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Analysis of differentially expressed transcripts from planthopper-infested wild rice (*Oryza minuta*)

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Abstract A subtracted library was constructed from planthopper-infested wild rice (*Oryza minuta*) by suppression subtractive hybridization in combination with mirror orientation selection. To screen the differentially expressed transcripts in the library, we applied a cDNA microarray containing 960 random clones in a reverse Northern blot analysis using cDNA probes prepared from the mRNAs of control and planthopper-infested samples. On the basis of the signal intensities and expression ratios obtained from experiments performed in triplicate, we selected 383 clones. The elevated expression levels and overall profiles over time were verified by Northern blot analysis. Although Southern blot analysis showed similar copy numbers of the screened genes in *O. minuta* and *O. sativa*, it also revealed that the expression profiles had a different pattern . Functional categorization placed the identified transcripts in the categories of subcellular localization, metabolism, and protein fate. The presence of these expressed sequence tags implies that resistance of *O. minuta* to insect infestation can be achieved not only by an elevated expression of defense-related genes but also by enhanced metabolic activities.

S.K. Cho and K.W. Jung contributed equally to this work.

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Keywords Wild rice · Planthopper · Suppression subtractive hybridization · Microarray · ESTs

blast: Basic local alignment search tool · *EST:* Expressed sequence tag · *MIPS:* Munich Information Center for Protein Sequences · *MOS:* Mirror orientation selection · *omii: Oryza minuta* insect-infested · *SSH:* Suppression subtractive hybridization

Introduction

Rice (*Oryza sativa* L.) is the world's most important crop and the primary food source for 35% of the world's population. However, the productivity of rice is adversely affected by a number of pathogens (viruses, bacteria, fungi, and insects) and abiotic (soil condition, wound, temperature, flooding, etc.) stresses (Brar and Khush 1997; Ronald 1997; Goff 1999). The brown planthopper (*Nilaparvata lugens*) is one of the most damaging rice pests (Karunaratne et al. 1999; Small and Hemingway 2000). The infested plants become shrunken and wilted and their productivity is much reduced. It is therefore important to establish strategies to overcome such biotic stressors and to use these in actual breeding programs (He et al. 1999; Chen et al. 2002). Genetic diversity in the cultivated rice has been severely narrowed by the concentration of many favorable alleles, such as high yield and quality, through intensive breeding among the elite lines.

The importance of wild relatives as a genetic resource can not be over-emphasized because wild species are known to contain many genetic variations and to display a wider spectrum of resistance to various stresses. The wild relatives of rice have diversified into a wide range of environments over 40 million years and thus could provide gene sources with economic characters not easily obtained in cultivated germplasms (Khush 1997). The rice genus, *Oryza*, consists of 23 species and nine recognized genome types, with genomes that are either diploid ($2n$=24) or tetraploid ($2n$=48), represented as AA, BB,

CC, BBCC, CCDD, EE, FF, GG, and HHJJ. These genomes are distantly related to that of *O. sativa* and represent an important reservoir of stress-related genes, thereby providing the breeder with a valuable strategy for controlling crop diseases or environmental stresses (Izawa and Shimamoto 1996; Brar and Khush 1997). *O. minuta*, a wild relative of rice, contains the BBCC genome, shows resistant to various kinds of pathogens and environmental stresses, and has been used as a donor of defense to blast and bacterial blight. Due to its potential agronomical importance, this species has become the primary focus of attention to rice breeders (Vaughan 1994).

In the investigation reported here, we constructed a subtracted library from planthopper-infested *O. minuta* by using a method based on suppression subtractive hybridization (SSH) combined with mirror orientation selection (MOS). To identify and screen the genes differentially expressed in *O. minuta* following insect infestation (results previously reported by Yang et al. 1999), we adapted the cDNA microarray technique as a form of reverse Northern blot analysis to eliminate false positive clones and to increase selection efficiency. Based on the triplicate microarray data, we selected 383 clones and analyzed their putative identities and functional groups. We also comparatively analyzed the genomic Southern blot to reveal not only the existence of the selected clones but also differences in the nucleotide sequences between *O. minuta* and *O. sativa*.

