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Full Length Research Paper

Identification of genes differentially expressed in maize (*Zea mays* L.) during *Rhizoctonia Solani* Kühn infection by suppression subtractive hybridization

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Banded leaf and sheath blight (BLSB) caused by *Rhizoctonia solani* Kühn is an emerging problem in maize worldwide. To study gene expression in maize plants infected by *R. solani* Kühn, we constructed two suppression subtractive hybridization (SSH) library using complementary DNA (cDNA) from uninoculated plants as a driver and those from inoculated plants as a tester. After two cycles of hybridization, 84 cDNA clones from the forward and reverse SSH library were obtained and sequenced. Gene analysis indicated that these clones represented 51 single genes, and the functions of 35 genes could be assigned using existing databases. Thirty-five expression sequences tags (ESTs) were classified in fuctional categories, such as: transcription, regulation, protein processing, metabolism, defense, disease response and other functions. The expression of 15 genes was analyzed by semi-quantitative reverse-transcription-polymerase chain reaction (RT-PCR) and found to be up-regulated or down-regulated in response to *R. solani* Kühn re-infection. In conclusion, this study provides a basis for a molecular understanding of host reaction in response to pathogen.

Key words: Banded leaf and sheath blight, expressed sequence tag, gene expression, maize, suppression subtractive hybridization.

INTRODUCTION

Rhizoctonia solani Kühn is a common soil borne pathogen with a great diversity of host plants range vegetables to crops. The pathogen attacks below ground plant parts such as the seeds, hypocotyls, and roots, but it is also capable of infecting above ground plant parts (for example, pods, fruits, leaves and stems) (Adams, 1988). Maize banded leaf and sheath blight (BLSB) caused by *R. solani* Kühn, which is a destructive disease results in significant yield losses in most maize-growing areas of the world (Wu et al., 1997). BLSB infection often leads to extensive necrosis of leaf sheaths of semi dwarf and nitrogen-responsive maize. In recent years, the BLSB-resistance line screening has been increased in maize breeding, especially in some rainy area. Although, this is a regional maize disease mainly occurring in China and Southeast Asia, it may spread to other parts of the world gradually (Bertus, 1927; Sharma and Saxena, 2002).

During the past decades, great efforts have been devoted to understand the molecular mechanism of the plants infected by *R. solani,* such as *Oryza sativa* L. and *Zea mays* L. (Liu et al., 2009; Zhang et al., 2010). Most scientists believed that resistance to *R. solani* is a typical quantitative trait controlled by polygenes, and there is partial resistance or tolerance existing in crop germplasm (Chen et al., 1993; Lee et al., 1999; Ronald, 1997). There are multiple minor genes or quantitative trait loci (QTLs) concerned with BLSB resistance in some crop varieties (Zhao et al., 2006; Zou et al., 2000). There are many catalytic enzymes involved in the *R. solani* infective

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response, including chitinase, glucanase and phenylanine ammonia lyase (Anuratha et al., 1996; Jedidah et al., 2000; Liu et al., 2009). Furthermore, a few pathogenesis-associated genes transiently exist in maize and resisted the pathogen (Alexander et al., 1993; Datta et al., 1999; Agrawal et al., 2001; Zhu et al., 2006). Although, these results suggest that some potential defense pathways are activated in response to *R. solani*; so far, no intensive study about gene expression in maize leaves induced by *R. solani* AG1-IA was reported, and our understanding of *R. solani* tolerance in maize still remains limited. Since efficacious maize varieties that are resistant to BLSB and none of QTL gene were cloned, therefore, finding alternative ways for understanding regulatory mecha-nisms of the disease are necessary. Gene expression profiling is a promising approach to study both regulatory mechanisms and signaling networks which underlie defense responses and pathogenesis of plants.

The purpose of our work is to study gene expression in maize leaf, with infection of *R. solani* AG1-IA at early post-penetration stages. Genes differentially expressed were identified by the suppression subtractive hybridization (SSH) (Diatchenko and Lau, 1996). The isolated expressing sequence tag (EST) clones were sequenced and compared with the existing databases. The identified genes provide an important genomic resource for future molecular analysis of the interaction between maize and *R. solani*.

MATERIALS AND METHODS

Plant materials

The maize inbred line R15, which exhibits high tolerance to BLSB (Yang et al., 2003), and high general combining ability (GCA) for yield and elite yield components, was used for constructing SSH library and gene expression analysis. The maize line Ye478 is high-susceptibility maize inbred line for gene expression analysis. Plants were cultivated in the Fenjiang Farm of Maize Research Institute in Sichuan Agricultural University and maintained in a growth room under the following conditions: 25°C, constant relative humidity, photoperiod; 16 h light to 8 h dark.

Inoculations with *R. solani*

The pathogenic fungus causing maize BLSB is *R. solani* Kühn in China, and the dominant microorganisms is Anastomosis group AG1-IA, which has high virulence and broad host spectrum (Xiao et al., 2002). Anastomosis group AG1-IA isolates were provided by the Plant Pathology Institute of Sichuan Agricultural University, maintained on potato dextrose agar (PDA: potato, 200 g; dextrose, 20 g; agar, 10 g; H_2O 1000 mL), and incubated at 26°C for 3 to 5 days before inoculating. Colonized wheat grains for used in the field inoculations were prepared by transferring the mycelium to sterilized wheat grain, and incubating at 26°C until mycelium covered the surface of the wheat grain. At the jointing stage of the maize plants, two colonized wheat grains were artificially inoculated into the third sheath of twenty plants. Uninfected plant was as the positive treatment. In this study, we collected samples at 0, 12, 24, 36, 48, 60, 72, 84 and 96 h after inoculation and stored at -70°C.

