed confirmed through reverse transcription-polymerase chain reaction (RT-PCR) during the time course of infection. The differentially expressed genes were inferred to demonstrate physiological functions and possible defense mechanisms in maize ear rot.

Materials and Methods

Plant Materials and Inoculation Procedures

Both maize inbred lines, the resistant Bt-1 and the susceptible Ye478, were identified in preliminary evaluation for many years for field responding to FV infection in southwestern China. The two inbred lines with completely different genetic backgrounds were used in this study for isolating differentially expressed genes. The spores of FV were cultured on potato dextrose agar media for 15 days prior to collection for inoculations. Inoculum was prepared by washing conidia from the cultures and diluting a final concentration to approximately 1.0×10^6 spores/mL in water. Milky stage maize plants were inoculated with 3 mL on each bract by injection. The inoculated plants and control plants were grown in the same growth chambers at Maize Research Institute, Sichuan Agricultural University, and each corn cob was wrapped separately using a kraft paper bag to prevent cross-contamination. The treated and control bract tissues were collected six times at 24-h intervals after inoculation.

Biochemical and Physiological Assays

The crude extracted proteins of phenylalanine ammonia-lyase (PAL) and peroxides (POD) were isolated from the inoculated and control bract tissues following Han et al. (2009) with three independent biological replicates. Total protein content of samples was determined by Bradford method (Bradford 1976) basing on a standard curve with known quantities of bovine serum albumin (Sigma). One gram of each inoculated and control bract tissues was triturated in 5 mL extraction buffer (0.2 M boric acid buffer, pH8.8, 0.5 M β-mercaptoethanol, 2 %*w*/*v* polyvinylpyrrolidone) for PAL extraction and in 0.05 M Na_2HPO_4/NaH_2PO_4 buffer (pH 7.0, 1 % Triton X-100) on ice for POD extraction. Homogenates were then centrifuged at 10,000×*g* for PAL

and 12,000×*g* for POD at 4 °C for 30 min. The supernatant that contained the crude enzyme extract with PAL was analyzed by spectrophotometer at 290 nm and at 470 nm for POD.

RNA Isolation and SSH Library Construction

Total RNA was isolated from the collected tissues using the extraction kit TRIzol Reagent (Invitrogen). mRNA was purified from the total RNA with the PolyATract mRNA Isolation Systems kit (Promega) following the manufacturer's instructions. The final concentration of mRNA was pooled as equal quantity after detection on the purity and integrity from six stages. Briefly, cDNA was synthesized from the pooled purified mRNA (2 μg). SSH was performed using the PCR Select cDNA Subtraction kit (Clontech) according to the manufacturer's instructions. In forward subtractive library, the cDNA from inoculated bracts were used as the "tester" and control as the "driver." The reverse subtractive library was performed with interchange of driver and tester. The obtained cDNA fragments were purified and condensed using Wizard SV Gel and PCR Clean-Up System kit (Promega). Then, the subtractive cDNA products were cloned into pMD18-T vector (Takara) to generate the SSH cDNA library. The competent cell JM109 was used in the transformation for differential screening. Aliquots (200 μL) of the transformation mixture were then spread on Luria–Bertani agar plates containing 100 μg/mL ampicillin, 80 μg/mL X-gal, and 50 μM IPTG and were incubated at 37 °C overnight. White clones were selected to generate a subtractive library and stored in 384-well plates at −80 °C.

Reverse Dot-Blot Hybridization of cDNA Arrays

Forward and reverse subtracted cDNA were digested with *Rsa* I and labelled as probes with digoxigenin (DIG) using DIG High Prime DNA Labelling and Detection Starter kit II (Roche) following the manufacturer's instruction. The cDNA inserts from SSH libraries were amplified by polymerase chain reaction using the nest primer 1 (5′-TCGAGCGGCCGCCCGGGCAGGT-3′) and nest primer 2R (5′-AGCGTGGTCGCGGCCGAGGT-3′) (Clontech) in a total volume of 20 μL reaction mixture. The inserted PCR products (1 μL) were spotted in parallel by reverse dot-blot onto two prewet nylon membrane (Hybond-N^+, Amersham). The membrane with DNA was placed on a wet filter paper rinsed with 0.4 M NaOH for 5 min to denature the DNA and then immediately briefly rinsed in 2× saline–sodium citrate buffer. After cross-linking in a baker at 80 °C for 2 h, the membrane was hybridized with a specific probe or conserved at room temperature for future use. The radioactive intensity of each spot was quantified, and the background with an empty pMD 18-T easy vector was subtracted for normalization. Screening was conducted as described previously (Diatchenko et al. 1999). Putative differential clones were selected from two independent biological experiments, and the standard deviation was calculated for each spot. For each cDNA library, poor quality spots were excluded, and spots were selected as average fold change of >2.