Materials and methods

Plant materials and insect infestation

Oryza minuta (accession no. 101144) was sown in a glasshouse at the National Crop Experiment Station (NCES) in Korea, and 4-week-old seedlings were used for the insect infestation. At least ten larvae of the planthopper (*Nilaparvata lugens*) Korean biotype1 were placed on each plant. *O. sativa* cv. Hwaseongbyeo, which is known to be vulnerable to planthopper infestation, was used as a reference. All plants were cultured at a constant temperature (28°C). Sampling occurred at 0 h (untreated control) and at 6, 24 and 72 h post-infestation, with the sampled plants being immediately frozen in liquid nitrogen and stored at –70°C.

RNA extraction and construction of the subtracted library

A planthopper infestation-induced transcript-enriched library (*O. minuta* insect-infested; *omii*) was constructed by the modified subtraction method, which combined SSH with MOS, to reduce the number of background clones in the subtracted library. Total RNA was prepared using TRIZOL reagent (Gibco/BRL, Gaithersburg, Md.; http://www.invitrogen.com), according to the manufacturer's instructions. The amount and quality of the total RNA was checked by spectrophotometer (O.D.=260/280) and by formaldehyde-containing 1.0% agarose gel electrophoresis. For the subtraction, poly(A)$^+$ RNA was extracted using Dynabeads Oligo $(dT)_{25}$ (Dynal, New Hyde Park, N.J.; http://www.dynal.net), according to the manufacturer's instructions. The double-stranded cDNAs of tester (the pooled samples of planthopper-infested) plants and driver (control) plants were synthesized from 2 μg of poly(A)$^+$ RNA. cDNA conversion and SSH were carried out according to the manual of the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, Calif.; http://www.bdbiosciences.com), and MOS was performed as previously described (Rebrikov et al. 2000), with minor modifications. Briefly, 150 ng of the secondary PCR product, amplified with nested primer 2 (NP2R) and biotin-labeled nested primer 1 (NP1), was digested with 10 U of restriction enzyme *Sma*I (NEB, Beverly, Mass.; http://www.neb.com) for 2 h at 25°C. To monitor complete digestion by *Sma*I, we used a 5′-end biotin-labeled nested primer. Biotin played two important roles in our modified MOS experiment. First, labeled biotins give assurance of proper *Sma*I digestion. Second, labeled biotins inhibit the ligation of undigested templates, which become false positive clones in this subtracted library. Therefore, we monitored *Sma*I digestion by detecting biotin signals. Following electrophoresis on a 0.8% agarose gel, uncut and *Sma*I-digested PCR products were transferred to a positively charged nylon membrane (Tropix, Applied Biosystems, Foster City, Calif.; http://www.appliedbiosystems.com). The biotin signal was detected using a Southern-Star biotin-labeled DNA detection kit (Tropix). After MOS hybridization for 4 h at 68°C using the *Sma*I-digested secondary PCR products, MOS PCR was carried out using the NP2Rs primer (short sequence of the NP2R used in secondary PCR in SSH). The sizes of the MOS PCR products ranged from 200 bp to 850 bp, with a median value of approximately 400 bp. The PCR products were inserted into pCR2.1 vectors and transformed into *Escherichia coli* strain TOP10 using an Original T/A Cloning kit (Invitrogen, Carlsbad, Calif.; http://www.invitrogen.com) to construct a subtracted library enriched with insect infestation-induced cDNAs.