RNA isolation and construction of subtractive cDNA library

Total RNA from different samples was collected at different time and control was extracted using Trizol® reagent (Invitrogen, USA) according to the manufacturer's protocol. Messenger RNAs were isolated using a PolyATract mRNA isolation systems (Promega, Madison, USA) according to the manufacturer's protocol. Double-stranded cDNA was synthesized from approximately 2 ug of poly $(A)^+$ mRNA.

SSH was performed with the polymerase chain reaction (PCR) select cDNA subtraction kit (Clontech) following the manufacturer's instructions. For the forward subtractive cDNA library, cDNA obtainned from R15 infected by BLSB from 12 to 96 h was defined as a tester, while cDNA from uninfected control plants R15 was used as a driver. In the reverse subtractive cDNA library, the tester and driver populations were interchanged. Therefore, up-regulated genes could be screened from the first library, and down-regulated genes could be identified from the second library. In short, we obtained two subtracted cDNA populations, one with the up-regulated fragments and one with the down-regulated fragments.

The subtracted cDNA fragments were cloned into the pGEM-T Easy Vector (Promega, Madison, USA) and transformed into competent cells of *Escherichia coli* strain DH5α (Promega). The transformed cells were plated onto Luria–Bertani (LB) containing 100 mg/L ampicillin, 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG), and 80 mg/L X-gal, then incubated at 37°C overnight to obtain a subtracted EST bank. Positive white colonies were selected and grown in LB medium containing ampicillin (50 mg/L) in 96-well plates at 37°C for 5 h and then kept at -70°C with 30% glycerol.

cDNA microarray as a form of reverse RNA gel blot analysis

The cDNA microarrays for screening were generated by using 1 uL of positive colonies culture as the template for the PCR reaction using the nested PCR primer 1 (5'-TCGAGCGGCCGCCCGGGCAGGT-3') and nested PCR primer 2R (5'- AGCGTGGTCGCGGCCGAGGT-3') (BD Biosciences Clontech, Palo Alto, CA) and *Taq* polymerase using the PTC-100 thermal cycler (Bio-Rad). 5 uL of the PCR product was combined with 5 mL of 0.6 N NaOH and 1 uL of the resulting mixture was transferred to the Hybond-N+ (Amersham Biosciences Corp., Piscataway, NJ) nylon membranes. Blots were neutralized in 0.5 M Tris–HCl (pH 7.4) and cross linked using the Spectrolinker XL-1000 UV cross linker (Spectronics Corporation, Westbury, NY). Uninfected and infected mixed cDNA probes were labeled with DIG-High Prime (Roche Applied Science, Mannheim, Germany). Dot blots were pre-hybridized for 1 h and hybridized overnight at 50°C in hybridization solution (50% formamide, 5 × SSC [1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 2% blocking reagent [Roche], 0.1% lauroyl sarcosyne, 0.02% SDS, 100 ng/mL of heat-denatured salmon sperm DNA). Membranes were then washed four times for 15 min in 2 × SSC–0.5% SDS at ambient temperature and twice for 30 min in 0.5% SSC–0.1% SDS at 60°C. CDP-Star was used as the detection substrate and the resulting chemiluminescence was documented using X-ray film fixation.

Sequence analysis and blast search

Sequencing of the plasmid cDNAs was performed using M13 forward and reverse primers by commercial DNA sequencing service providers (Biotech, Shanghai, China). Base calling and quality assessment were performed using DNAMAN. Low-quality (Phred score < 20), short (< 100 bp), vector and linker sequences were removed.

The BLAST algorithms at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) were used to

Table 1. Oligonucleotide probe sequences.

Number	Forward primer (5´ - 3´)	Reverse primer (5´ - 3´)	Ta (°C)	Size of amplicon (bp)
MRSF1	TCCCTATTGGTGGGTGAACAA	ATACGAACCGTGAAAGCGTG	58	207
MRSF6	GCGAACACAAGCAGCACTA	GAGAAGCACCCAGTCAACCT	59.1	236
MRSF16	CCTGAAGAAGGACAACACCT	CAGCCAGTGACACCATAATG	56.3	186
MRSFc5	AGGCTCTCCTGAACTGTATCA	ACATTCTATCCTACGCTTGGG	56.6	148
MRSFc6	TGCCTACTCAGTCCTGTTCA	CGAGCACGCAGTCAAATCT	57.9	125
MRSFc8	TCCTCGTCGCTGGATTCCTT	CCACTCCCTCTTTGCCTACTT	58.7	184
MRSFc9	TGTCGTAGAGAAGTTCAG	GAAGTTAGAATGTGAGCG	49.5	160
MRSF7	TGAGCGAGTTCCAAGTGACC	ACCAACACAAACAGTCCGC	58.4	292
MRSF37	GCCACTCGCAAACTTGTT	CAGTAGACAGGGATGACCA	54.8	285
MRSR94	AAAGGATTACACTGGGCGAG	TTTATGTCATTCGGCAGGAGG	57.6	312
MRSR95	GGACTTGCTCCGTCTGTAATG	ATCCTCAATCCCTTGGTCAGC	59.3	286
MRSR102	CAGAGATACCATCAGACCAGT	TCCAATACGACCAAGAGTAGC	56.7	410
MRSr2	TCAGGGTGAGGATGTGGTT	AGTGGCTCTCCAGGATGTT	56.8	119
MRSRr6	CGGTCAACTCCTTCCCTTCA	GCATCCTACCCTATTGTCGC	57.6	153
MRSR67	AACATCTGGCACTGACTCAC	CGATAACGCTGACACTCTACA	57.1	183

initially identify sequences in the non-redundant GenBank database (Altschul et al., 1997). A match was considered to be significant if the expected (E) value was less than $1e^{-3}$, and for this analysis only the most significant matches were examined.

