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RESEARCH ARTICLE

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Early stage SSH library construction of wheat near isogenic line *TcLr19* under the stress of *Puccinia recondita* f. sp. *tritici*

Abstract cDNA library of wheat near isogenic line *TcLr19* was constructed with suppression subtractive hybridization (SSH) 16 h after inoculation with race 366 of *Puccinia recondita* f. sp. *tritici.* This SSH library included 1337 positive clones and the insert sizes ranged from 200 bp to 600 bp, 237 clones were selected according to the result of reverse northern blotting, and then 35 ESTs were sequenced. EST similarity analysis was finished by comparing sequences with BLAST software in the non-redundant database of GenBank. The results showed that they were related to many biological processes including signal transduction, transcription regulation and hypersensitive response.

Keywords *TcLr19*, leaf rust, suppression subtractive hybridization (SSH), early stage, ESTs

1 Introduction

Wheat leaf rust caused by an obligate biotrophic fungus, *Puccinia recondita* f. sp. *tritici*, is an economically important wheat disease in the world. Many aspects of this disease, such as disease epidemic, fungus morphology, pathogenicity diversion, eruption rules, resistance identification, screening the molecular mark and chemical defense methods, have been carried out (Xu et al., 2005). In contrast, studies at the molecular level are limited to some resistance gene cloning or protein function identification because of lacking of corresponding skills and technologies, and few results of system research on host resistance to leaf rust fungus are reported although there are many progresses in cytology and histological host-pathogen recognition. The suppression subtractive hybridization (SSH) technology can solve the problem. It has been widely used in the signal transduction and functional gene expression of tomato, maize and lucerne (Mathews et al., 2003; Bassani et al., 2004; Bouton et al., 2005), but the global transcriptional profiling in response to leaf rust fungus has not been reported in wheat.

Upon contact with an epidermal cell, a biotrophic conidiospore germinates to form an appressorium that breaches the stoma. Subsequently, an infection structure, termed a haustorium, is formed within the mesophyll cell through which a dynamic exchange of signals and metabolites occurs between pathogen and host cell, which means the formation of parasitism relationship between host and pathogen. Moreover, this is the important stage that the *R* gene recognizes the *Avr* gene and activates the resistance response downstream (Heintz and Blaich, 1990; Rumbolz et al., 2000).

To isolate differentially expressed genes associated with incompatible combination, choices of cultivars and time point after inoculation with pathogen are major considerations. Huang et al. (2003) showed, using differential interference microscope and electron microscope, 16 hours post inoculation (hpi) is a key point in studying resistance response because most part of the infected fungus hyphae can develop into haustoria. And most importantly, global transcriptional profiling and signal transduction in response to leaf rust have not been reported in the world. Our aim was to determine molecular events associated with the leaf rust-responsive gene expression patterns in wheat, by isolating cDNA sequences that are induced in a highly resistant cultivar near isogenic line (NIL) *Lr19* after infection with leaf rust race 366, which could avoid the disadvantages of complex genetic background of other materials. It would be more significant to learn the wheat-leaf rust resistant mechanism system and signal transduction, and identification of relative resistant genes expressed at the early infection stage.

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2 Materials and methods

2.1 Materials and treatment

Wheat (*Triticum aestivum*) cultivars NIL *Lr19* and leaf rust race 366 were used in the present study. The wheat seeds and fungal spores were provided by the College of Life Science, Agricultural University of Hebei. The incompatible combination consisted of wheat cultivar NIL *Lr19* plus race 366.

According to Qi et al. (2008), wheat seeds were planted in organic soil in 10-cm pots and grown under greenhouse conditions at 24°C with a 16-h-day and 8-h-night cycle. Seven-day-old seedlings were used for infection with leaf rust fungus. Fresh rust urediospores were suspended in water to a final concentration of 3×10^5 spores·mL^{-1}. Then, the plants were inoculated with the spore suspension by brushing it on the surface of the first leaves of seedlings with a paintbrush. Control plants were treated with water only, referred to as mock inoculation. Then water was sprayed on the surface of the first leaves with a spray gun to simulate high humidity conditions. Finally, inoculated seedlings were kept in a humid chamber for 16 h in the dark at 25°C to allow infection to occur, after 16 h, the seedlings were returned to the greenhouse with the conditions as described above. The mock and treated plant leaves were sampled at 16 hpi, and the samples were quickly frozen in liquid nitrogen, and then stored at −80°C.

