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Construction and Analysis of SSH-cDNA Library from Leaves of Susceptible Rubber Clone Resistant to Powdery Mildew Induced by BTH*

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ABSTRACT

To understand the mechanism of benzothiadiazole (BTH)-induced susceptible rubber clone resistance to powdery mildew on gene level, a differentially expressed cDNA library was constructed by suppression subtractive hybridization (SSH) with rubber Reyan 7-33-97 clone. The constructed cDNA library was high integrity through detection of the critical processes of SSH, such as efficiency of adaptor connection, subtraction and conversion, as well as the type of recombinant genes. The positive rate was 99% after identification with random 400 white spots. The size of the cDNA clone inserted fragments was various but most in 400 bp - 1000 bp. There were 23 cDNA sequences matching the function of energy and basic metabolism, signal transduction, membrane and transport, secondary metabolism and so on after detection of the 42 positive clone sequences selected randomly from the cDNA library and comparison on nucleic acid sequences in Genbank. 7 ESTs were logged in Genbank and accession numbers were GW873071 and GW874604-GW874610. The results implicated that BTH could effectively induced rubber tree resistance to powdery mildew through increasing expresses of defense-related genes in leaves of rubber tree susceptible clone. It should provide a new approach for rubber disease management.

Keywords: Benzothiadiazole; ESTs; *Hevea brasiliensis*; Induced Resistance; *Oidium heveae*; Suppression Subtractive Hybridization

1. Introduction

Rubber tree (*Hevea brasiliensis*) is a very important tropical industrial crop. Powdery mildew disease caused by *Oidium heveae* is one of the most important leaf diseases and impacts severely to the growth and latex production of rubber trees [1]. To control this disease, rubber resistance clone breeding and chemical control were employed generally [2]. Recently, induced rubber resistance to powdery mildew by oligosaccharin [3] and BTH (benzothiadiazole-7-carbothioic acid S-methyl ester or acibenzolar-S-methyl) [4] and to anthracnose (*Colletotrichum gloeosporioides*) by BTH [5] was studied.

BTH, analogs of salicylic acid, is an excellent chemical inducer that can induce plant resistance to pathogens [6], insects [7], nematodes [8] and parasitic weeds [9]. The mechanism of BTH induced resistance is activation of the plant defense genes and expression resistance-related proteins or enzymes [10]. The defense-related genes of BTH induced diseases resistance to rice, wheat [11], cucumber [12], papaya [13], coffee [14], cocoa [15] and so on were identified and analysed. In rubber tree susceptible clone against the diseases, the peroxidase, phenylalanine aminolyase and β-1,3 glucanase are increasing significantly in leaves after BTH treatment [4,5]. No report has been found so far on BTH induced defense genes in rubber tree susceptible clones against the disease. To understand the mechanism of BTH induced rubber tree resistance to powdery mildew in molecular knowledge, a cDNA library of BTH-induced resistance to the disease of rubber tree was constructed through SSH (suppression

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subtractive hybridization) and the function of differentially expressed genes induced by BTH was analysed.

2. Materials and Methods

2.1. Plant Growth and Pathogen Collection

Rubber budding seedlings (Reyan 7-33-97 clone, susceptible to powdery mildew [16]) were provided by Rubber Research Institute of CATAS. The seedlings were grown in plastic bags with leaves bronze to light green stage and kept in plant growth chamber with temperature at 25˚C, humidity at 80% and 12 h/12 h at light/dark. Fresh conidia of powdery mildew (*Oidium heveae*) were collected in suspension from infected rubber tree leaves and adjusted to 5×10^4 spores/ml under a microscope.

2.2. BTH Treatment and the Pathogen Inoculation

The healthy leaves of rubber budding seedlings were sprayed uniformly with BTH (Bion 50%WG, Syngenta) at 250 mg a.i. l^{-1}. The spore suspension was sprayed on the leaves after 5 days of BTH spray. The leaves were collected after 4 days of inoculation. Blank control was the some clone and only inoculated with the pathogen. The leaves were stored at −70˚C till for total RNA extraction.