Data validation by semi-quantitative RT-PCR

Semi-quantitative RT-PCR was used for gene expression assay. In this study, according to ESTs putative functions, we chose 15 important ESTs for expression profiles analysis, which were involved in disease-resistance/defense, signal transduction, transcription regulation, protein modification and storage, protein transportation, energy metabolism etc. Primers were designed for 9 ESTs from the forward subtractive library and 6 ESTs from the reverse library (Table 1). First strand cDNAs of different samples with or without infection, were reverse transcribed from 2.5 µg of the total RNA in the presence of oligo d(T) primer (Promega), 20 µM each of dNTPs, and 200 units of M-MLV reverse transcriptase (Promega). This was done in a total of 50 µL at 42°C for 2 h. PCR reactions were performed by mixing 1 µL of first-strand cDNAs, 0.2 µM gene specific 5' and 3' primers, and 5 units *Taq* DNA polymerase (Promega) in a total of 50 µL. Thermal cycling parameters were as follows: 1 cycle at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 56 to 58°C for 30 s, and 72°C for 1 min, using a PTC-100 thermal cycler (Bio-Rad). *Actin* was also amplified and used as an internal control for comparing relative levels of target gene expression. RT-PCR products were resolved by gel electrophoresis and images were captured using a Bio-Rad Gel Doc system.

RESULTS

Suppression subtractive hybridization

Two maize leaf subtracted libraries have been constructed to identify specific gene expression in response to AG1-IA infection. To identify genes up-regulated by AG1-IA infection, a library named SF was generated using mRNAs from control plants as driver, while genes down-regulated by AG1-IA infection were screened in a library named SR that was generated with mRNAs isolated from plants with AG1-IA infection as driver.

The efficiency of the SSH procedure was assessed by depletion of the housekeeping gene (GADPH) and determined by PCR amplification over a graded increase in the number of cycles. Expression of the GAPDH gene was not detected by the subtracted SSH-cDNAs from 18 to 33 cycles of amplification; however, it has been detected by 23 cycles of PCR amplification from non subtracted cDNAs (data not shown). The pattern of GADPH amplification clearly indicated that subtractions were effective.

After the cloning of the individual SSH-generated cDNA production into pGEM-T Easy Vector, 480 clones were isolated from the forward hybridization SF and 288 clones from the reverse subtraction SR with cDNA from 51 to 910 bp. These clones were pre-screened using PCR primers specific to the oligonucleotide adapters for ensuring that only different inserts induced differential screening (data not shown).

Differential screening of genes identified by SSH

Using our reciprocal SSH libraries, the screened clones were spotted on two sets of micro-arrays, that is, reverse Northern blots were employed by cDNA from the control (uninfected) and fungus-treated samples as probes to screen the differential cDNA fragments from the two libraries. Tester and driver cDNAs were labeled with digoxin (DIG) by random priming DIG DNA labeled kit