Sequence Analysis and Blast Search

All positive clones were subjected to sequencing by the Invitrogen Ltd. The nucleotide and expressed sequence tags (ESTs) were annotated based on GenBank database (http://www.ncbi.nlm.nih.gov/) using the Blastn or Blastx algorithm. Classification of annotated sequences was categorized according to the putative function of genes. Meanwhile, the differentially expressed sequences from SSH were submitted into MaizeGDB database (http://www.maizegdb.rog/). The positions of the ESTs on maize chromosome were obtained by comparing with the B73 reference genome.

Confirmation of Differentially Expressed Genes by Performing Semiquantitative RT-PCR

First-strand cDNA synthesis was performed with 1 μg total RNA from resistant and susceptible inbred lines using a M-MLV reverse transcriptase (Promega). The cDNA products were normalized for RT-PCR using actin gene as a control. Specific primers used in sets for PCR were designed for target genes using Primer Premier 5.0 and listed in Table 1. The PCR reactions were performed as 30 cycles at 94 °C for 30 s, 30 s at the primer-specific annealing temperature, and 72 °C for 30 s. After separation on 1 % (*w*/*v*) agarose gels, the intensity of each band (area) was calculated using the software Image J (USA). Each PCR reaction was repeated at least three times.

Results

Photographic Record of Pathogen Invasion Between Bt-1 and Ye478

Infected kernels on the cob were observed through photographing to demonstrate pathogen progress in both resistant and susceptible inbred lines Bt-1 and Ye478. The symptom for *Fusarium* ear rot recorded was that a white or light pink mold could be observed on the kernels and bracts in both inbred lines. As shown in Fig. 1, a distinct difference was observed for the infected degree in response to the FV inoculation. The infected area in the resistant inbred line Bt-1 was smaller than that in the susceptible line Ye478. The

Table 1 Primers used for semi-quantitative RT-PCR analysis

Target gene	Primer sequence	Tm (°C)
Osmotin (PR-5)	5′-GTGCCCCAAGGGCGGGCCG-3′; 5′-CTTCAAGGTTGGGAATTAAT-3′	56
MYB family transcription factor (MYB)	5′-GAGGGCGCCGTGCTGCGAGA-3′; 5′-CTAGAGATTGTCCAGGAAGA-3′	55
RAB GTP binding (RAB)	5′-GCATTGGACGCCAACAAGTT-3′; 5′-AGAGCATGAGCGCGATCAGG-3′	57
Small ubiquitin-like modifier (SUM)	5′-GCGGCGAGCAGACCCCTG-3′; 5′-CAGAAGACACTGGGTCCAAC-3′	56
Pathogenesis-related protein 1 (PR-1)	5′-CTCCAAACCCCACATTTGAT-3′; 5′-GGAACGGTCCTGCTTGTTAC-3′	56
Ethylene-responsive protein (ERF)	5′-CACCAAAGAATGCTGGGCTG-3′; 5′-CTGCCGGGCGGCCGCTCGAA-3′	57
S-adenosylmethionine synthase (SAMS)	5′-GAGCGGCCGCCCGGGC-3′; 5′-CTCGGCCGCGACCACGA-3′	57
Abscisic stress protein (ABA)	5′-ACGTATGTATGCGCTCGTG-3′; 5′-GAAGAGTTCGATAGATGGT-3′	56
Actin (GenBank no. gi121211756)	5′-GTGACCTTACCGACAACC-3′; 5′-CCAATACCAGGGAACATAG-3′	56

smaller infected area indicates that certain defense mechanisms were probably activated in the inbred line Bt-1.

Variation of PAL and POD Activities in Bt-1 and Ye478

Both PAL and POD activities were examined during a 6-day time period to understand FV defense responses in maize. As shown in Fig. 2a, a distinct difference was observed in PAL activity in response to the FV infection. PAL activity in the susceptible line Ye478 was higher and increased much quicker during the first 72 h post-inoculation (hpi), compared to that in the resistant line Bt-1, while there was no significant difference in their control groups. PAL activity in Ye478 increased promptly at the first 24 h and peaked at 48 h. Instead, PAL activity in Bt-1 showed a slow increase in the first 48 hpi and a peak at 72 hpi. Meanwhile, POD activity in both inbred lines increased regularly and peaked at 72 hpi and then declined slowly. Compared to their control groups, POD activity in Ye478 was higher than that in Bt-1 (Fig. 2b). POD activity curve was almost the same in both inbreds, but the level of POD activity in Ye478 was higher than that in Bt-1. As a whole, PAL and POD activities negatively correlated with the resistance of the inbred lines after FV infection.