2.2 RNA extraction

Total RNAs were extracted from the samples harvested above, and the extraction method using TIANGEN RNAPlant and the isolation of poly (A) RNA using oligotex were operated according to the manufacturer's protocol (Qiagen, Germany). The quality and quantity of RNA and mRNA were measured by nucleonic acid and protein detection instrument. As the initial material, mRNA was cadenced to 0.5 μg·μL^{-1}.

2.3 Generation of a subtracted library by SSH

Suppression subtractive hybridization was performed between treated sample (Tester) and mock (Driver) using the PCR-selected cDNA subtractive hybridization kit according to the manufacturer's recommendations (Clontech). The plants inoculated with fungus were used as testers and the mock plants were used as drivers. The tester and driver cDNA of the leaves were prepared from 2 μg mRNA extracted from the treated and the control samples. The tester cDNA was digested with *Rsa* at 37°C for 1.5 h and then ligated to adaptors 1 and 2 in separate reactions at 16°C overnight. Then driver cDNA was added to each of the test samples, which were subsequently re-suspended in the hybridization buffer. After heat-denaturizing, the mixture was allowed to anneal at 68°C for 8 h. Then, the two samples from the first hybridization were mixed together and the freshly denatured driver cDNA was added to the sample followed by incubation at 68°C overnight for the second hybridization. PCR amplification with two different nested primers was conducted to amplify differentially expressed cDNAs. After evaluation of the subtraction efficiency, the subtracted library cDNA was cloned directly into pMD19 vector (TaKaRa, Japan), and transformed into *E. coli* strain DH5α cells by heat shock. The library was plated onto 22 cm agar plates containing ampicillin (100 mg·mL^{-1}), IPTG (100 mol·L^{-1}) and X-Gal (50 mg·mL^{-1}). Plates were incubated at 37°C until small colonies were visible, then incubated further at 4°C until blue/white staining could be clearly distinguished. The recombinant white clones were randomly picked to construct the subtracted cDNA library.

2.4 Insert fragment detection

The primers included in the kit (Nested PCR primer1 5′-TCGAGCGGCCGCCCGGGCAGGT-3′ and Nested PCR primer2R 5′-AGCGTGGTCGCGGCCGAGGT-3′) were used to amplify the insert fragments in pMD-19 vector. Individual recombinant clones were picked and used to inoculate 1.5 mL sterile eppendorf tubes containing LB medium and ampicillin at 100 mg·mL^{-1}. After incubation of bacteria on a gyratory shaker for 4 h at 37°C, samples of 1 μL bacterial lysate were used to amplify cloned inserts in 20 μL reactions using standard PCR buffer, 2 μL 10×PCR buffer, 1.5 μL 2.5 mmol·L^{-1} dNTP, 1 μL bacterial liquid, 1 μL 1 μmol·L^{-1} primers, 0.1 μL 5 U·μL^{-1} *Taq* polymerase. Cycling parameters were as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 2 min and 72°C elongated for 10 min. Five μL PCR product was loaded in 1% agarose gel, dyed with EB. Pictures were taken to identify the fragment lengths.

2.5 Reverse northern high density blots and screening

The mixture of phenol:chloroform:isoamyl alcohol (25:24:1) was added into the amplified product mentioned above to remove impurity, then 1/10V NH_4Ac (3 mol·L^{-1}) and 2 V dehydrated alcohol were added to the upper solution to get precipitation which was resolved by 15 μL ddH_2O finally. The product was denatured in boiling water for 10 min after being detected by agarose gel electrophoresis; subsequently 1 μL was loaded onto a nylon membrane, which was baked for 30 min at 120°C to fix DNA. The filters were hybridized under stringent conditions with equivalent amount probes made by approximately equal DIG-labeled double-stranded cDNA, derived from the driver and tester mRNA respectively. Next, all hybridization steps followed the introduction of DIG-High Prime DNA Labeling and Detection Starter Kit I (ROCHE).

2.6 Sequencing and analysis of EST fragments

Selected EST fragments were sequenced in Shanghai Sangon Biological Engineering Technology & Services Co., Ltd, and the results were compared and analyzed with the data in GenBank by BLASTx and BLASTn.

