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Construction of suppression subtractive hybridization libraries and identification of brown planthopper-induced genes

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Summary

A suppression subtractive hybridization technique was used to screen for brown planthopper (BPH)-inducible genes in rice (*Oryza sativa*). cDNAs from a BPH-resistant rice line (B5) infested by BPH were used as the tester population, and mixed cDNAs from a BPH-sensitive line (MH63) and a control (uninfested B5) as the driver population. After hybridizing and cloning, forward and reverse subtraction cDNA libraries were obtained, containing 5700 clones. These clones were further analyzed by differential gene expression screening, and 154 clones that were clearly induced by BPH were identified. Sequencing analysis and homology searching showed that these clones represent 136 single genes, which were assigned to functional categories, including 10 putative cellular functions, according to categories established for *Arabidopsis*. The 136 genes include 21 known to be related to disease, wound and other stresses, most of which were found to be up-regulated in BPH feeding responses. In addition, an *Oryza* cysteine inhibitor and a β-glucosidase belonging to the 21 genes group were found in the rice response to BPH feeding, these two genes have previously been shown to be induced in plant responses to chewing insects. Our results not only confirm that several identical genes are activated in defense mechanisms against both sucking and chewing insects, but also show that genes have overlapping functions in both pathogen and insect resistance.

Abbreviations: BPH, brown planthopper; CHI, chalcone isomerase; CHS, chalcone synthase; DEPC, diethylpyrocarbonate; EST, expressed sequence tag; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; RDA, representational difference analysis; SAM decarboxylase, S-adenosylmethionine decarboxylase; SSH, suppression subtractive hybridization; X-gal, 5-bromo-4-chloro-3-indolyl bd-galactopyrano-side

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Introduction

The brown planthopper (BPH), *Nilaparvata lug*ens, is one of the most serious pests of rice throughout Asia. In China, widespread outbreaks across the rice planting area have often occurred in the 1990s. In addition, the damage in some areas is so severe as to cause whole plants to become yellowish and rapidly dry, a pattern known as 'hopper-burn'. BPH causes direct damage to rice plants by sucking assimilates from the phloem (Sogawa, 1973) and secondary damage by transmitting viral diseases (Ling et al., 1970, 1978). B5 (*Oryza sativa*), an introgression line from *Oryza officinalis*, shows high resistance to BPH and has been used as gene donor for BPH resistance studies (Yang et al., 1999a). The combination of B5 and BPH has become a model system for studying resistance interactions between rice plants and sucking insects. Understanding the mechanism of rice–BPH interactions will help to develop resistant rice varieties and environmentally friendly insecticides.

In the past decade, strenuous efforts have been made to study the characteristics of genes related to insect feeding. However, most of this research has focused on chewing insects such as caterpillars and beetles, which chew and tear tissues, extensively damaging the host plants. These investigations have provided us with new insights into the molecular mechanisms of the defense response in plants. A systematic and thorough search for genes related to resistance sucking insects is essential to better understand the concerted response of cells to herbivore invasion.

Recently, the polymerase chain reaction (PCR)-based suppression subtractive hybridization (SSH) technique has been developed for the rapid and sensitive comparison of mRNA expression pattern between 'tester' and 'driver' populations (Diatchenko et al., 1996; von Stein, 2001; Xiong et al., 2001). To better understand the molecular mechanism involved in rice response to BPH feeding, we employed the SSH in the present investigation since this technique would enable us to identify rarely expressed but specifically BPH-inducible genes. These defense genes collections provide us with an important genomic resource for profiling gene expression during interaction between rice and BPH.