2.3. Total RNA Extraction and mRNA Purification

The BTH treatment and the pathogen inoculation were as the tester and the only pathogen inoculation was as the driver. The total RNAs of tester and driver leaves were extracted with the modified Bugos extract [100 mmol·l^{-1} Tris, 200 mmol·l^{-1} Nacl, 15 mmol·l^{-1} EDTA, 0.5% (W/V) SDS]. The total RNA was dissolved in DEPC-H_2O after treatment with DNase I (TaKaRa, Dalian, China) for 30 min at 37˚C. The extracted RNA was kept in cryopreservation for integrity examination with 1.2% agarose gel electrophoresis and amount and purity detection with UV at $A_{260/280}$. mRNAs of tester and driver from the RNAs were purified with Oligotex™-dT30 <Super> mRNA Purification Kit (TaKaRa, Dalian, China) and used for cDNA library construction.

2.4. Differentially Expressed cDNA Library Construction, Adaptor Ligation Detection and Transformation

The differentially expressed cDNA library of BTH induced resistance to the pathogen was constructed with PCR-Select™ cDNA Subtraction kit (TaKaRa, Dalian, China). The adaptor ligation efficiency before and after SSH was detected by primer of Actin gene [17] (a housekeeping gene of rubber, designed as ACTIN-F: 5'-CAGTGGTCGTACAACTGGTAT-3' and ACTIN-R: 5'-ATCCTCCAATCCAGACACTGT-3', synthesized by SBS Genetech, Beijing, China). Adaptor ligation 1 or 2 was used as a template for the tester cDNA. PCR amplification was conducted respectively with the primer Actin 3' as one side and Actin 5' or adaptor ligation 1 as another side. The purpose cDNA fragments 3 μl, pMD18-T Vector (TaKaRa, Dalian, China) 1 μl, Ligation Solution I 5 μl and ddH_2O 1 μl were mixed in microcentrifuge tube and slightly centrifuged, then transformed into *E. coli* DH5*α*. The droplets on the tube wall were put down on the bottom of test tube and overnight at 16˚C. 50 μl of competence was poured into 1.5 ml centrifuge tube with 10 μl ligation product. The tube was placed on ice bath for 20 min, shocked at 42˚C for 90 sec and immediately placed on the ice bath for 2 min again. The tube was shook at 37˚C for 45 min after addition of 700 μl LB medium.

2.5. cDNA Library Reorganization Rate Detection

The conversion products were coated on LB ampicillin plates with X-gal and IPTG and cultured at 37˚C under dark conditions for 12 - 16 h. The positive clones were identified after coloration at 4˚C when the colony size was suitable. Recombination rate of cDNA library was calculated.

2.6. Fragment Length Recognization and Recombinant Sequence

A 25 μl reaction system [10 × Buffer (Mg^{2+}) 2.5 μl, dNTP (2.5 M) 2 μl, rTaq (5 U/μl) (Sangon Biotech, Shanghai, China) 0.25 μl, primer 1 (20 pm) 1.5 μl, primer 2 (20 pm) 1.5 μl, template 0.5 μl, sterile water 16.75 μl] was prepared in 0.2ml PCR tube and immediately accessed to the following cycle after centrifugation: 4.5 min at 94˚C, 35 sec at 94˚C, 30 sec at 66˚C for 30 cycles; then 1.5 min at 72˚C and 5 min at 72˚C. The reactant of 5 μl was loaded on 1.2% agarose gel electrophoresis. The inserted fragments of positive clones identified by PCR were sequenced.

3. Results

3.1. The Total RNA Extraction

2 bright bands of total RNAs were appeared after agarose gel electrophoresis, corresponding to 28S and 18S rRNA with a ratio of intensity at 2:1 (**Figure 1**). The UV absorbance of the RNAs was at 1.89 - 2.03 (common value at 1.9 - 2.1). This indicated that the extracted total RNAs were high quality, and corresponding to build

library.

3.2. The Adaptor Ligation Efficiency

The connection efficiency of the adaptors 1 and 2 was more than 25% (**Figure 2**). The efficiency met the requirement.