3 Results

3.1 RNA detection

The total RNA of tester and driver were electrophoresized in 1× TAE to test the extraction quality (Fig. 1). There are 5 bands in each line: 28S, 23S, 18S, 16S and an obscure 5S rRNA. 28S and 18S rRNA were cytoplasmic ribosome RNA, 23S and 16S rRNA were ribosome RNA in chloroplast. The 28S rRNA had equal brightness with 18S rRNA in total RNA of two samples, which means the RNA had not degraded. Meanwhile, the concentration of the RNA was determined by the absorption value of A260, and the results showed that A260/280 value was between 1.7–2.1. It suggested that RNA sample may be polluted by protein or other organic solvent if the A260/280 value is lower than 1.7. The A260/280 value of the total RNA indicated the purity that can satisfy the requirement mentioned above.

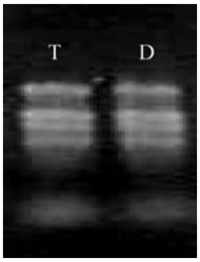

Fig. 1 Integrity detection of total RNA of wheat leaves
Note: T and D represent tester and driver, respectively.

3.2 dscDNA synthesis

Six μL synthesized dscDNA was analyzed by 1.2% TAE agarose gel electrophoresis, with the band smear over 200–5000 bp (Fig. 2) which satisfied the requirement of SSH library construction.

3.3 Evaluation of subtraction efficiency

Subtraction efficiency determined the success or failure of the library construction, only after the abundant constitutive expressed genes were removed from the high-quality SSH, the probability of gaining rare genes would be elevated greatly. The wheat actin gene, as a report gene, was amplified from the subtracted and nonsubtracted dscDNA. The product of the subtracted samples after 35 cycles was as bright as that nonsubtracted one after 35 cycles (Fig. 3). It meant a decrease in the actin abundance of the subtracted sample.

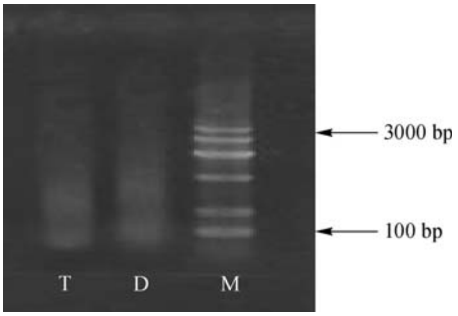

Fig. 2 dscDNA synthesis
Note: T means tester, D means driver, and M means marker.

Fig. 3 Reduction of actin abundance by PCR-select subtraction
Note: 1–4 mean the actin product of the subtracted samples after 20, 25, 30 and 35 cycles, respectively; 5–8 represent the actin product of the nonsubtracted samples after 20, 25, 30 and 35 cycles, respectively.

3.4 Inserts identification

Selected positive clones were cultured in LB 5 mL liquid medium under 37°C overnight, and then used as template in colony PCR. The result of agarose gel electrophoresis showed that the insert lengths ranged differently from 150 to 1000 bp, with the most between 300–500 bp (Fig. 4).

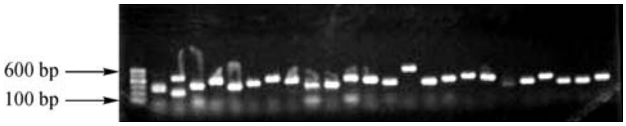

Fig. 4 Insert fragment detection

3.5 Reverse northern blotting

The products of colony PCR were loaded on two nylon membranes respectively to test positive points (Fig. 5). cDNA of both tester and driver were used as probes to hydride. There were 3 conditions in the membranes: (1) the point color on the tester membrane was significantly darker than that on the driver's; (2) the points were on tester membranes but not on driver membranes; (3) the points were of the same color on both membranes. One hundred and ninety-two colonies corresponded with (1), and 45 points corresponded with (2). Thirty-five different points were selected randomly to sequence.