Fig. 1 Symptoms of *Fusarium* ear rot of the hyphea invasion. *Left*, FV-infected inbred line Bt-1; *right*, inbred line Ye478. The infected area is indicated as a *white circle*

Construction and Differential Screening of Subtractive Libraries from Two Inbred Lines

Forward and reverse subtractive libraries were constructed simultaneously from both inbred lines after inoculation. A total of 6,560 clones were first identified from SSH four libraries. The lengths of inserts were reexamined by PCR, between 0.2 and 1.0 kb. Then, the PCR products were dot blotted on membrane and hybridized with four different DIG-labelled probes. As a result, 145 positive clones were obtained from the four subtractive libraries, which included 66 clones from the resistant inbred line Bt-1 and 79 clones from the susceptible inbred line Ye478. These cDNA clones were further analyzed by sequencing and similarity searches in NCBI database, resulting in identification of 93 unique ESTs. Among them, 68 were identified to exhibit high similarity to the genes with known functions, while 24 were genes with unknown functions (Table 2). Moreover, one gene could not be matched with any known sequences. Sequence data in the study have been deposited at GenBank under accession numbers GH201185–GH201258 and GH295163–GH295181. Meanwhile, the 93 unique ESTs were also submitted into MaizeGDB database, resulting in five ESTs (GH201188, GH201190, GH201192,

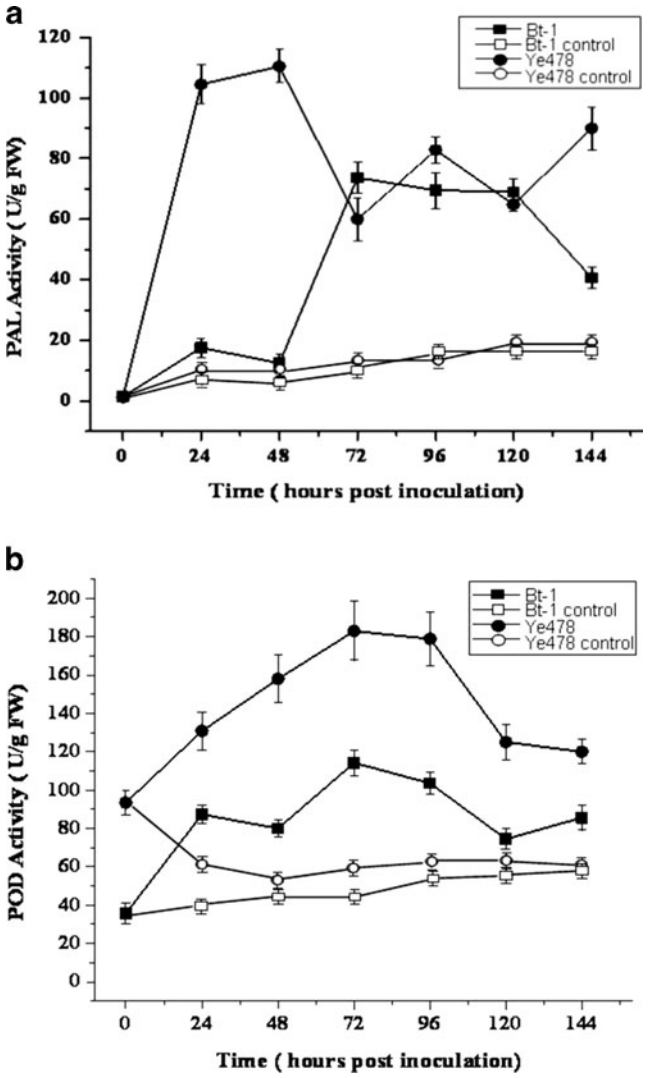

Fig. 2 Defense enzymes change during a 6-day time period in both Bt-1 and Ye478 inbred lines. PAL (**a**) and POD (**b**) activity after inoculation. Values are average of three biological samples for each time point

GH295175, and GH201211) closely linking to ear rot resistance QTL. The located ESTs associating with resistance QTL regions should be paid more attention for their resistant physiological functions.

Functional Categorization Between Bt-1 and Ye478

The unique ESTs with significant protein similarity were grouped into function categories by using functional classification scheme of Gene Ontology. A total of 40 unique ESTs were obtained from the forward and reverse libraries of resistant inbred line Bt-1 (Table 2). Among the forward subtracted library, 21 ESTs exhibited high sequence similarity to genes with known functions, six ESTs were identified to show similarity to genes with unknown functions, and one gene without a significant similarity to any identified genes in the GenBank database. Among the reverse subtracted library, six ESTs showed similarity to genes with known functions, and six ESTs were similar to genes with unknown functions. The cellular functions for the 40 ESTs from Bt-1 can be classified into disease defense (eight distinct proteins encoded by eight ESTs, 20.0 %); gene destination and transcription, which could participate in modification or regulation processes at transcriptional level (six distinct proteins encoded by seven ESTs, 17.5 %); signal transduction (five distinct proteins encoded by five ESTs, 12.5.0 %); protein destination and storage, which could participate in modification or regulation processes at translational level (three distinct proteins encoded by three ESTs, 7.5 %); metabolism (two distinct proteins encoded by two ESTs, 5.0 %); energy (one distinct proteins encoded by one EST, 2.5 %); and unknown (32.5 % including 13 ESTs) (Fig. 3a).