Materials and methods

Plant material

Rice (*O. sativa* L.) line B5 is highly resistant to BPH, which derived its resistance genes from wild rice, *O. officinalis* Wall ex. Watt (Yang et al., 1999a). Rice line MH63 is a sensitive cultivar to BPH feeding (Huang et al., 2001). All BPH insects used were second or third instar nymphs reared on the susceptible rice variety Taichung Native 1 in the Genetics Institute of Wuhan University.

mRNA isolation and subtractive cDNA library construction

Tissue samples were pulverized with a mortar and pestle. Total RNA from different BPH feeding times (from 2–52 h) was extracted according to Chen et al. (2002). Both tester and driver poly $(A)^+$ RNA were purified with the Messagemaker reagent assembly kit (Gibco). Double-stranded cDNA was produced from approximately 2 μg of poly $(A)^+$ RNA. SSH was performed with cDNA from young and mature leaves using PCR-select cDNA Subtraction Kit (Clontech, Palo Alto, CA) following the manufacturer's instructions. For the forward subtractive cDNA library, cDNA obtained from B5 infested by BPH from 2 to 52 h was used as a 'tester' population and cDNA from the susceptible line MH63 and uninfested B5 was used as a 'driver' population. 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. Total RNAs isolated after 2 and 52 h BPH feeding were pooled equally, and converted into cDNA, using a cDNA synthesis kit from Invitrogen (Carlsbad, CA). In addition, to evaluate the efficiency of the cDNA subtraction, we compared the amplification levels of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene products by TR-PCR in subtracted and unsubtracted cDNA populations.

Construction of the subtracted cDNA libraries (T/A cloning)

The SSH libraries enriched for differentially expressed cDNA were constructed by ligating the subtracted cDNAs into the pGEM-T vector (Promega, Madison, WI) and transferring them, by electroporation, into DH10B *Escherichia coli* cells (Invitrogen) that were plated onto LB agar containing 100 μg mL^{-1} ampicillin, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 80 μg mL^{-1} 5-bromo-4-chloro-3-indolyl bd-galactopyranoside (X-gal), then incubated at 37 °C overnight to obtain a subtracted expressed sequence tag (EST) bank. Individual recombinant white colonies were picked and grown in LB medium

containing ampicillin (50 μg mL^{-1}) on 96-well microtiter plates.

Differential screening of the subtracted libraries

Individual clones from the two subtractive libraries were randomly picked and cultured in 96-well plates. The plasmids were then extracted and transferred to Hybond-N$^+$ nylon membranes (Amersham, Piscataway, NJ) for dot blot hybridization. Duplicate membranes with equal amounts of plasmids were hybridized with either the forward-subtracted probe or the reverse-subtracted probe. Ubiquitin cDNA and cDNA clones of CHI (chalcone isomerase), CHS (chalcone synthase) and S10981 (calmodulin) were used as internal controls since it has been shown that their levels are not significantly changed by BPH infection (Zhang et al., 2004). G3PDH cDNA, supplied with the kit, served as a negative control. In the case of the forward-subtracted probe, cDNA was synthesized from the tester cDNA-subtracted mRNAs obtained from BPH-feeding B5 leaves. Conversely, the reverse-subtracted probe was prepared from driver cDNA subtracted by tester cDNA. This probe was enriched for transcripts that were induced in susceptible responses. The forward and reverse unsubtracted cDNA populations were synthesized from the tester and driver mRNA, respectively, by reverse transcription. After the first screening, all clones showing apparent intensity changes were selected, transferred to 96-well plates and rescreened using the same set of probes.

EST sequencing and analysis

Using a BigDye™ Terminator v3.0 ready reaction cycle sequencing kit (Perkin–Elmer-Applied Biosystems), positive plasmid DNA selected by dot-blot hybridization was amplified by M13 forward and reverse primers to generate partial sequences. Amplified products were then sequenced in a GeneAmp PCR System 9700 Thermal Cycler (Perkin–Elmer, USA). Based on the qualified sequences, the predicted amino acid sequences were used to search for similar peptide sequences in public databases (http://www.ncbi.nlm.nih.gov), using the BlastX search algorithm (Altschul et al., 1997). The similarity scores between the cDNA clones and known sequences were represented by the BlastX probability *E*-values. The cDNAs were classified according to the *E*-values generated in the BLAST searches. *E*-values < 1e-10 were deemed to indicate significant homology and each EST with such a value was manually assigned the same putative cellular function as the protein giving the lowest *E*-value, following the annotation of the *A. thaliana* genome published by Mewes et al. (1997). ESTs with *E*-values > 1e-10 were deemed to have no significant homology to any known protein, and assumed to be novel (Yamamoto and Sasaki, 1997; Riley, 1993).