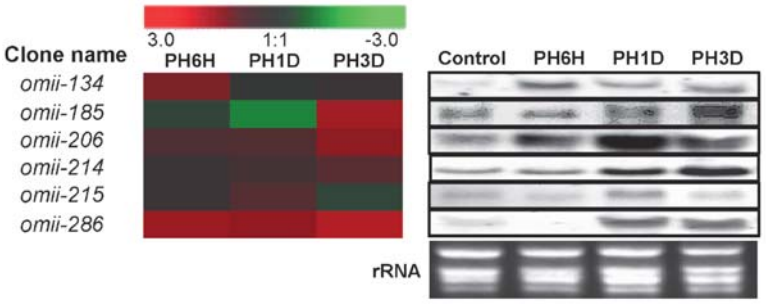
Microarray as a form of reverse Northern blotting

A total of 960 randomly chosen clones were used as PCR templates. Following re-amplification of the colony PCR products with T7 and M13R primers, the PCR products were purified using an AccuPrep PCR Purification kit (Bioneer, Seoul, Korea; http://www.bioneer.co.kr), dissolved in a spotting solution (6× SSC) at a concentration of 0.5 μg/μl, and then dotted onto a poly-L-lysine-coated slide glass using a HT-Arrayer (Bioneer). All of the steps for cDNA chip manipulation were performed as described by the Brown Laboratory (Stanford University, Calif.; http://cmgm.stanford.edu/pbrown/protocols). To prepare probes, we first labeled the target cDNAs, as described by Eisen and Brown (1999) with some modifications, using 1 μg poly(A)$^+$ RNA of tester and driver plants. Tester and driver cDNAs were incorporated with 0.1 m*M* Cy5-dUTP and 0.1 m*M* Cy3-dUTP (Amersham Pharmacia Biotech, Piscataway, N.J.; http://www.amershambiosciences.com). The purified labeling mixture was diluted with 1.25× ArrayIt UniHyb solution (TeleChem, Sunnyvale, Calif.; http://www.arrayit.com) and hybridized at 55°C for 16 h. The slides were then washed twice—with the first washing solution [0.4× SSC, 0.1% sodium dodecyl sulfate (SDS)] at 55°C for 30 min and with the second washing solution (0.04× SSC, 0.1% SDS) at room temperature for 10 min—following which the slides were scanned with GENEPIX 4000B (Axon, Union City, Calif.; http://www.axon.com).

Each hybridization experiment was repeated in triplicate with a newly labeled cDNA probe of the corresponding RNA sample. After scanning, data were merged and sorted by signal intensity and expression ratio. The data obtained were initially selected using a "flag" column (the indicator of a good signal) of each clone produced by a scanning image-analyzing program, GENEPIX PRO 3.0 (Axon). All clones maintaining good flags, in triplicate, were selected, sorted by signal intensity, classified by quartile, and sorted by expression ratio (the median of the triplicate). Colonies with an expression ration of more than 1.5 were uniformly selected from the lower, median, and high quartiles.

Northern blot analysis

Aliquots (15 μg) of total RNA were fractionated on a 1.0% agarose-formaldehyde gel in a MOPS buffer, transferred onto a Tropilon-plus membrane (Tropix) by the alkaline transfer method (Chomzynski 1992), and crosslinked with UV irradiation. The



Clone name		PH6H	PH1D	PH3D
omii-134	Microarray	2.29	0.96	1.14
	Northern blot	4.81	2.80	3.74
omii-185	Microarray	0.76	0.45	2.87
	Northern blot	0.95	1.92	3.24
omii-206	Microarray	1.63	1.68	2.50
	Northern blot	2.67	4.21	1.81
omii-214	Microarray	1.10	1.38	1.80
	Northern blot	1.16	2.89	4.23
omii-215	Microarray	1.12	1.76	0.75
	Northern blot	0.67	4.21	1.81
omii-286	Microarray	2.69	2.57	3.26
	Northern blot	0.71	5.43	5.04