A total of 53 unique ESTs were obtained from the two libraries of susceptible inbred line Ye478 (Table 2). Among the forward library, 21 ESTs exhibited high sequence similarity to genes with known functions, and six ESTs were similar to genes with unknown functions. Among the reverse library, 20 ESTs showed high similarity to genes with known functions, and six ESTs were similar to genes with unknown functions. The cellular functions for the 53 ESTs from Ye478 can be classified into disease and defense (ten distinct proteins encoded by 13 ESTs, 24.5 %), gene destination and transcription (eight distinct proteins encoded by nine ESTs, 16.9 %), signal transduction (four distinct proteins encoded by five ESTs, 9.4 %), protein destination and storage (six distinct proteins encoded by seven ESTs, 13.2 %), metabolism (three distinct proteins encoded by three ESTs, 5.7 %), energy (three distinct proteins encoded by three ESTs, 5.7 %), intracellular trafficking (one distinct proteins encoded by two ESTs, 3.8 %), and unknown (22.6 % including 12 ESTs) (Fig. 3b).

Confirmation of Differentially Expressed Genes by Semiquantitative RT-PCR

To confirm responsive genes specifically expressed during the FV infection, expression patterns of eight representative genes, including pathogenesis-related protein 1 (PR-1), osmotin (PR-5), MYB family transcription factor (MYB), RAB GTPase, ethylene-responsive protein (ERF), small ubiquitin-like modifier protein (SUM), S-adenosylmethionine synthase (SAMS), and abscisic stress protein (ABA), were investigated by using RT-PCR at each time point for both genotypes. As expected on the basis of the SSH results, all the representative genes were indeed differentially expressed during FV invasion, and most of them were significantly induced in two inbred lines, while actin levels did not differ between samples (Fig. 4). For PR-5 gene, upregulation was observed in both

Table 2 Putative functions of isolated genes in the resistant and susceptible SSH libraries