RNA gel blot analysis

RNAs were isolated from samples representing each control and treatment with TRI-ZOL reagent (Gibco BRL, USA), and dissolved in diethylpyrocarbonate (DEPC) treated water. RNA was quantified with BIO 20/1.0 nm UV/VIS spectro photometer (Perkin–Elmer, USA). For RNA gel blot analysis, total RNAs (20 μg) were separated on a denaturing 1.5% formaldehyde agarose gel and then transferred to Hybond N+ membranes (Amersham-Pharmacia, USA).

Results

Evaluation of subtraction efficiency

Differences in amplification patterns between the subtracted and unsubtracted cDNA samples indicative of successful subtraction were visually observed. The subtraction efficiency was subsequently evaluated by measuring G3PDH abundance in the subtracted and unsubtracted populations. For this purpose, portions of the PCR products obtained after 18, 23, 28 and 33 cycles were examined on a 2.0% agarose/EtBr gel. The amount of G3PDH decreased significantly after subtraction, indicating that the subtraction had worked well.

Characterization of the subtracted cDNA library

Subtractive cDNA libraries were constructed for the two phenotypes (resistance and sensitive to BPH). Two mRNA populations were prepared. The first, extracted from the B5 line after BPH feeding as tester and the second (extracted from a mixed sample containing both leaves from B5 plants that had not been confronted with BPH insects and leaves from the MH63 cultivar following BPH feeding) as the driver population. The libraries consisted of approximately 5700 positive clones, with insertions ranging from 160 to 1100 bp. After deleting the vector sequence and poor-quality

sequences, the average readable coding sequence was approximately 400 bp long. A set of four identical nylon membranes was prepared for dot-blot hybridization in a 2×2 factorial design (forward/reverse subtracted libraries, to be hybridized with subtracted and unsubtracted probes). The DNA blots contained four control cDNAs: ubiquitin cDNA and cDNA clones of CHI, CHS and S10981. Differentially hybridized signals on the membranes were compared after hybridization with ^{32}P-labeled subtracted/unsubtracted cDNA probes, respectively. For the majority of the clones, the expression levels differed less than twofold. Clones detected with a twofold or greater signal intensity compared with the internal controls were considered to be BPH-resistance-related genes. A total of 120 clones hybridized with the forward subtracted probe were found to be up-regulated, and considered to be BPH-induced resistance-related genes and 34 appeared to be up-regulated (and thus are presumably related to BPH sensitivity) when hybridized to the reserve subtracted probe. Alternatively, they can be considered BPH down-regulated genes.

EST sequencing and analysis

More than 154 recombinant clones induced by BPH were sequenced, and 146 qualified sequences with insertions longer than 100 bp were used in the Blast search. The search results revealed that of the 146 positive clones, 136 represented unique genes and the other 10 were duplicates. Of the 136 genes, 66 had high homology with plant genes of known function (Table 1), 61 were homologous to unknown proteins or cDNA clones from rice or other plants, and nine were novel ESTs. These novel ESTs were highly homologous (80%) to rice scaffold proteins, but were not found in the public databases consulted. Because numerous genes could potentially be classified into more than one category defined on the basis of the biochemical processes they are associated with, the results of BlastX comparisons of the subtractive ESTs were classified into 10 putative cellular functions (Fig. 1), based on the functional categories established for *Arabidopsis* (Mewes et al., 1997). These functions concerned carbon metabolism, the cell cytoskeleton, disease and abiotic stresses, electron transport, fatty acid metabolism, photosynthesis, protein structure and synthesis, signal transduction, unclear functions, and novel ESTs.

RNA blot analysis

RNA gel blot analysis was used to verify whether the SSH technique combined with high-throughput array hybridization is an effective method to isolate genes that are specifically induced by BPH feeding. Genes related to disease, wound, biotic and abiotic stresses were selected. Among the 21 selected genes, 16 were confirmed by RNA gel blotting to be up-regulated and four to be down-regulated (Fig. 2). The 16 up-regulated genes included two known genes that encode oryzacystatin and beta-glucosidase. Oryzacystatin has been shown to be a proteinase inhibitor that induces digestive compensation in a natural insect predator and thus indirectly contributes to plants' anti-feedant defenses (Bouchard et al., 2003). β-glucosidase is an elicitor, which has been identified in the oral secretions of insect herbivores that can trigger the production of volatile compounds in response to herbivore damage (Agrawal, 1998; Falco et al., 2001). The other 14 up-regulated genes have known roles in responses to biotic and abiotic stresses, such as wounding, disease and waterlogging, as well as their apparent role in responses to insect feeding. The four down-regulated genes were also identified as having roles in other stress responses (Table 2). It should be noted that one of these four clones was not detected in the RNA gel blot analysis, possibly because its presence in the SSH library was too low.