3.3. cDNA Library Subtractive Efficiency

There was appearance of lighter bands after the 18 cycles on the samples before subtraction and after the 28 cycles on the samples after subtraction (**Figure 3**). The more than 10 cycles meant that the most of expressed constitutive genes were effectively removed. The subtractive library was high quality.

3.4. Differentially Expressed cDNA Examination

A total of 18,350 clones were collected in cDNA library, thereinto, 15,180 clones was white spots. The positive rate was 99% after PCR identification with random 400 white spots. The recombination rate was 82.7%. The cDNA library met the general requirement.

The size of cDNA inserted fragment was different and most in 400 to 1000 bp after PCR amplification with the random differentially expressed cDNA clones. The constructed cDNA library insert size was consistent after electrophoresis (**Figure 4**).

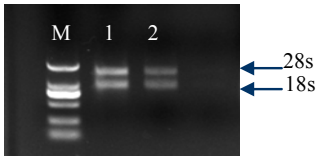

Figure 1. Total RNAs integrity from rubber tree leaves; Lane 1: tester, Lane 2: driver, Lane M: Marker DGL2000 (TakaRa).

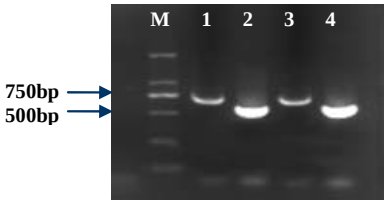

Figure 2. Efficiency of the adaptor ligation with Actin; Lane 1: tester1-1 as the template and the actin3' primer, PCR primer1; Lane 2: tester1-1 (adaptor1-ligated-cDNA fragment) as the template and the actin3', 5' primer; Lane 3: tester1-2 as the template and the actin 3' primer, PCR primer1; Lane 4: tester1-2(adaptor 2R ligated-cDNA fragments) as the template and the actin 3', 5' primer; Lane M: Marker DGL2000.

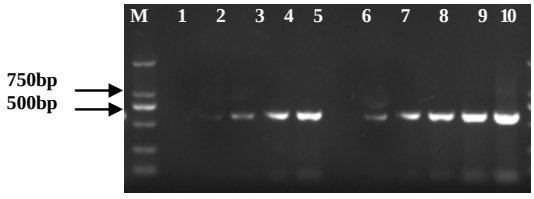

Figure 3. Evaluation of subtraction efficiency; Lanes 1-5: Subtracted samples after 18, 23, 28, 33 and 38 cycles of amplification; Lanes 6-10: Unsubtracted samples after 18, 23, 28, 33 and 38 cycles of amplification; Lane M: Marker DL2000.

3.5. The Sequence Analysis and Gene Functional Annotation

There were 23 cDNA sequences and 2 repeats known as function as energy and basic metabolism, signal transduction, membrane and transport, secondary metabolism and so on, 13 sequences unknown of function, 2 sequences no significant match and 2 clones no needed sequences after detection of the sequences of 42 positive clones randomly selected from the cDNA library and comparison on nucleic acid sequences in Genbank (**Table 1**). 7 ESTs were logged in Genbank and accession numbers were GW873071 and GW874604-GW874610. Parts of the sequences and their functions were attached in Appendix, including disease resistance and defense response gene—zinc finger protein, signal transduction protein—kinase and ion channels—membrane water channel protein.

4. Discussion

During the plant growth process, it would be attacked by many pathogen microorganisms, appearing as disease susceptible or resistant. The process of disease resistant of plant is an outcome by a series of signal recognition, signal transduction, defense reaction activation and the coordinate action of defense gene expression. SSH technology has obvious advantage in gene expression of concentration difference. Therefore, it has been used broadly for understanding the gene differences of plant

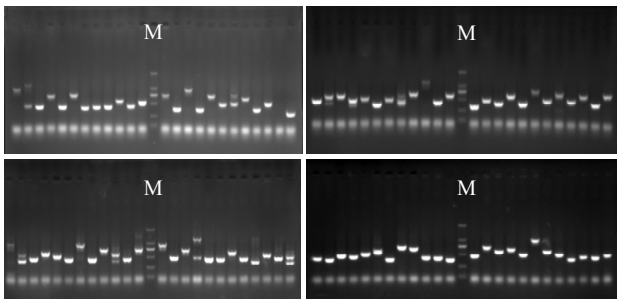

Figure 4. Identification of inserted fragments in plasmids of subtractive cDNA library by PCR; lane M: Marker DGL-2000, Others: inserted fragments in subtractive cDNA library.