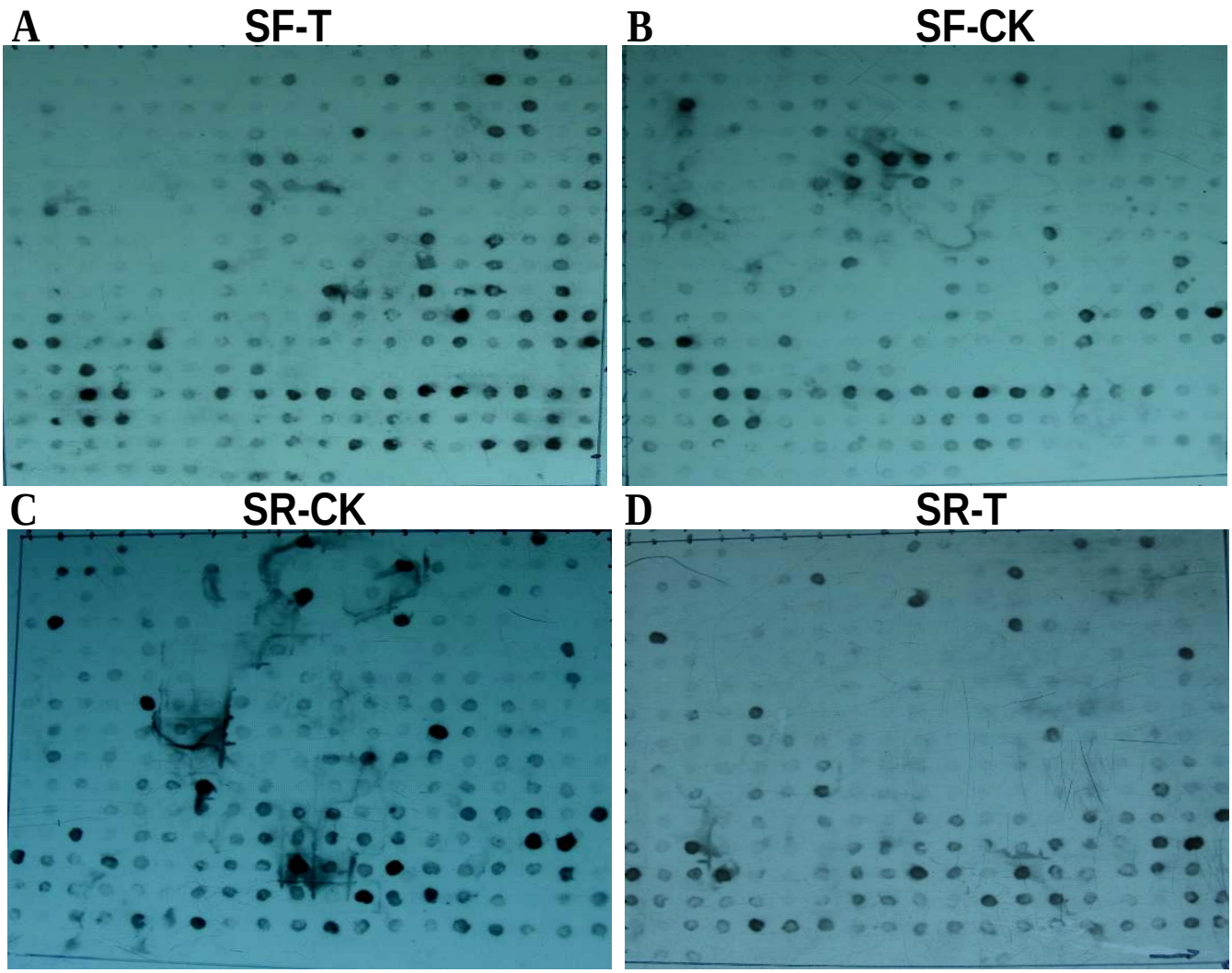

Figure 1. Representative differential screening images of SSH library macroarrays. Four identical sets of membranes were dot blotted with PCR-amplified cDNA fragments obtained by suppression subtracted hybridization. Hybridization of macroarrays was then performed with four different probes: (**A**) SF-T cDNAs of the inoculation treatment, (**B**), SF-CK cDNAs of the uninoculated treatment, (**C**) SR-CK cDNAs of the inoculated treatment, and (**D**) SR-T cDNAs of the uninoculated treatment.

(Roche Applied Science, Indianapolis, IN). The result reveals differential screening has yielded 54 SF-specific clones (Figure 1A, B) and 30 SR-specific clones (Figure 1C, D). Most clones from two libraries exhibited differential expression that may warrant further investigation.

Sequence analysis and functional classification of ESTs

For gene identification, the screened clones were sequenced and analyzed to reduce the redundant sequence after trimming the adapter and vector ends. Using the BLAST program VecScreen, we identified 54 SF clones, in which 30 showed to be non-redundant sequences (Table 2), and 21 non-redundant sequences were from the 30 reverse hybridization SR clones (Table 3). BLAST results of up-regulated and down-regulated clones showed 64% of them had significant homology to genes of known function in available public databases. By comparison, 30 up-regulated EST sequences were obtained from the forward-library, in which the functions of 22 are predictable. Twenty-one down-regulated ESTs were obtained from the reverse-library, in which the functions of 13 genes are predictable. All the ESTs were submitted to the dbEST of Genbank (except 3 sequences). The authorized serial numbers were listed in Tables 2 and 3.

Most plant disease-residence traits are quantitative traits which are controlled by polygenes. Compared with natural disease stress, although inoculation with *R. solani* as pathogenic fungus is a comparatively single stress, the response at the mRNA level remains to be poorly

Table 2. Identification of genes that were up-regulated in response to the infection by *R. solani.* cDNA clones were screened by suppression subtractive hybridization for maize leaf inoculated with pathogen AG1-IA.

Clone	Accession	Putative function	Score	Expected value	Identity	Source organism
MRSF1	DY742722	Putative senescence-associated protein	403	$2e^{-38}$	75/84 (89%)	*Pisum sativum*
MRSF6	DY742723	Putative receptor-associated protein	337	$1e^{-30}$	79/106 (74%)	*Oryza sativa*
MRSF16	DY742724	Ferredoxin	330	$6e^{-30}$	60/60 (100%)	*Zea mays*
MRSFc5	DY742725	Serine carboxypeptidase I, putative	225	$9e^{-18}$	38/70 (54%)	*Oryza sativa*
MRSFc6	DY742726	Phosphatidylinositol-4-phosphate 5-kinase	274	$2e^{-23}$	53/59 (89%)	*Oryza sativa*
MRSFc8	DY742727	Ferritin	192	$6e^{-14}$	37/37 (100%)	*Zea mays*
MRSFc9	DY742728	Thioredoxin	148	$8e^{-09}$	29/59 (49%)	*Fagopyrum tataricum*
MRSF7	DY742729	Putativ ethylene-responsive transcriptional coactivator	322	$6e^{-29}$	65/68 (95%)	*Oryza sativa*
MRSF37	DY742730	Dual-specificity protein-like phosphatase 1	257	$3e^{-21}$	50/50 (100%)	*Zea mays*
MRSF47	DY742731	Cytochrome c oxidase subunit II	153	$2e^{-09}$	47/51 (92%)	*Homo sapiens*
MRSF11	DY742732	SER/Thr receptor-like kinase	384	$4e^{-36}$	76/91 (83%)	*Zea mays*
MRSF2	DY742733	FP-10_H08.SEQ cDNA library of Phaeosphaeria nodorum grown on wheat cell walls Phaeosphaeria nodorum cDNA, mRNA sequence *	70	$8e^{-30}$	145/162 (89%)	*Phaeosphaeria nodorum*
MRSF45	DY742734	Mitogen-activated protein kinase 12	255	$3e^{-21}$	53/73 (72%)	*Homo sapiens*
MRSF49	DY742735	Similar to zinc finger protein 258 isoform 2	73	$1e^{-12}$	60/64（94%）	*Canis familiaris*
MRSF54	DY742736	G protein-coupled receptor TGR7	74	$1e^{-14}$	60/64 (94%)	*Canis familiaris*
MRSF3	DY742737	Similar to KH domain containing, RNA binding, signal transduction associated 3 isoform 9	376	$3e^{-35}$	72/73（98%）	*Canis familiaris*
MRSF5	DY742738	1000054-B04.GAD10-F UGI-Reseq Zea mays cDNA, mRNA sequence *	81	$4e^{-37}$	81/81 (100%)	*Zea mays*
MRSF72	DY742739	Zea mays BSP1 mRNA for bundle sheath cell specific protein 1, complete cds *	159	$3e^{-83}$	162/163 (99%)	*Zea mays*
MRSF8	DY742740	Zea mays CL20291_1 mRNA sequence *	42	$6e^{-14}$	42/42 (100%)	*Zea mays*
MRSF21	DY742741	MEST1095_F05.T7-1 UGA-ZmSAM-XZ2 Zea mays cDNA, mRNA sequence *	38	$1e^{-11}$	38/38 (100%)	*Zea mays*
MRSF30	DY742742	MEST109-E02.T3 ISUM4-TN Zea mays cDNA clone MEST109-E02 3', mRNA sequence *	188	$2e^{-100}$	197/200 (98%)	*Zea mays*
MRSFc2	DY742743	Zea mays partial ivr2 gene for vacuolar invertase, exons 1-2	51	$1e^{-19}$	51/51 (100%)	*Zea mays*
MRSF43	DY742744	NADH dehydrogenase subunit 1	399	$2.e^{-37}$	123/133（92%）	*Homo sapiens*
MRSF12	DY742745	HF0010_I10:A Triphysaria versicolor (parasitic plant) root-tip, host forward SSH library Triphysaria versicolor cDNA, mRNA sequence *	234	$5e^{-128}$	234/234 (100%)	*Triphysaria versicolor*

Table 2. Conitnues.