Discussion

Many methods such as differential display (Liang and Pardee, 1992; Sokolov and Prockop, 1994) and serial analysis of gene expression (Velculescu et al., 1995) have been developed to study differentially expressed genes. A notable example is SSH, an improved method involving selective amplification by representational difference analysis (RDA, Lisitsyn et al., 1993), which has provided new insights to isolate genes that are specifically expressed under diverse sets of conditions. Yang et al. (1999b) showed that a combination of SSH and cDNA microarray techniques can be successfully applied to identify differentially expressed genes. Clearly, application of SSH could provide a good way to find mRNA species that are differentially expressed in rice in response to BPH feeding, and knowledge of such genes would contribute to a better understanding of the molecular responses of plants to insect feeding.

Table 1. Identification of BPH-inducible genes in rice (*Oryza sativa*) matched with plant genes of known functions

Clone	BLAST sequence similarity	Related accession number[a]	*E*-value	Regulation pattern
Carbon metabolism				
BPHiw116[b]	Enoyl-CoA-hydratase	AAK00451	1e-55	Up
BPHiw073	4-hydroxyphenylpyruvate dioxygenase	AF251065	e-140	Down
Cytoskeleton				
BPHiw036	Actin	AA062546	3e-69	Up
BPHiw121	Clathrin heavy chain	GMU42608	7e-42	Up
BPHiw125	OsRAc1 mRNA for actin	AB047313	e-123	Up
BPHiw096	Ankyrin-kinase-like	BAB9019	3e-34	Down
Disease, wound and stress response				
BPHiw004	DNA binding protein tWRKY4	AF193770	2e-71	Up
BPHiw005	Catalase	Q59296	5e-10	Up
BPHiw021	Glyceraldehyde-3-phosphate dehydrogenase	AAM00227	2e-71	Up
BPHiw023	GSH-dependent dehydroascorbate reductase	BAA90672	9e-50	Up
BPHiw026	Cellulose synthase-8	AF200532	2e-39	Up
BPHiw028	Beta-glucosidase	NP199277	2e-17	Up
BPHiw044	S-adenosylmethionine decarboxylase	AF067194	9e-96	Up
BPHiw051	*O. sativa* hsp70 gene	X67711	9e-83	Up
BPHiw052	*Campylobacter jejuni* catalase	CAA59444	e-112	Up
BPHiw057	Water channel protein RWC3	AB029325	e-133	Up
BPHiw065	RHD3	BAB90264	5e-19	Up
BPHiw068	Vacuolar targeting receptor bp-80	AF161719	7e-87	Up
BPHiw070	Shaggy-like kinase etha	Y13437	1e-37	Up
BPHiw076	O-Methyltransferase ZRP4 (OMT)	P47917	3e-33	Up
BPHiw079	P-type ATPase	CAC40036	2e-52	Up
BPHiw085	Oryzacystatin	OSU54702	e-132	Up
BPHiw105	Lipoic acid synthase	NP179682	4e-23	Up
BPHiw016	EDR1	AAG31141	9e-38	Down
BPHiw042	IMP	BM422132	0.0	Down
BPHiw045	Polyubiquitin	RUBQ2	6e-82	Down
BPHiw109	Retroelement	AAM01070	1e-45	Down
Electron transport				
BPHiw012	Potassium transporter	CAD21000	5e-22	Up
BPHiw037	Cyc07	BAA89498	5e-97	Up
BPHiw060	FH protein interacting protein FIP2	XP470249	4e-30	Up
BPHiw064	Mitochondrial F1-ATPase	Q01859	3e-79	Up
BPHiw067	Cytosolic monodehydroascorbate reductase	D85764	8e-67	Up
BPHiw104	Coproporphyrinogen III oxidase	Q42840	2e-48	Up
BPHiw111	Choline-phosphate cytidylyltransferase	T06558	8e-26	Up
BPHiw059	ATPases	NP188608	e-143	Down
BPHiw093	Peroxisomal multifunctional protein	AF442962	3e-60	Down
BPHiw123	ABC transporter	AAK98701	1e-32	Down
Fatty acid signaling and metabolism				
BPHiw033	Fatty acid alpha-oxidase	AF229813	0.0	Up
BPHiw077	Acyl-CoA synthetase-like protein	AF503770	1e-59	Up
BPHiw089	Acyl carrier protein I	P02902	7e-41	Down
BPHiw126	Malate dehydrogenase	Q42973	5e-37	Down
Photosynthesis-related				
BPHiw099	Brassinolide responsible protein	BAC07002	2e-41	Down
BPHiw131	Photosystem I (LHCa) mRNA	AF010321	e-128	Down
Signal perception/transduction				
BPHiw008	MEK1	AF080436	2e-42	Up
BPHiw025	Serine protease-like protein	AAM13204	2e-33	Up
BPHiw043	RH3	AF467728	6e-81	Up