Clone	Accession number	Putative function/similar GenBank accession	Organism	Chr.	Position (cm)	Length (bp)	e value
Genes from resistant forward SSH library							
SSHBF1	GH201185	Phosphoenolpyruvate carboxykinase (ATP)/CX944983	*Arabidopsis thaliana*	9	1.53	252	3e-04
SSHBF4	GH201186	Methyladenine glycosylase family protein/FK978831	*Arabidopsis thaliana*	1	18.27	452	7e-13
SSHBF6	GH201187	Similar to Copia protein (Gag-int-pol protein)/DT464889	*Canis lupus*	1	29.04	490	9e-112
SSHBF12	GH201188	Inositol phosphorylceramide synthase 1/AI666066	*Arabidopsis thaliana*	3	17.92	167	2e-59
SSHBF13	GH201189	Cyclin-dependent kinase C (CDKC)/DN228315	*Arabidopsis thaliana*	1	27.09	156	2e-74
SSHBF14	GH201190	ATSBT5.4; subtilase/EE181383	*Arabidopsis thaliana*	7	13.10	88	4e-08
SSHBF15	GH201191	Arginyl-tRNA synthetase, putative/DY239734	*Arabidopsis thaliana*	6	12.47	345	9e-155
SSHBF16	GH201192	Serine/threonine-protein kinase WNK11/ AW360726	*Arabidopsis thaliana*	4	1.48	127	2e-57
SSHBF18	GH201200	60S ribosomal protein L7 (RPL7C)/DN560702	*Arabidopsis thaliana*	7	16.13	133	1e-60
SSHBF21	GH201194	Small ubiquitin-like modifier 1 (SUM-1)/FM187906	*Arabidopsis thaliana*	8	16.21	279	9e-60
SSHBF180	GH295163	Myb family transcription factor/EX451196	*Arabidopsis thaliana*	1	0.78	247	4e-05
SSHBF181	GH295164	RAB1C; GTP binding/CD448027	*Canis lupus*	9	1.15	532	0.0
SSHBF183	GH295166	Fructose-bisphosphate aldolase, putative/DY306985	*Arabidopsis thaliana*	8	1.63	251	3e-99
SSHBF184	GH295167	Protein disulfide isomerase/CX068197	*Zea mays*	4	6.98	307	1e-57
SSHBF185	GH295168	Protease-associated zinc finger family protein/CO499271	*Arabidopsis thaliana*	9	1.53	191	2e-06
SSHBF186	GH295169	Metallothionein 2A (MT2A)/BI135315	*Arabidopsis thaliana*	3	1.98	142	8e-35
SSHBF187	GH295170	ATOSM34 (osmotin 34)/EE046233	*Arabidopsis thaliana*	1	25.84	499	4e-06
SSHBF188	GH295171	ATP-binding/hydrogen ion-transporting ATP synthase/BM349622	*Arabidopsis thaliana*	3	13.82	388	9e-19
SSHBF189	GH295172	Allene oxide cyclase 3 (AOC3)/EC859839	*Arabidopsis thaliana*	10	0.93	389	2e-13
Genes from resistant reverse SSH library							
SSHBR38	GH201201	Ribosomal protein S8/EC859826	*Zea mays*	4	17.82	131	2e-61
SSHBR46	GH201202	Protein kinase, putative/CK161090	*Arabidopsis thaliana*	2	14.10	65	5e-19
SSHBR192	GH295175	Dehydrin/DN830738	*Zea mays*	4	15.43	124	9e-20
SSHBR193	GH295176	HSP81-2; ATP binding/unfolded protein binding/EC613420	*Arabidopsis thaliana*	2	1.93	408	3e-143
SSHBR194	GH295177	FliG-like family protein/DT465112	*Arabidopsis thaliana*	5	0.82	290	8e-53
SSHBR195	GH295178	Wound-induced protein/DY398257	*Zea mays*	8	1.22	392	5e-05
Genes from susceptible forward SSH library							
SSHYF51	GH201206	Pseudouridine synthase family protein/DV943322	*Arabidopsis thaliana*	1	1.16	101	7e-25
SSHYF56	GH201207	Photosystem I subunit L/FD471445	*Arabidopsis thaliana*	9	1.30	146	4e-29
SSHYF65	GH201208	Pathogenesis-related protein 1/DY742760	*Zea mays*	7	0.85	168	4e-30
SSHYF68	GH201209	Glutaredoxin, putative/EC868338	*Arabidopsis thaliana*	4	19.43	165	3e-80
SSHYF70	GH201210	FTSH10 (FtsH protease 10); ATPase/BM267059	*Arabidopsis thaliana*	8	11.62	314	2e-135
SSHYF73	GH201211	Alcohol dehydrogenase 1/CK986330	*Zea mays*	1	27.39	245	2e-124
SSHYF76	GH201212	Glutathione peroxidase/DV493967	*Zea mays*	2	1.86	338	6e-81
SSHYF79	GH201213	Glycosyltransferase/DT640241	*Arabidopsis thaliana*	8	15.76	362	1e-118
SSHYF88	GH201215	Tonoplast membrane integral protein ZmTIP4-1/ CO520069	*Zea mays*	6	13.33	234	1e-116
SSHYF90	GH201217	Ribosomal protein S11/EG183621	*Triticum aestivum*	2	21.21	113	8e-15
SSHYF91	GH201218	Cylicin-1/CF012562	*Oryza sativa*	2	23.39	134	2e-61
SSHYF95	GH201220	Ribosomal protein s6 RPS6-2/EG168708	*Zea mays*	4	23.26	88	2e-35
SSHYF97	GH201221	S-adenosylmethionine synthetase 1 (SAM-1)/CD975152	*Arabidopsis thaliana*	3	16.38	129	6e-42
SSHYF104	GH201222	Abscisic stress protein homolog/EE162766	*Zea mays*	10	0.88	274	8e-44
SSHYF105	GH201223	Trigalactosyldiacylglycerol2 (TGD2)/CD437090	*Arabidopsis thaliana*	8	15.64	287	5e-146
SSHYF108	GH201224	Paused (PSD)/BM347828	*Arabidopsis thaliana*	6	9.10	322	7e-131
SSHYF109	GH201225	Staurosporin and temperature-sensitive 3-like A/BM349622	*Arabidopsis thaliana*	5	12.05	342	3e-16
SSHYF120	GH201226	Ethylene-responsive protein/EE292785	*Arabidopsis thaliana*	5	20.92	320	2e-125

Table 2 (continued)

Clone	Accession number	Putative function/similar GenBank accession	Organism	Chr.	Position (cm)	Length (bp)	e value
Genes from susceptible reverse SSH library							
SSHYR127	GH201235	ATNUDT3 (nudix hydrolase homolog 3)/FE896374	*Arabidopsis thaliana*	2	1.64	129	3e-05
SSHYR134	GH201236	Nucleolar histone deacetylase HD2-p39/EG175529	*Zea mays*	4	14.93	540	2e-53
SSHYR135	GH201237	Heat shock protein 91 (HSP91)/CV222834	*Arabidopsis thaliana*	5	9.12	242	2e-15
SSHYR148	GH201239	40S ribosomal protein S20 (RPS20B)/CF050938	*Arabidopsis thaliana*	4	21.23	358	1e-68
SSHYR149	GH201240	Metallothionein 2A (MT2A)/EG287814	*Arabidopsis thaliana*	6	0.37	132	3e-50
SSHYR152	GH201242	Sucrase related/DT464698	*Arabidopsis thaliana*	8	10.74	115	3e-44
SSHYR156	GH201243	Ferredoxin family protein/CD439943	*Arabidopsis thaliana*	3	22.43	587	0.0
SSHYR158	GH201244	Hypothetical protein/EC590769	*Canis lupus*	6	1.34	533	9e-121
SSHYR161	GH201245	SET domain protein 123/EE163216	*Zea mays*	2	20.39	928	0.0
SSHYR163	GH201247	Putative sesquiterpene cyclase/CA626136	*Zea mays*	6	0.80	128	1e-38
SSHYR164	GH201248	Myb family transcription factor/DW290923	*Arabidopsis thaliana*	1	20.43	376	6e-07
SSHYR166	GH201249	Similar to Copia protein (Gag-int-pol protein)/DT464780	*Canis lupus*	8	7.81	627	0.0
SSHYR176	GH201250	AtRABG3f/AtRab7B/AW134428	*Arabidopsis thaliana*	8	12.29	80	1e-20
SSHYF55	GH201228	No hit					