Fig. 1 Expression patterns of *omii* clones screened by microarray analysis. Differential expression patterns over time (6, 24, 72 h post-infestation) were compared by microarray and Northern blot analysis. The *colored image* indicates the level of gene expression from the microarray analysis: *red* elevated levels, *green* repressed levels. The *white and black* image shows the expression pattern from the Northern blot analysis. Each transcript is indicated by a *single row*, and each sampling time is presented by a *single column*. While the expression profiles of these partial transcripts were quite different over time, all of the clones tested exhibited elevated gene expressions following infestation. rRNA was used as a control to ensure equal loading and the integrity of the Northern blot analysis. *Lanes*: *Control* no insect infestation, *PH6H* 6 h post-insect infestation, *PH1D* 24 h post-insect infestation, *PH3D* 72 h post-insect infestation

probe was prepared by incorporating biotin-labeled deoxycytidine triphosphate (dCTP) during the touchdown PCR and then hybridized with the membrane at 68°C for 16 h. A Southern-Star biotin-labeled DNA Detection kit (Tropix) was used to detect signals.

Southern blot analysis

Aliquots (15 μg) of genomic DNAs of *O. minuta* and *O. sativa* cv. Hwaseongbyeo were digested (*Eco*RV or *Hin*dIII), fractionated on a 0.8% agarose gel, and transferred onto a Tropilon-plus membrane (Tropix) as described for the Northern blot analysis. The biotin-incorporated probe was hybridized with the membrane at 65°C for 16 h. A Southern-Star biotin-labeled DNA Detection kit (Tropix) was used to detect the signals.

Sequence analysis and BLAST search

Following sorting of the microarray data, nucleotide sequences of the selected cDNA clones were determined using a BigDye Termination Cycle Sequencing Ready Reaction kit (Applied Biosystems). Electrophoresis was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequence homologies were determined using the BLAST Network Service (National Center for Biotechnology Information (NCBI), Bethesda, Md.; http://www.ncbi.nlm.nih.gov) (Altschul et al. 1997). Expressed sequence tags (ESTs) were classified based on the alignments of translated amino acid sequences with the Munich Information Center for Protein Sequences (MIPS; http://www.mips.biochem.mpg.de) (Frishman et al. 2001). All EST data are publicly available through the NCBI; GenBank dbEST accession nos. CD026344–CD026512 and CV133208–CV133211.

Results and discussion

Construction of an insect infestation-induced transcript-enriched library from wild rice

We have constructed a planthopper infestation-induced transcript-enriched library (*omii*) using the modified subtraction method, which combined SSH with MOS, to reduce the number of background clones in the subtracted library. The MOS method is based on the fact that background molecules have only one orientation for the adaptor sequences, whereas target molecules represent both sequence orientations. Subtraction by SSH combined with MOS allowed the efficient and rapid cloning of differentially expressed transcripts. However, these sequential procedures are not totally free of false positive clones. Screening by Northern blot is labor-intensive, and the traditional reverse Northern blot analysis barely detects transcripts present in low abundance. To overcome these major drawbacks, we adapted the microarray as a high-throughput method of performing reverse Northern blot analysis. Amplified templates of 960 colonies, which were randomly chosen from the subtracted library, were dotted onto a poly-L-lysine coated slide glass for hybridization. Cy5 and Cy3 dye were incorporated during cDNA synthesis for the planthopper-infested and control tissue samples, respectively. Hybridization was repeated in triplicate with the newly labeled cDNA probes of the corresponding RNA samples. We subsequently selected 383 cDNA clones that matched the criteria described in the Materials and methods and characterized these further.

Confirmation of expression level between microarray and the Northern blot analysis

To verify the elevated expression levels of the screened clones, Northern blot analysis was performed with the total RNAs in the manner described for the microarray probe. The transcription pattern of six *omii* clones—*omii-134*, *-185*, *-206*, *-214*, *-215*, and *-286*—were analyzed and compared with their expression patterns in the microarray data (Fig. 1). To exclude any biased selection of clones for the Northern blot, we selected clones on the basis of a hierarchical clustering, and not on either sequence information or functional category. Four clones showed homology with different genes in the GenBank database