Table 1. Sequence alignment and annotation of gene function.

Sequence ID	Query length (bp)	Highest homology (organism)	E-value	Gene function
XM_002521974	250	Ricinus communis zinc finger protein, putative, mRNA	2e−60	Relative gene in expression and regulation
XM_002279330	689	Vitis vinifera aquaporin PIP2;2 (PIP2;2), mRNA	2e−180	Relative genes in translocator
AJ534339	151	Agaricus bisporus partial mRNA for putative inorganic phosphate transporter (ipt gene), clone pm127	5e−04	
XM_002512535	449	Ricinus communis Aspartic proteinase precursor, putative, mRNA	5e−115	Relative genes in protein synthesis
XM_002516652	366	Ricinus communis Pectinesterase PPE8B precursor, putative, mRNA	7e−99	
XM_002525601	622	Ricinus communis cyclophilin, putative, mRNA	1e−179	
EU002153	500	Manihot esculenta 26S ribosomal RNA gene, partial sequence	0.0	Relative genes in cellular structure
XM_002533297	244	Ricinus communis 60S ribosomal protein L10, putative, mRNA	2e−66	
EF135610	337	Endospermum moluccanum 26S ribosomal RNA gene, partial sequence.	1e−146	
EF135606	617	Bischofia javanica 26S ribosomal RNA gene, partial sequence	0.0	
CU227494.1	459	Populus EST from severe drought-stressed leaves	4e−167	Relative genes in disease resistance and defense
CU232928	278	Populus EST from severe drought-stressed opposite wood	1e−30	
XM_002516535	284	Ricinus communis Dehydration-responsive protein RD22 precursor	2e−50	
XM_002518446	298	Ricinus communis multidrug resistance pump, putative, mRNA	3e−46	
XM_002338984	459	Populus trichocarpa histone H4 (HFO913), mRNA.	3e−60	Relative genes in signal transduction
XM_002522100	287	Ricinus communis pyridoxine kinase, putative, mRNA	1e−89	
AY741496	313	Juanulloa aurantiaca SAMT (SAMT) mRNA, partial cds	2e−11	
XM_002514771	622	Ricinus communis calreticulin, putative, mRNA	4e−165	
XM_002521856	303	Ricinus communis aldo-keto reductase, putative, mRNA	4e−69	Relative genes in energy metabolism
XM_002515921	381	Ricinus communis malic enzyme, putative, mRNA	7e−126	
XM_002514184	515	Ricinus communis malic enzyme, putative, mRNA	2e−177	
XM_002524020	473	Ricinus communis ATP-dependent RNA helicase, putative, mRNA	4e−134	Relative gene in RNA helicases
XM_002511797	593	Ricinus communis DNA binding protein, putative, mRNA	0.0	Relative gene in DNA bindingprotein
XM_002320749	579	Populus trichocarpa predicted protein, mRNA	2e−178	unknown
XM_002309420	215	Populus trichocarpa predicted protein, mRNA	1e-43	
XM_002308330	311	Populus trichocarpa predicted protein, mRNA	2e−61	
XM_002300289	551	Populus trichocarpa predicted protein, mRNA	2e−42	
XM_002303489	297	Populus trichocarpa predicted protein, mRNA	3e−46	
XM_002321119	458	Populus trichocarpa predicted protein, mRNA	2e−67	
XM_002323527	526	Populus trichocarpa predicted protein, mRNA	3e−165	
AY090953	929	Arabidopsis thaliana unknown protein (At2g06530) mRNA, complete	5e−176	
HBU40402	458	Hevea brasiliensis hydroxynitrile lyase (hnl) mRNA, complete cds	0.0	
XM_002515525	669	Ricinus communis conserved hypothetical protein, mRNA	0.0	