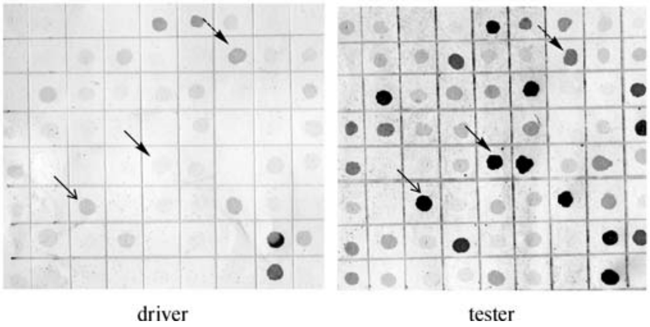

Fig. 5 Reverse northern blotting

Note: → represents the point color on tester membrane that was significantly darker than that on driver's;
⟶ represents the points on tester membranes but not on driver membrane;
- - → represents the points in the same color on both membranes.

3.6 Sequence analysis of positive colons

The sequenced ESTs were blasted for sequence homology with BLASTn and BLASTx soft on GenBank, 7 ESTs were not hit, and they may be new genes. The other 28 ESTs showed the known homologization sequences. Functional classification was carried out according to Bevan standard of plant genes (Bevan et al., 1998), the result showed that of the 28 ESTs, glutathione-S-transferase, Cytochrome P450 and S-adenosylmethionine decarboxylase were involved in plant resistance and defenses (showed randomly in Table1). All of the homologous sequences of 28 ESTs could be found in the dbEST library of GenBank, and the homologous sequences came from libraries of biotic and abiotic stress conditions such as pathogen, low temperature, salt, oxygen and SA inducing, etc. Some ESTs corresponded with those induced by many stress conditions.

4 Discussion

A basal disease resistance is generally induced during the initial interaction between a host and a virulent pathogen as

Table 1 Similarity analysis (BLASTx) of ESTs with the function identified genes in GenBank

accession	length/bp	putative identification	*E* value
SL1	288	ADP-ribosylation factor (*Triticum aestivum*)	2.00E-176
SL2	504	chloroplast psbA gene for herbicide-binding protein D1 (*Secale cereale*)	1.00E-178
SL3	374	vacuolar ATP synthase subunit E (VATE) (*Triticum aestivum*)	1.00E-81
SL10	681	partial CA4 gene for P-type ATPase (*Hordeum vulgare*)	1.00E-90
SL15	457	NADP-ME2 for NADP dependent malic enzyme (*Oryza sativa*)	3.00E-189
SL16	425	Cytochrome P450 (*Triticum aestivum*)	1.00E-140
SL18	787	glycine max plamsma membrane-associated AAA-ATPase	5.00E-174
SL21	319	multifunctional protein (*Oryza sativa*)	1.00E-82
SL25	404	MBD2 (*Triticum aestivum*)	7.00E-175
SL27	179	calcium-dependent protein kinase 3-like (CDPK3) (*Triticum aestivum*)	3.00E-122
SL30	130	metallothionein-like protein (Wali1) (*Triticum aestivum*)	3.00E-49
SL37	607	S-adenosylmethionine decarboxylase (*Triticum monococcum*)	0
SL38	419	gstA2 (*Triticum aestivum*)	0
SL39	158	glutamine-dependent asparagine synthetase (ASN1) (*Triticum aestivum*)	2.00E-66
SL42	268	putative adenine phosphoribosyl transferase (*Triticum aestivum*)	3.00E-117
SL48	323	4-hydroxyphenylpyruvate dioxygenase (*Triticum aestivum*)	8.00E-109
SL50	331	arabinoxylan arabinofuranohydrolase isoenzyme AXAH-II (*Hordeum vulgare*)	4.00E-157
SL67	322	putative E2 SUMO conjugating enzyme (*Triticum turgidum*)	6.00E-110
SL60	257	glutathione S-transferase (*Triticum aestivum*)	1.00E-90
SL69	363	vacuolar proton-inorganic pyrophosphatase (*Triticum aestivum*)	3.00E-173