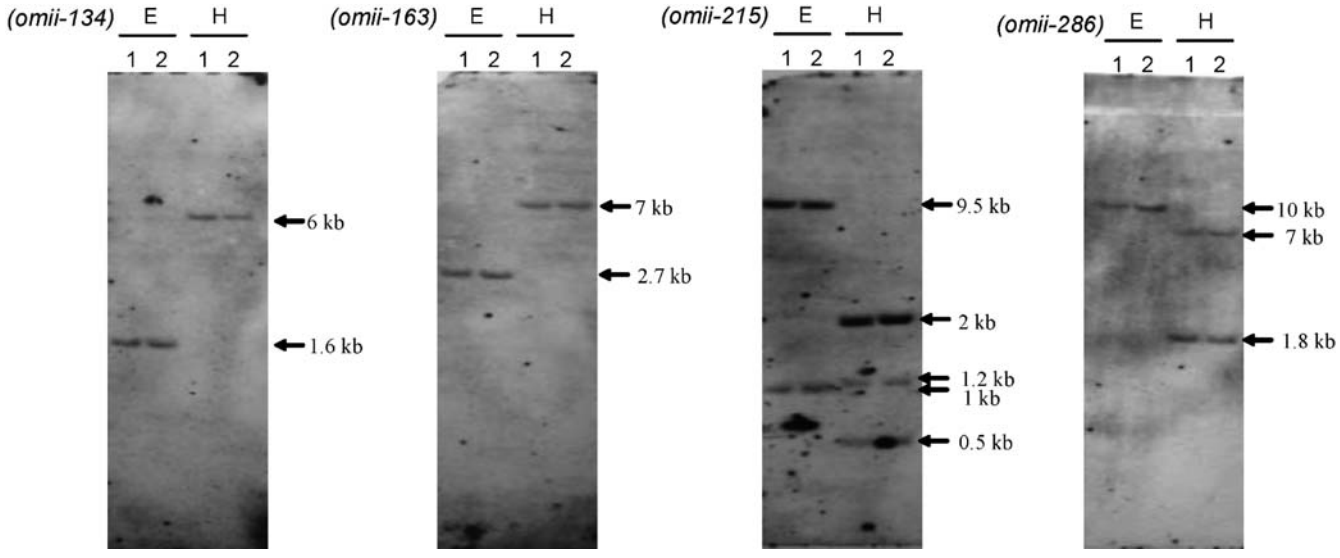


Fig. 2 Comparative Southern blot analyses between *Oryza minuta* (*lane 1*) and *O. sativa* cv. Hwaseongbyeo (*lane 2*). Fifteen-microgram samples of genomic DNAs were digested with *Eco*RV (*E*) or *Hin*dIII (*H*)

(cysteine proteinase inhibitor-1, putative protein, vacuolar targeting receptor, and oryzain gamma chain precursor), and functional categorization showed that these transcripts are mainly related to transcription, protein fate, cellular transport and transport mechanisms, defense, and subcellular localization. In the case of no-match clones, the transcripts were grouped into the functional category of metabolism (*omii-215*), transcription, and subcellular localization (*omii-134*). These latter groups could play important roles in the resistant system against various kinds of stresses.

The Northern blot data showed expression patterns that were similar, but not the same, to those shown by the microarray data. There may be a number of reasons explaining this result, including the following. (1) The targets dotted on the glass slide are cDNA fragments of target genes, and the dye-labeled probe is a mixture of complete transcripts. However, in the Northern blot analysis, the targets on the membrane are mixtures of total RNA or mRNA, and the probe is a labeled full or partial single-gene transcript. (2) The chemical and physical properties of the fluorescent dyes used for the microarrays and the biased incorporation of particular dyes for certain sequences may affect the data. (3) Another possibility is that differences in the specificity of the hybridization create problems of consistency between the microarray and Northern blot analysis (Chen et al. 1998; Taniguchi et al. 2001).

Despite the appearance of these slight differences, our strategy to eliminate the false positive clones using microarray analysis was more than adequate for our purpose, and although the gene expression profiles of the six *omii* clones were dependent upon infestation time, all of the clones tested exhibited elevated gene expression following insect infestation, indicating that the *omii* library reliably identified insect-induced transcripts.