Table 1. (*continued*)

Clone	BLAST sequence similarity	Related accession number[a]	*E*-value	Regulation pattern
BPHiw055	Serine/threonine phosphatase	AAO26213	2e-62	Up
BPHiw058	Serine/threonine kinase	CAA73067	2e-97	Up
BPHiw100	EF-1 alpha	BAA23657	6e-45	Up
BPHiw001	Dof zinc finger protein	BAA78575	3e-34	Down
BPHiw002	Signal recognition particle receptor	XP482983	1e-42	Down
BPHiw019	Phosphoglycerate kinase	Q42961	4e-54	Down
BPHiw020	ER66	AAD46410	9e-30	Down
BPHiw092	Pyruvate dehydrogenase kinase (PDK)	AY026039	0.0	Down
BPHiw103	MAPK3 epsilon protein kinase	AAL87195	6e-84	Down
Structural proteins and protein synthesis				
BPHiw011	Membrane related protein CP5	XP463949	1e-43	Up
BPHiw029	Ribosomal protein S2	BE644511	2e-76	Up
BPHiw056	Membrane protein	BAD46353	2e-18	Up
BPHiw069	Permease 1-like protein	NP201094	9e-67	Up
BPHiw072	26S proteasome regulatory particle	BAB78490	9e-21	Up
BPHiw098	WD domain protein-like	BAB02803	7e-54	Up
BPHiw106	Acidic ribosomal protein P2b	O24415	3e-23	Up
BPHiw119	*Nilaparvata lugens* 16S ribosomal	AF158038	1e-97	Up
BPHiw128	*Rhizobium* sp. JEYF14 gene	AB069651	4e-89	Up
BPHiw137	T30a-17 partial 16S rRNA	AJ585959	e-163	Up

[a]Database accession number of related sequences.
[b]Identified clones were termed brown planthopper-induced (BPHi).

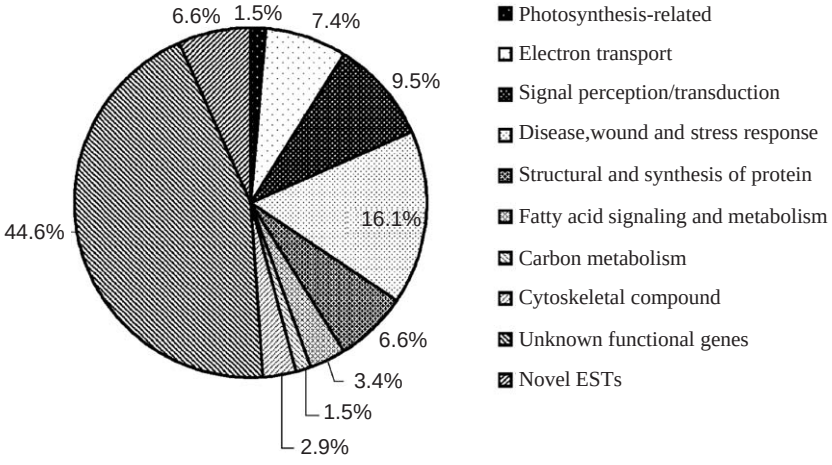

Figure 1. ESTs among various functional categories. ESTs were classified into 10 functional categories, unclear function, and novel ESTs. Percentages represent the percentages of genes belonging to a particular functional group with total number of genes including genes with known functions, unknown functions and sequences that did not have any homology to known sequences while homology to rice databases.