Unknown (24 clones)

inbred lines, but more pronounced in Bt-1. MYB transcriptional factor was significantly upregulated in the Bt-1 inbred line at 40 h after the FV inoculation. Similar early upregulation phenomenon was also observed for SUM and ERF genes in Bt-1. Although SAMS and ABA genes exhibited a similar pattern in both inbred lines, Bt-1 showed a higher expression level after 12 hpi than that in Ye478. These results suggest that expression of the genes involved in the FV infection were differentially regulated in the resistant and susceptible maize inbred lines.

Discussion

Maize ear rot is a pathogen-induced disease that affects multiple cellular activities, including various physiological changes, membrane integrity, DNA–protein interaction, and gene expression (Eulgem 2005). Upon recognition of the pathogen infection in plant, it is usually accompanied by an oxidative burst, which has been proposed to orchestrate the establishment of different defensive barriers against the pathogens (Delledonne et al. 2001). As observed in this study, significant increase of PAL and POD activities strongly suggest that defense responses were activated in the FV-infected host. Furthermore, we found that enzymatic activities in response to FV infection were distinctly different between the resistant maize inbred line Bt-1 and the susceptible maize inbred line Ye478 (Fig. 2). Activities of defense enzymes in the susceptible inbred responded stronger than in the resistant inbred, indicating a negative correlation between defense enzyme activity and the resistant inbred. The correlation in our research may be different from some other reports that the different correlations between protective enzyme activities and cultivars might result in researchers using different experimental materials, tissues, or treatments (Li et al. 2003; Liang and Hou 2004). Our results are consistent to some previous reports on pathogen infection that limited pathogen growth in susceptible plants leads to programmed cell death (PCD) (Greenberg 1997). This phenomenon is consistent with previous microscopic observation that the FV invaded into tissue cells of the susceptible inbred slightly earlier than that of the resistant inbred (Yuan et al. 2012). It is likely that the pathogen invasion into bract tissues was delayed by unknown mechanisms in the resistant inbred. This result is also consistent with photographic record that the infected area was smaller in the resistant inbred than that in the susceptible line. A smaller infected area and a delay in invasion of FV in the resistant inbred line may contribute to the prevention of pathogen invasion and progression.

As a first step towards the identification of genes differentially induced by FV attack, resistant and susceptible inbred lines used in this study have been proven to be a suitable system for isolation of genes involved in the disease defense. A total of 93 unique ESTs were identified from 145 positive clones with possible functions in defense from four subtractive libraries (Table 2). To date, this is the most important EST collection generated simultaneously from maize cultivars resistant and susceptible to ear rot. In detail, the 65 unique ESTs associated with defense activities, such as PR-1, MYB family transcription factor, RAB GTPase (RAB), alcohol dehydrogenase, small ubiquitin-like