a result of interactions among pathogen-associated molecular pattern-triggered immunity, effector-triggered susceptibility, and weak effector-triggered immunity (Jones and Dangl, 2006). As a result of immunity, hypersensitive response (HR) is an important form of plant defense reaction. And some genes involved in the early stage HR have been sequenced recently (Yammanoto and Sasaki, 1997). Caldo et al. (2006) reported that some grapevine defense genes induced by powdery mildew had a similar express tendency in susceptible and resistant combination at the early stage. The expression of these genes declined to lower levels in the compatible interaction at 24 hpi with fungal conidiospores. These results suggest that the fungus may interfere with signaling events involved in the host's signal transduction and substance metabolism process (Hahn and Mendgen, 2001). And 13% sequences induced in a late-blight-resistant potato cultivar undergoing the hypersensitive response 24 hpi were previously characterized as either defense-, stress-, or senescence-associated (Birch et al., 1999). cDNA library of wheat near isogenic line *TcLr19* was constructed using suppression subtractive hybridization (SSH) at 16 hpi under the stress of *Puccinia recondita* f. sp. *tritici*, and some wheat defense genes involved in the early stage resistance were found from preliminary screening.

It has been proven that, cytochrome P450 and glutathione-S-transferase, of the ESTs obtained from this experiment, played an important role in SAR and HR. The identification of these defense response enzymes at the time point of 16 hpi proved that they were already expressed at a high level at the initial disease resistance. Meanwhile, the signals starting the defense response were already transformed before the leaf rust colonization and haustorium formation. This is consistent with the results of grapevine-, barley-, or wheat-PM systems in which the majority of conidia penetrated host cells and began to form functional haustorium at 16 hpi (Panstruga and Schulze, 2002). These new discoveries suggested that systemic researches on the signaling and the expression of resistance gene in defense response of wheat leaf rust should be done in future experiments.

Programmed cell death (PCD) occurs in response to pathogen attack, such as leaf rust fungus to wheat, and these inducible PCD forms called hypersensitive response (HR) are intensively studied due to their experimental tractability. Some genes involved in signaling and transcript regulation in the ESTs were acquired in our work. For example, ADP-ribosylation factor (ARF) is a GTP-binding protein, which is a member of ARF subfamily. One report about this factor suggested that ARF plays an important role in signaling transduction (Hou et al, 2007), but the exact function is still not well understood. However, in the report of Ma and Jiang (2005), ARF has an important role in the apoptosis of the tumor cell. Since the small ADP ribosylation factor (Arf) GTP-binding proteins are major regulators of vesicle biogenesis in intracellular traffic, they may be helpful for changing host cell permeability to improve wheat resistance to leaf rust. V-ATPase plays an important role in plant resistance against abiotic stress. The activity of V-ATPase of *Mesembryanthemum crystallinum* under NaCl stress was significantly raised (Smart et al., 1998) and the research results of cotton (*Gossypium hirsuturm* L.) vacuolar revealed that over expression of H^+-ATPase was capable of promoting fission yeast cell tolerance to high NaCl and high pH stresses (Xiao et al., 2008). There was also some evidence that the plasma membrane ATPase specifically expressed in broad bean rust involved the pathogens' absorption of nutrients from the host cell (Hahn et al., 1997). NADP-ME (Ec1.1.1. 40) is a kind of oxidation decarboxylase which is widespread in animals, plants, and prokaryotes. It catalyzes malic acid into pyruvate, CO_2 and NADPH. NADPH is involved in many metabolic processes (Maurio et al., 2001). Recent studies have also confirmed that they may also play an important role in plant defensive response and fruit maturation. But more researches are to discover how these genes discussed above affect wheat resistance to leaf rust.

SSH technology is used in many aspects of botany. According to the instruction in the SSH kit, many up-regulated positive colonies were selected while down-regulated ones were lost, and they may be important to the plant's entire global regulation system to pathogens. Of course, there are many methods to resolve the problem. For example, reverse library construction by taking the original tester dscDNA as driver dscDNA, can select higher expression fragments in original driver sample which means the down-regulated expressions can be detected in the original tester. In addition, the combination of gene chip and SSH is a convenient method. Different genes expression can be resolved by different grades of fluorescence.

In conclusion, genes such as signal transduction, transcription factor and secondary metabolism which are up-regulated in different plant materials and different stress types were found in our experiment, the evidence indicated the consistency of plant basal defenses and common physiological functions to different environmental stress reported as a system antireversion force. In addition, the pathway is not well understood in signaling during plant defense. Therefore, further sequencing of clones in this library and more information up-stream of unidentified clones obtained by rapid amplification of 5′RACE will reveal pathways involved in resistance to leaf rust fungus and in the HR in general.

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