Continued

XM_002529738	659	Ricinus communis hypothetical protein, mRNA	2e−93
AF516352	267	Hevea brasiliensis eukaryotic translation initiation factor 5A isoform III (eIF-5A) mRNA, complete cds	1e−47
XM_002509641	423	Ricinus communis NAD dependent epimerase/dehydratase, putative, mRNA	6e−137
Unknown	365	No significant similarity found	
Unknown	303	No significant similarity found	

disease resistance [10] and environment adversity stress [18]. The present study we constricted BTH inducing positive and negative SSH-cDNA library of the rubber blade. There were 13 sequences that their function was unknown. It would possibly imply some important genes related to the disease resistant. There were 2 sequences that had no obvious match. It might be represented novel unknown genes or high variable cDNA noncoding region sequences. All these are valuable to be further study.

In the present study, constructed SSH-cDNA library has coded zinc finger protein gene occurring (ESTs accession numbers: GW874607). Zinc finger protein recognizes transcription factor structure of special alkaline residue sequence. The function of lant C_2H_2 zinc finger protein is possible related to plant bio-organism or biological stress. The C_3H group of RAR1 and HvRar1 zinc finger protein genes from *Arabidopsis thaliana* has been proved to be located in the relative tight squeezed location of disease resistance [19-21]. In constructed SSH-cDNA library, it also has water channel protein gene (ESTs accession numbers: GW874608) appearing. Water channel protein belongs to membrane intrinsic protein. Its expression is influenced by many hormones (ABA, GA) and environment factors (blue light, water stress and pathological infection). Yamada *et al.* (1995) [22] found that under high salt stress, level of mRNA expressed water channel protein in ice plant declines rapidly. The mRNA level would gradually recover to its original level or much higher level as along with accumulation of osmosis active substances (such as sucrose and polyamine) in the cell. So, it can enhance resistance after expression of inducing water channel protein [23]. In addition, we fund that inorganic phosphate transferring and light harvesting proteins were excited. It is sure after BTH induction, the transferring activity of the host is more active, the function of substances transferring is enhanced, even discover of the presence of inorganic phosphate transfering protein and light harvesting proteins are unable make sure their genes relating to disease resistant directly. In this study, the sequences encoding aspartic protease precursor, calreticulin and cyclophilin were appertaining. These proteins all have disease resistant role in animals and plant, as well as in human [24,25]. We presume these protein genes likely participate in the reaction of rubber resistant to powdery mildew. We also fund some genes (The factors causing stress include cold, salt, drought, and pathogenic bacteria) relating to biological and non-biological stress. For example, the genes of coded stress response protein are considered to play a role in coordination of resistant outside stress in plant.

In conclusion, BTH induces resistant to rubber powdery mildew disease relating to many ways including recognizing process of pathogen and host, translocating process of signal substances, starting various disease resistance pathways in host and final producing large amount of resistant-related substances against powdery mildew invasion and hypha extension. Our results demonstrated BTH could effectively induce resistant to rubber powdery mildew disease, it provides a new way for controlling such disease, and also provides an useful information for further study on the expression and signal transduction of rubber defense genes.

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Appendix

Parts of the Sequences and Their Functions

1. Disease Resistance and Defense Response Gene—Zinc Finger Protein

TCGAGCGGCCGCCCGGGCAGGTACAAGCAATTG
GAATACTTCCAACAATACCAGCAAAGGGTTACT
GGGCTTATTGGAGCTGAGCAAACTCAGAGATTA
GTTAATGAAGCACTTGTCCTTATGACCGTGGGA
GGCAATGACTTTGTTAACAACTACTATTTGGTCC
CCTTCTCTGCTAGATCTCGCCAATTCTCCCTCCC
AGACTATGTAGTCTACGTCATCTCCGAGTACC-
TCGGCCGCGACCACGCTA