MRSF25	DY742746	HR0011_M05:A Triphysaria versicolor (parasitic plant) root-tip, host reverse SSH library Triphysaria versicolor cDNA, mRNA sequence *	276	$6e^{-153}$	279/280 (99%)	*Triphysaria versicolor*
MRSF15	DY742747	Catalase (EC 1.11.1.6) CAT-2	183	$7e^{-13}$	33/34 (97%)	*Zea mays*
MRSFc3	DY742769	IV-3524-1A-H01.T7-1 UGIV-3524-Reseq Zea mays cDNA, mRNA sequence *	115	$3e^{-57}$	115/115 (100%)	*Zea mays*
MRSF31		Lysis protein	294	$9e^{-26}$	57/57（100%）	*Coliphage phiX174*
MRSF24		Similar to PHD finger protein 20-like 1 isoform 1 isoform 14	397	$1e^{-37}$	91/96（94%）	*Canis familiaris*
MRSF9		SNF1-related protein kinase; SnrK1	140	$7e^{-08}$	30/87 (34%)	*Zea mays*

Identity represents the percent homology estimates of equine cDNA fragments with nucleotide sequences in GenBank. * Means of homology results from Genbank [NR or expressed sequence tag (EST) databases].

Table 3. Identification of genes that were down-regulated in response to the infection by R. solani. cDNA clones sceened by suppression subtractive hybridization were used for gene identification by Blast search.

Clone	Accession	Putative function	Score	Expected value	Identity	Source organism
MRSR70	DY742748	Putative glutathione reductase	439	$4e^{-122}$	213/233 (91%)	*Oryza sativa*
MRSR72	DY742749	Putative legumain	311	$4e^{-84}$	147/149 (98%)	*Zea mays*
MRSR94	DY742750	Alanine aminotransferase	318	$6e^{-86}$	168/173 (97%)	*Zea mays*
MRSR95	DY742751	Glutathione S-transferase	150	$6e^{-09}$	64/79 (80%)	*Haloarcula marismortui*
MRSR96	DY742752	Triose phosphate/phosphate translocator	521	$8e^{-147}$	275/276 (100%)	*Zea mays*
MRSR102	DY742753	Pyruvate, phosphate dikinase (EC 2.7.9.1) precursor	610	$2e^{-62}$	116/118 (98%)	*Zea mays*
MRSR58	DY742754	Heat shock protein 70	498	$1e^{-139}$	266/295 (90%)	*Cucumis sativus*
MRSR67	DY742755	Inosine-5'-monophosphate dehydrogenase	165	$1e^{-10}$	55/88 (62%)	Thermococcus kodakarensis
MRSRr6	DY742756	Cysteine protease	160	$1e^{-38}$	71/72（98%）	*Zea mays*
MRSRr2	DY742757	Probable cytochrome P450 monooxygenase	226	$3e^{-58}$	130/175（74%）	*Zea mays*
MRSR71	DY742758	Antitermination NusB domain-containing protein	369	$2e^{-34}$	73/118 (61%)	*Arabidopsis thaliana*
MRSR76	DY742759	Glyceraldehyde-3-phosphate dehydrogenase	190	$1e^{-47}$	92/93 (98%)	*Homo sapiens*
MRSR137	DY742760	Za59b12.g1 Maize Glume cDNAs Library cDNA cloneza59b12 5', mRNA sequence *	316	$2e^{-176}$	388/412 (94%)	*Zea mays*
MRSR129	DY742761	Unknow sequence				
MRSR97	DY742762	Unknow sequence				
MRSR86	DY742763	Unknow sequence				

Table 3. Conitnues.

MRSR69	DY742764	Unknow sequence				
MRSR89	DY742765	AU197207 Rice callus cDNA clone C62241, mRNA sequence *	593	$2e^{-166}$	347/363 (95%)	*Triphysaria versicolor*
MRSR80	DY742766	Za61g03.g1 Maize Glume cDNAs Library cDNA clone za61g03 5', mRNA sequence *	170	$7e^{-90}$	177/178 (99%)	*Oryza sativa*
MRSR127	DY742767	C19715 Rice panicle at ripening stage cDNA clone E10827_1A, mRNA sequence *	170	$7e^{-90}$	177/178 (99%)	*Oryza sativa*
MRSR79	DY742768	Ubiquitin carboxyl-terminal hydrolase 24	201	$8e^{-51}$	94/94 (100%)	*Homo sapiens*

Identity represents the percent homology estimates of equine cDNA fragments with nucleotide sequences in GenBank. * Means of homology results from Genbank [NR or expressed sequence tag (EST) databases].

known. An amount of research showed that the response of plant to pathogen has inner relativities with that to other stress. Especially, those down-stream anti-stress reactions might be identical. In order to further understand the EST-involved metabolism reaction, we assorted the 35 known genes according to their putative functions (Figure 2). Interestingly, most of the genes were up-regulated in response to *R. solani* infection which were identified to be involved in disease-resistance/defense (8 ESTs, 23%), signal transduction (7 ESTs, 20%), transcription regulation (6 ESTs, 17%), and secondary metabolism (5 ESTs, 14%). Also, other up-regulation genes are involved in primary metabolism (6%), protein modification and storage (6%), protein transportation (6%), energy metabolism (6%), and cell structure. All these results suggest that in response to *R. solani* AG1-IA infection, there were totally 9 pathways to be involved and multiple genes were regulated either with up-regulation or down-regulation. It also proved that disease-resistance mechanism of plant is a complex process, which might involve the cooperation of multi-pathways of metabolism.