The BPH is one of the most serious rice pests in tropical and temperate Asia. The interactions between plants and insects have attracted much attention, and many studies now focus on the physiological effects of insects on plants. Researchers have succeeded in identifying resistance genes against BPH and located them on genetic maps of rice. To date, at least 12 genes for resistance to BPH have been identified and mapped (Huang et al., 2001). However, the molecular mechanisms involved in plants' resistance to insect feeding remain unexplained. The present study represents the first published systematic isolation and characterization of BPH-induced genes in rice, which

are important initial steps towards understanding the mechanisms of BPH resistance.

According to recent data from several research groups, hundreds of plant genes are induced by insect feeding (Reymond et al., 2000) and since

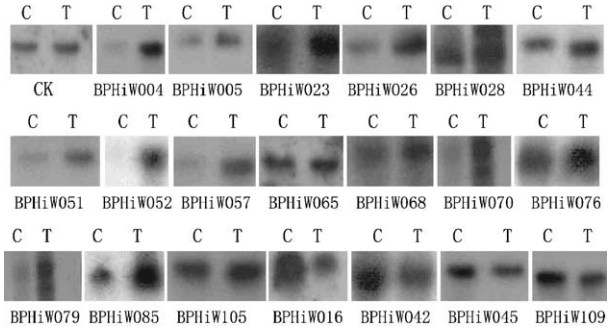

Figure 2. RNA gel blot verification of BPH-induced biotic and abiotic stress-related genes. For the BPH-treated and control groups, RNAs from each time course were equally mixed to constitute the corresponding RNA pools. Twenty micrograms of RNA from each pool was separated in a formaldehyde gel and blotted onto a Hybond N^+ membrane. RNA gel blotting was used to hybridize probes with the corresponding cDNA and ubiquitin cDNA. After sequential hybridizing to the pooled cDNA and ubiquitin cDNA, the signal was then standardized. "C" indicates the control RNA pool, and "T" the BPH-infected RNA pool. The corresponding genes are indicated by the clone names, in which "BPHi" is omitted.

plants present a wide variety of defense strategies against insect damage, several genes must be involved in defense mechanisms against insect feeding. Proteinase inhibitors (of the serine, cysteine and aspartic families) have been classified as defense proteins, cysteine and aspartic acid proteinases are frequently found in insects with relatively acidic contents (e.g., Coleopteran) (Terra and Ferreira, 1994). It is assumed that proteinase inhibitors interfere with the digestive process of insects by inhibiting the proteolytic activity of midgut enzymes, thus depressing the synthesis of proteins necessary for growth, development, and reproduction (Broadway and Duffey, 1986). Interestingly, expression of cysteine inhibitor defensive proteins (oryzacystatin, BPHiw085) has been observed in rice plants in the BPH infection process, which may increase the level of protection in the tissues where it is induced, and similar defense mechanisms may exist in different plant species.

In addition, many plant species release volatile compounds in response to herbivore damage. In cabbage leaves, for instance, a β-glucosidase from *Pieris brassicae* caterpillars elicits the release of volatile compounds (Mattiacci et al., 1995), thus the finding that BPH feeding induced the expression of β-glucosidase in our study suggests that a similar blend of volatile terpenoids may be released when rice seedlings are damaged by BPH feeding. While

Table 2. Identification of BPH-inducible genes related to stress in rice (*Oryza sativa*)