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
XM_002521974.1	Ricinus communis zinc finger protein, putative, mRNA	219	219	84%	8e-54	85%	G
XM_002268057.1	PREDICTED: Vitis vinifera hypothetical protein LOC100243401 (LOC	163	163	84%	4e-37	81%	UG
AM453300.2	Vitis vinifera contig VV78X167183.5, whole genome shotgun sequer	163	163	84%	4e-37	81%	
AY491975.1	Agave americana GSDL-motif lipase (AEL) mRNA, AEL1 allele, comc	119	119	49%	8e-24	84%	

2. Signal Transduction Protein—Kinase

TAGCGTGGTCGCGGCCGAGGTACATAAAGCTTC
CCTTCATCACCCATTACTGGATCACAAACATATG
TAAGTTTGGGATTTATGAAGCGAAGCTTGTTGA
CAACTTCCAATACAGTGTCCAAAAATGAAACTG
AACCAATATAACCTGTTAACAAATGAGTATAAT
ACAGCAAGTCATTTGCTTCAAGGCCTTCTATTAA
ATCCCATAGTTGCTGTCCATTCAAAACTTGGCCT
TTAAAGGAAGGATATCCTGTGTGATTTGA-
GACCTGCCCGGGCGGCCGCTCGAA

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
XM_002522100.1	Ricinus communis pyridoxine kinase, putative, mRNA	324	324	85%	2e-85	90%	G
XM_002302915.1	Populus trichocarpa predicted protein, mRNA	209	209	79%	6e-51	83%	G

3. Ion Channels—Membrane Water Channel Protein

TAGCGTGGTCGCGGCCGAGGTAAGGAGGTGAGT
GAAGAAACGCAGCCTACCCATGGGAAGGACTAT
GTTGATCCACCACCAGCTCCTCTCATTGACGTGG
CTGAGCTCAAGCTCTGGTCTTTCTACCGTGCTCT
TATAGCTGAGTTCATAGCCACTCTTCTTTTCCTC
TACATCACTGTAGCTACTGTAATTGGCTACAAG
AAACAAGCTGACCCTTGTGGCGGAGTTGGGCTT
CTGGGTATTGCATGGGCCTTTGGTGGCATGATTT
TTATCCTTGTTTACTGCACTGCTGGTATCTCTGG
TGGTCATATTAACCCAGCGGTCACTTTTGGACTT
TTCTTGGCGAGGAAGGTGTCACTGATTAGGGCA
GTGGCTTACATGGTGGCTCAGTGCTTGGGTGCA
ATCTGTGGTGTTGGGTTGGTGAAGGCATTTATGA
AGCATCCATATAATGCTCTTGGAGGCGGTGCTA
ACTCCGTGGCTCATGGTTACAACAAAGGCACCG
CTTTGGGTGCTGAGATCATAGGCACTTTTGTGCT
TGTCTACACTGTTTTCTCTGCAACTGACCCTAAG
AGGAGTGCACGTGACTCTCACGTCCCTGTGTTG
GCTCCTCTTCCAATTGGGTTTGCTGTGTTCATGG
TCCACTTGGCAACAATCCCCATCACTGGTACC-
TGCCCGGGCGGCCGCTCGAA

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
GQ479824.1	Hevea brasiliensis plasma membrane aquaporin 2 (PIP2) mRNA, co	937	937	94%	0.0	92%	
EU599222.1	Manihot esculenta aquaporin (AQP) gene, complete cds	920	920	93%	0.0	92%	
XM_002521246.1	Ricinus communis Aquaporin PIP2.7, putative, mRNA	780	780	93%	0.0	88%	G
AJ605575.1	Ricinus communis mRNA for aquaporin (Pip2-1 gene), clone pPSL	780	780	93%	0.0	88%	
EF030420.1	Jatropha curcas aquaporin mRNA, complete cds	702	702	93%	0.0	86%	
XM_002306151.1	Populus trichocarpa aquaporin, MIP family, PIP subfamily, mRNA	682	682	93%	0.0	85%	G