Semi-quantitative RT-PCR analysis of 15 selected ESTs

To confirm the hybridization results of the differential screening, expression patterns of 15 selected genes (such as: senescence-associated protein, receptor-associated protein and ferredoxin) by semi-quantitative RT-PCR. The results of RT-PCR assay are summarized in Figure 3. The forward library was enriched with up-regulated genes induced by *R. solani* AG1-IA, such senescence-associated protein, receptor-associated protein and ferredoxin (Figure 3A). In general, expression of those selected genes was clearly up-regulated in 24 h after pathogen inoculation. Those genes were likely associated with BLSB resistance; while the reverse library was observed to be enriched with down-regulated genes induced by BLSB. It seems that genes have down-regulated in several physiological and metabolic processes in maize in response to the *R. solani* infection. The results of RT-PCR show down-regulated expression of the six selected genes were obvious after 12 h pathogen infection (Figure 3B). However, different gene exhibited different response during infection.

DISCUSSION

In this study, we constructed two subtracted cDNA libraries from the maize inbred line R15 after inoculating by pathogen AG1-IA. A number of ESTs presented in this work are homologous to pathogenesis-related protein, senescence-related protein, receptor-associated protein etc.; furthermore, expression profile analysis of 15 important genes from SSH libraries showed up-regulation or down-regulation after inoculation. Selected genes possibly related to AG1-IA infection will be discussed in the following sections.

Gene involved in signal transduction

Based on the SSH screening, 20% of screened genes were involved in stress signaling, such as, signal perception (receptor-associated protein and receptor kinases) and signal transmission (G proteins, protein kinases, phosphatases protein).

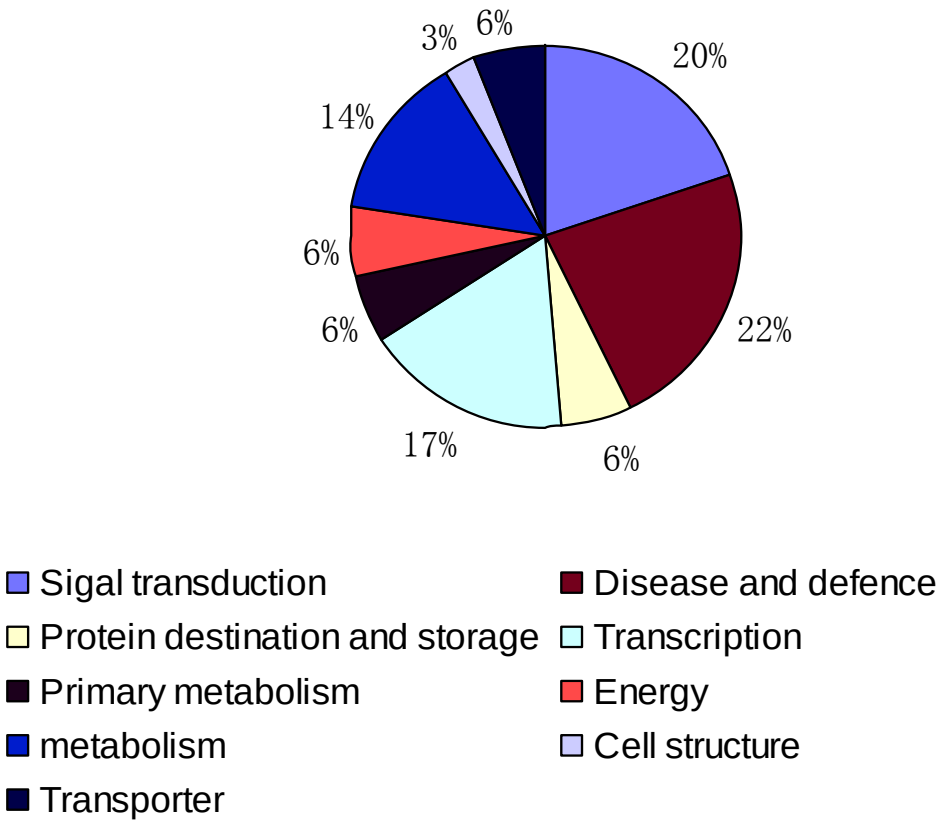

Figure 2. Functional distribution of the *EST sequences* derived from the SSH on the basis of their homology.

Overall, signal conductive pathway plays an important role in the disease resistance system. Six clones of EST sequence associated genes obtained in this research related to the signal conduction induced by BLSB. These genes encoded mitogen-activated protein kinase, dual-specificity protein, phosphatidylinositol-4-phosphate 5-kinase, Ser/Thr receptor-like kinase, receptor-associated protein, and G protein-coupled receptor. Among them three are receptor associated proteins which are presumed to relate to signal recognition. Another three are protein kinases involved in many signal conductions, which revealed many signal recognition and conduction are involved in disease resistance system.

Gene involved in defense and stress reactions

In maize plants infected by AG1-IA, expression of many genes known to be involved in defense and stress reactions might be induced. In this study, this category include genes encoding pathogenesis-related protein, senescence - related protein. These results are consistent with data obtained from other studies on plant-pathogen interaction (Die et al., 2007; Li et al., 2009). Although, host responses maybe different, it is likely that different plants have the same defense systems. Pathogenesis-related proteins are proteins which are involved in plant defense against pathogen, such as fungi. Furthermore, the past study on pathogenesis-related protein revealed that PRs was related to biotic and abiotic stress (Mitsuhara et al., 2008; Niu et al., 2007; Sarowar et al., 2005).