Clone	Putative identity	Related accession number[a]	*E*-value	Regulation pattern
BPHiw004[b]	DNA-binding protein tWRKY4	AF193770	2e-71	Up
BPHiw005	Catalase	Q59296	5e-10	Up
BPHiw023	GSH-dependent dehydroascorbate reductase	BAA90672	9e-50	Up
BPHiw026	Cellulose synthase-8	AF200532	2e-39	Up
BPHiw028	Beta-glucosidase	NP199277	2e-17	Up
BPHiw044	SAM decarboxylase	AF067194	4e-76	Up
BPHiw051	*O.* sativa hsp70 gene	X67711	9e-83	Up
BPHiw052	*Campylobacter jejuni* catalase	CAA59444	e-112	Up
BPHiw057	Water channel protein	AB029325	e-133	Up
BPHiw065	RHD3	BAB90264	5e-19	Up
BPHiw068	Vacuolar targeting receptor bp-80	AF161719	7e-87	Up
BPHiw070	Shaggy-like kinase etha	Y13437	1e-37	Up
BPHiw076	O-Methyltransferase ZRP4	P47917	3e-33	Up
BPHiw079	P-type ATPase	CAC40036	2e-52	Up
BPHiw085	Oryzacystatin	OSU54702	e-132	Up
BPHiw105	Lipoic acid synthase	NP179682	4e-23	Up
BPHiw016	EDR1	AAG31141	9e-38	Down
BPHiw042	IMP	BM422132	0.0	Down
BPHiw045	Polyubiquitin	RUBQ2	6e-82	Down
BPHiw109	Retroelement	AAM01070	1e-45	Down

[a]Database accession numbers of related sequences.
[b]Identified clones were termed brown planthopper induced (BPHi).

in another experiment, a plant β-glucosidase was also present in crushed leaves with large amount of juice, volatiles are potentially important in plant interactions because they can attract parasitoids; but it should be noted that the cabbage β-glucosidase is different from that of *P. brassicae* (Mattiacci et al., 1995).

It can be speculated that rice β-glucosidase is involved in a process other than volatile emission. For instance, it may directly activate defense pathways in response to herbivory since β-glucosidase up-regulation was detected. However, regardless of whether or not the β-glucosidase found in rice was directly defense-related, and whether it originated from the plant or the insects, it is worthy of further study.

Although little information is available on the mechanisms in rice plants that defend against BPH damage, the response of plants to herbivory has been extensively studied in other plant species. It has been estimated that more than 500 genes of *Nicotiana attenuata* are related to herbivory, and about half of these genes with known functions are also involved in plant–pathogen interactions (Hermsmeier et al., 2001). In the present study, a large number of genes identified in the subtractive analyses were pathogen-, disease- or stress-related. For instance, shaggy-like kinases (like BPHiw070, which was upregulated by BPH feeding) are known to be involved in osmotic stress responses of *Arabidopsis* (Piao et al., 1999). Similarly, S-adenosylmethionine decarboxylase (BPHi044) has been shown to be involved in salt and drought stress responses (Li and Chen, 2000). Some of the other genes found in our study to be affected by BPH feeding, such as EDR1 and RH3, have been reported to encode pathogenesis-related (PR) proteins (Frye et al., 2001; Senthilkumar et al., 1999). The DNA-binding protein tWRKY4 (BPHiw004) reportedly plays an important role in defense responses to pathogens, and also responds to changes in salicylic acid levels (Chen and Chen, 2000). These findings suggest that a significant amount of genes have overlapping functions in both pathogen and insect attack responses.

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References

Agrawal AA. Induced responses to herbivory and increased plant performance. Science 1998;279:1201–2.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 1997;25:3389–402.

Bouchard É, Cloutier C, Michaud D. Oryzacystatin I expressed in transgenic potato induces digestive compensation in an insect natural predator via its herbivorous prey feeding on the plant. Mol Ecol 2003;12:2439–45.

Broadway RM, Duffey SS. Plant proteinase inhibitors: mechanism of action and effect on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exiqua*. J Insect Physiol 1986;32:827–33.

Chen CH, Chen ZX. Isolation and characterization of two pathogen- and salicylic acid-induced genes encoding WRKY DNA-binding proteins from tobacco. Plant Mol Biol 2000;42:387–96.

Chen XP, Yuan HY, Chen RZ, Zhu LL, He GC. Isolation and characterization of triacontanol regulated genes in rice (*Oryza sativa* L.): Possible role of triacontanol as a plant growth stimulator. Plant Cell Physiol 2002;43:869–76.