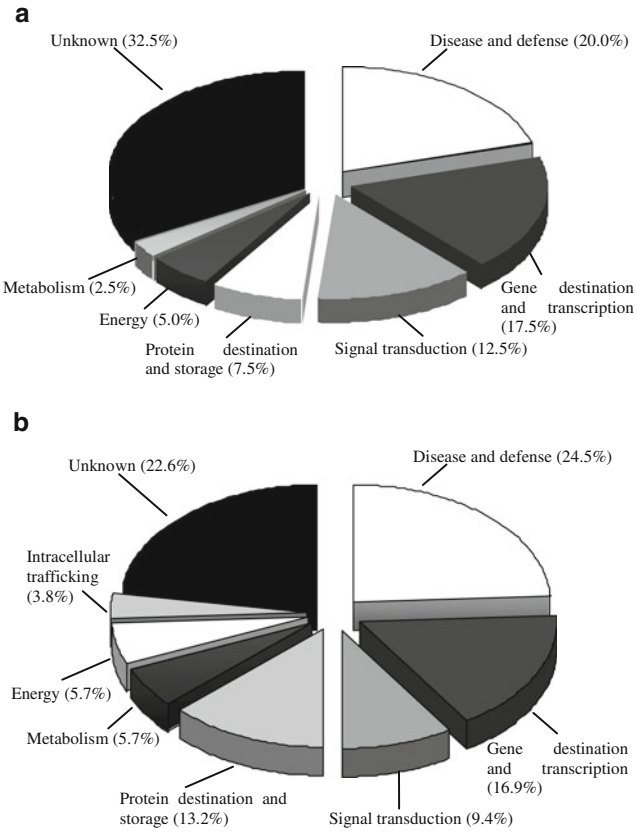

Fig. 3 Functional categorization and percentage of FV inoculation ESTs based on their putative functions. Differentially expressed genes under FV infection in the resistant inbred line Bt-1 (**a**) and those in the susceptible inbred line Ye478 (**b**). A total of 40 EST sequences from forward and reverse libraries from the resistant genotype were classified into seven unique categories, while 53 EST sequences from the two susceptible genotype libraries were classified into eight unique categories. The proportions of EST sequences in the total genes from resistant genotype libraries or susceptible genotype libraries were showed as percentages

modifier protein, S-adenosylmethionine synthase, and abscisic stress protein, were also found in previous reports on the FV infection response (Yuan et al. 2012). However, other 28 unique ESTs were specially identified in this study, including osmotin (PR-5) and sucrase-related ferredoxin family protein, which may play defense roles in preventing pathogen invasion. By comparing expression profiles of the eight representative genes in the two genotypes, the selected genes from different physiological functional categories were modulated with a much weaker response in the susceptible Ye478. It is possible that the constitutively elevated expression of these defense-related genes in the resistant Bt-1 may play important roles in modulating the response to FV infection or enhancing protection system. This strong transcriptional response in pathogen-affected tissues is consistent with previous findings in other plant–pathogen interactions (Schenk et al. 2000; Tao et al. 2003). There is no doubt that the genes identified in this study should provide very valuable information for our understanding on the maize defense mechanism against FV infection and will streamline the community effort in elucidating the functions of many defense response genes in maize. Furthermore, additional experiments will be required to provide evidence for the cause–effect relationship between expression levels of these genes and ear rot resistance.

Our data showed that several transcripts encoding PR proteins were expressed at elevated levels in two genotypes. As shown in Fig. 4, PR-1 and PR-5 were significantly induced after FV infection. PR-1 is often used as markers for the enhanced defensive state conferred by pathogen-induced systemic acquired resistance and has been reported extensively for its antifungal activities (Lebel et al. 1998; Edreva 2005; Sekhon et al. 2006; Casacuberta et al. 1991; Cordero et al. 1992). PR-5 (thau-l proteins), which is an antifungal cytotoxic agent causing rapid cell death, was described as inhibition of the hyphae growth and lysis of sporangia (Yun et al. 1998; Capelli et al. 1997; Kim et al. 2009; Thompson et al. 2006; Husaini and Rafiqi 2011). Moreover, several defense-related regulatory genes associating with potentially defense response were also identified in our study. The upregulated ERF could regulate the ET signalling pathway and also may upregulate the downstream defensive genes resulting in HR cell death and preventing pathogen spread (Yang and Klessig 1996; Singh et al. 2002; Chen et al. 2012). Expression of MYB gene was significantly upregulated in the early stage in Bt-1 upon the FV infection, associating with cellular morphogenesis and regulation of meristem formation and the cell cycle (Jin and Martin 1999). In addition to the genes in the defense, several other types of genes were identified at elevated levels in our experiment, such as RAB, SUM, SAMS, and ABA, suggesting that these genes should be involved in the maize defense system against FV infection. It is reported that RAB can induce the expression of PR proteins by wounding and/or attempted invasion of pathogens (Sano et al. 1994; Sano and Ohashis 1995). SUM protein could cause a rapid plant defense response in plants, leading to PCD (Edelmann and Kessler 2008; Fraire-Velazquez and Lozoya-Gloria 2003; Zhang et al. 2012). SAMS may play an important role in ethylene biosynthesis during pathogen infection (Li et al. 2009). ABA can affect disease resistance mainly negatively by interfering at different levels with biotic stress signalling (Mauch-Mani and Mauch 2005; Jiang et al. 2012). In addition, genes involved in other physiological processes are also useful for understanding the molecular processes in maize ear rot during the FV infection.