Gene involved in transcriptional regulation

Transcription factors play key roles in gene regulation during various developmental stages and cellular responses to environmental factors (Liu et al., 2008). In this study, we identified both zipper (bZIP) transcription factors and Plant Homeo Domain (PHD) finger transcription factors in the stress cDNA collection. The bZIP transcription factors can be activated by light, UV radiation, pathogens, elicitors, wounding, abscisic acid (ABA) and salicylic acid (SA) (Despres et al., 2000; Kim et al., 2001). Certain Really Interesting New Gene (RING) finger proteins are rapidly induced by elicitors of maize and may be involved in the rapid degradation of regulatory proteins during early stages of pathogen attack (Salinas-Mondragón et al., 1999). We have also isolated two regulated domains in the stress cDNA collection, such as KH domain, anti-termination NusB domain. They would be helpful in implementation of the plant defense response (Todd et al., 2004), and play the important roles

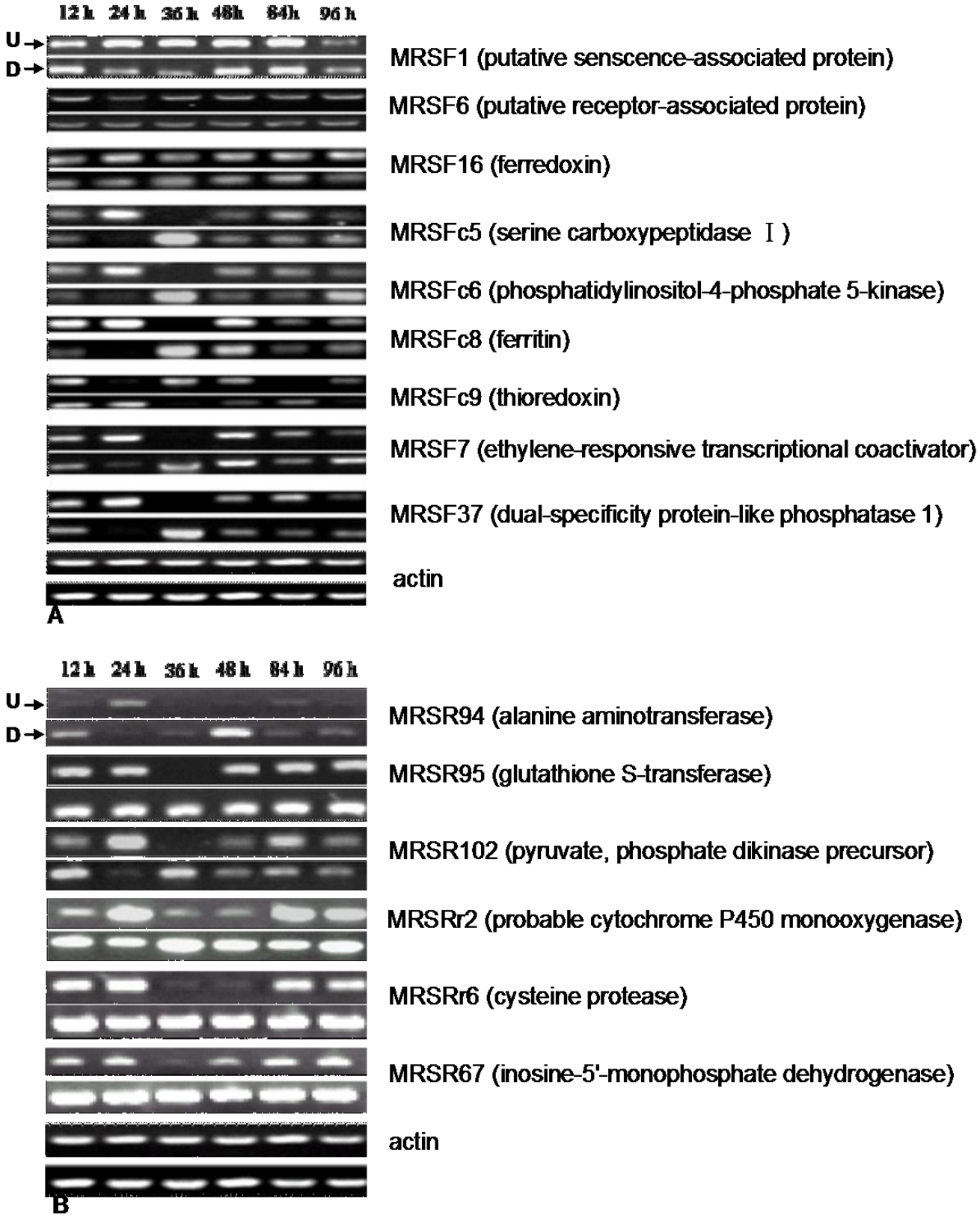

Figure 3. RT-PCR analysis of mRNA expression with: **A**: Expression clones from SF, **B**: Expression clones from SR.. Total RNAs were extracted from maize leaves at 12, 24, 48, 84 and 96 h with or without AG1-IA inoculation. Expression of the actin gene is chosen as control. For each clone there were two gel electrophoresis ladders, the upper ladder was expression result from inoculated samples' cDNAs, the down ladder was from uninoculated cDNAs.