Diatchenko L, Lau YFC, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov K, Gurskaya N, Sverdlov E, Siebert PD. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci USA 1996;93:6025–30.

Falco MC, Marbach PAS, Pompermayer P, Lopes FCC, Silva-Filho MC. Mechanisms of sugarcane response to herbivory. Genet Mol Biol 2001;24:113–22.

Frye CA, Tang D, Innes RW. Negative regulation of defense responses in plants by a conserved MAPKK kinase. Proc Natl Acad Sci USA 2001;98:373–8.

Hermsmeier D, Schittko U, Baldwin IT. Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. I. Large-scale changes in the accumulation of growth- and defense-related plant mRNAs. Plant Physiol 2001;125:683–700.

Huang Z, He G, Shu L, Li X, Zhang Q. Identification and mapping of two brown planthopper resistance genes in rice. Theor Appl Genet 2001;102:929–34.

Li ZY, Chen SY. Differential accumulation of S-adenosylmethionine decarboxylase transcript in rice seedlings in response to salt and drought stresses. Theor Appl Genet 2000;100:782–8.

Liang P, Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 1992;257:967–71.

Ling KC, Aguiero VM, Lee SH. A mass screening method for testing resistance to grassy stunt disease of rice. Plant Dis Rep 1970;54:565–9.

Ling KC, Tiongco ER, Aguiero VM. Rice ragged stunt, a new virus disease. Plant Dis Rep 1978;62:701–5.

Lisitsyn NA, Lisitsyn NM, Wigler M. Cloning the differences between two complex genomes. Science 1993;259:946–51.

Mattiacci L, Dicke M, Posthumus MA. Beta-glucosidase: an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. Proc Natl Acad Sci USA 1995;92:2036–40.

Mewes HW, Albermann K, Bähr M, Frishman D, Gleissner A, Hani J, Heumann K, Kleine K, Maieri A, Oliver SG. Overview of the yeast genome. Nature 1997;387:7–65.

Piao HL, Pih KT, Lim JH, Kang SG, Jin JB, Kim SH, Hwang I. An arabidopsis *GSK3/shaggy*-like gene that complements yeast salt stress-sensitive mutants is induced by NaCl and abscisic acid. Plant Physiol 1999;119: 1527–34.

Reymond P, Weber H, Damond M, Farmer EE. Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. Plant Cell 2000;12:707–19.

Riley M. Functions of the gene products of *Escherichia coli*. Microbiol Rev 1993;57:862–952.

Senthilkumar P, Parani M, Lakshmi M, Jithesh MN, Parida A. Histone H3 gene from *Porteresia coarctata* (Accession No. AF109910)(PGR99-023). Plant Physiol 1999; 119:806.

Sogawa K. Feeding of the rice plant and leafhoppers. Rev Plant Protein Res 1973;6:31–43.

Sokolov BP, Prockop DJ. A rapid and simple PCR-based method for isolation of cDNAs from differentially expressed genes. Nucleic Acids Res 1994;22: 4009–15.

Terra WR, Ferreira C. Insect digestive enzymes: properties, compartmentalization and function. Comp Biochem Physiol 1994;109:1–62.

Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. Serial analysis of gene expression. Science 1995;270:484–7.

von Stein OD. Isolation of differentially expressed genes through subtractive suppression hybridization. Methods Mol Biol 2001;175:263–78.

Xiong L, Lee MW, Qi M, Yang Y. Identification of defense-related rice genes by suppression subtractive hybridization and differential screening. Mol Plant Microbe Interact 2001;14:685–92.

Yamamoto K, Sasaki T. Large-scale EST sequencing in rice. Plant Mol Biol 1997;35:135–44.

Yang CJ, Yang ZH, Hu JF, He GC, Shu LH. Study on the brown planthopper resistance in introgressive lines from wild rice. Acta Phytophyla Sin 1999a;26:197–202.

Yang GP, Ross DT, Kang WW, Brown PO, Weigel RJ. Combining SSH and cDNA microarrays for rapid identification of differentially expressed genes. Nucleic Acids Res 1999b;27:1517–23.

Zhang F, Zhu L, He G. Differential gene expression in response to brown planthopper feeding in rice. J Plant Physiol 2004;161:53–62.