In summary, SSH approach has allowed us to generate four cDNA libraries, highly enriched for defense-associated ESTs from maize bracts after FV infection. By comparing the responses of resistant and susceptible inbred lines upon FV infection, we have identified 93 unique ESTs from 145

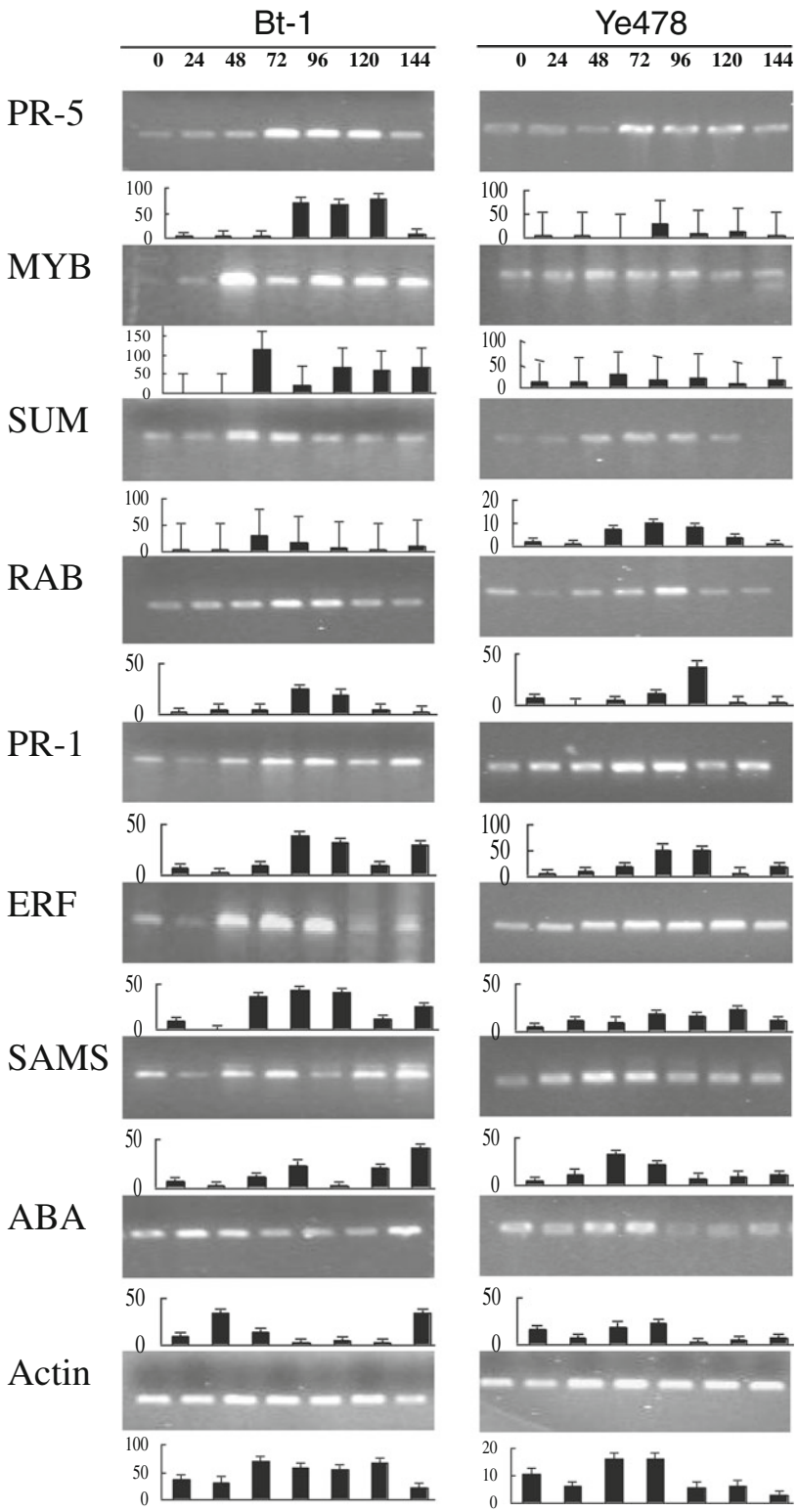

Fig. 4 Expression profiles of eight differentially expressed genes from the resistant and susceptible SSH libraries. Maize bract tissues collected at 0, 24, 48, 72, 96, 120, and 144 h after FV inoculation were analyzed by RT-PCR in resistant and susceptible genotypes. Control was carried out similarly. *Histograms* below each gel image represent relative intensity of transcript level of both genotypes. Actin genes were amplified as RT-PCR controls

positive clones with possible functions in defense of the FV infection. Although it is unclear whether these genes play the active role in cell response or as regulators in the reaction, these discoveries point toward future experiments that can uncover the molecular mechanisms of the host defense response to FV. The FV-responsive genes from SSH libraries from both inbred lines are a valuable resource for further functional genomics studies addressing resistance

mechanisms to maize ear rot. Such information can be used by breeders for selection and transfer of candidate genes to agronomically important maize inbred lines.

Acknowledgments We thank Dr. Yunbi Xu and Dr. Xiaoyang Zhu for their comments on this manuscript. This project was financially supported by grants from the Natural National Science Foundation of China (nos. 30571173 and 31201274), Program for Changjiang Scholars and Innovative Research Team in University of China (grant no. IRT0453), and National High Technology Research and Development Program of China (863 Program) (no. 2012AA10A307).

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