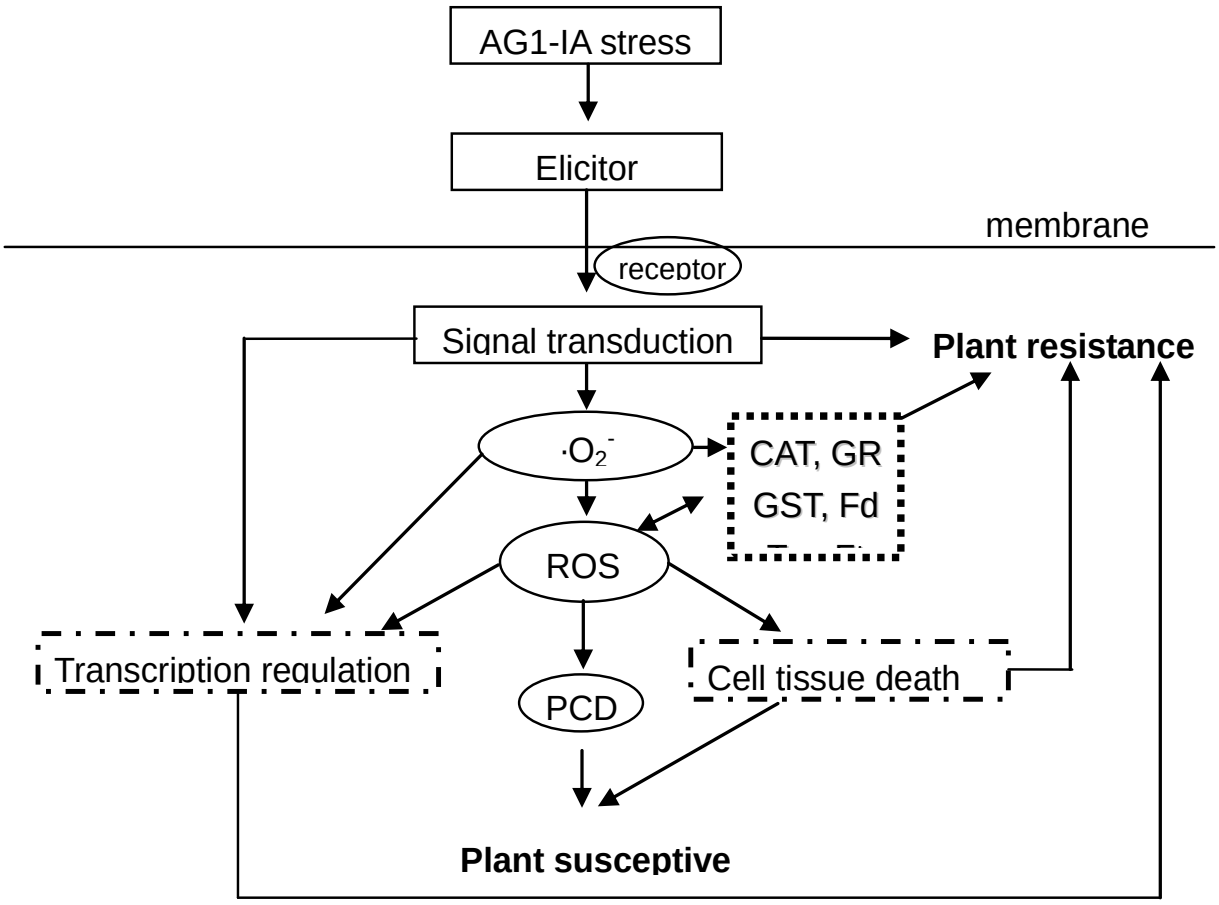

Figure 4. Putative disease-protection mechanistic model for maize induced by *R. solani* AG1-IA. *CAT*, Catalase; *GR*, glutathione reductase; *GST,* glutathione-S-transferase; *Fd,* ferredoxin; *Trx,* thioredoxin; *Ft,* ferritin; *ROS*, reactive oxygen species; *PCD*, programmed cell death.

against pathogenic infection by *R. solani*.

Gene involved in protein modification

Genes of maize plant involved in protein modification after inoculation with AG1-IA were identified in this study. Example of this category is ubiquitin carboxyl-terminal hydrolase, triose phosphate/phosphate translocator and serine carboxypeptidase. Among these proteins, serine carboxypeptidases (SCPs) are proteins belonging to the hydrolase family (Feng et al., 2006) and share characteristic structural features. It has been shown that SCPs have broader functions including response to wound and environmental stress (Moura et al., 2001; Cercos et al., 2003). This study revealed that SCPs may play a crucial role in protecting plants against stress by fungi.

In addition, this study obtained ESTs which involved in physiological metabolism; energy processes transporting, storing and degrading proteins. Based on the discussion above, it is obvious that the resistance mechanism of the pathogen AG1-IA was complex which involves signal transduction, transcription, resistance reactive oxygen species (ROS) and eliminates defense mechanism. This work represents to our knowledge, the first attempt to investigate gene expression in maize plants subjected to pathogen AG1-IA at the molecular level. Further work is in progress in our laboratory in order to study the molecular function of these genes.

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(2006AA100103).

Abbreviations

ABA, Abscisic acid; **AG1-IA**, anastomosis group IA; **BLSB**, banded leaf and sheath blight; **bZIP**, basic zipper; **CAT**, catalase; **cDNAs**, complementary DNA; **DIG**, digoxigenin; **EST**, expressed sequence tag; **Fd**, ferredoxin; **Ft**, ferritin; **GCA**, general combining ability; **GR**, glutathione reductase; **GST**, glutathione-S-transferase; **IPTG**, isopropyl-b-D-thiogalactopyranoside; **ivr2**, vacuolar invertase 2; **LB**, Luria–Bertani; **MA**, malic acid; **MDH**, malate dehydrogenase; **PCD**, programmed cell death; **PCR**, polymerase chain reaction; **PDA**, potato dextrose agar; **PEP**, phosphoenolpyruvate; **PPDK**, pyruvate phosphate-dikinase; **QTL**, quantitative trait loci; **ROS**, reactive oxygen species; **PR**, pathogenesis-related; **RT-PCR**, reverse transcription polymerase chain reaction; **SA**, salicylic acid; **SDS**, sodium dodecyl sulfate; **SNF1**, sucrose non-fermenting-1-related protein; **Snrk1**, sucrose non-fermenting-1-related protein kinase 1; **Trx**, thioredoxin; **X-gal**, 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside; **SSH,** suppression subtractive hybridization.

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