Southern blot analysis of the selected clones

Southern blot analysis was applied to discriminate the origin of the selected clones (from the wild rice genome, not from the planthopper), identify the copy numbers of the screened genes, and compare polymorphic patterns of genomic sequences between *O. minuta* and *O. sativa*. All of the clones tested that showed elevated expressions of the clones under the planthopper infestation in both the microarray and Northern blot analysis—*omii-134* (no match), *-163* (putative cathepsin B-like cysteine proteinase), *-215* (starch-associated protein R1), and *-286* (oryzain gamma chain precursor)—were determined to be a single or low copy in both *O. minuta* and *O. sativa* (Fig. 2). However, the comparative Northern blot analyses of the planthopper-infested *O. sativa* showed a different expression than those of *O. minuta* (Fig. 3), indicating that the subtracted clones, which initially appeared to be sequences unique to the wild species, were in fact common to both wild and cultivated rice. The different levels of gene expression and common patterns of genomic contents between insect-infested wild and cultivated rice revealed that, globally, genes of the wild rice per se are not very novel, with a few exceptions, but that the sequences of the nucleotides and amino acids are different between wild and cultivated rice, and probably quite different in the promoter sequences. Consequently, it should be possible to adjust the defense system of *O. minuta* by controlling gene expression rather than by controlling the expression of the novel defense-related genes that are unique to wild germplasm.

Characterization of the *omii* library

The nucleotide sequences of the 383 selected clones were analyzed and their putative functions identified by car-

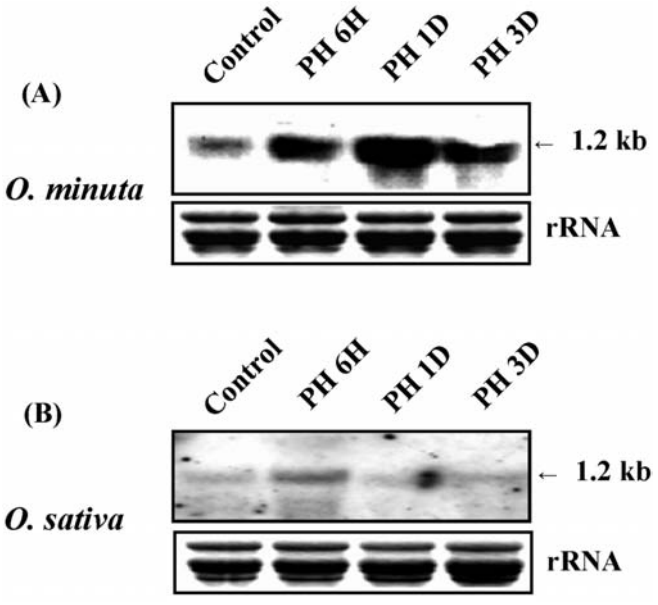


Fig. 3 Comparative Northern blot analyses between *O. minuta* (**a**) and *O. sativa* cv. Hwaseongbyeo (**b**). Northern blot analyses were performed using the *omii-206* clone as a probe. rRNA was used as a control to ensure equal loading and the integrity of the Northern blot analysis. *Lanes*: *Control* no insect infestation, *PH6H* 6 h post-insect infestation, *PH1D* 24 h post-insect infestation, *PH3D* 72 h post-insect infestation

Table 1 Summary of ESTs from the *omii*-subtracted library

	Number	Percentage (%)
Total clones	383	
BLAST match clone	347	90.6
BLAST no-match	36	9.4
Non-redundant sequence	153	39.9
Cluster	50	
Redundancy	278	72.6
Singlet	103	
BLAST match	137	89.5
BLAST no-match	16	10.5

rying out BLASTX and BLASTN searches. As a result of homology searches, 347 clones (90.6%) were matched to previously known genes with high significance (*E* value $\leq$ 9.00E-04). The 383 clones of the *omii* library included 153 unique clones (39.9%) of cDNA fragments, which in turn consisted of 50 assembled sequences and 103 singletons (Table 1). Transcript redundancy (ESTs assembled in clusters/total ESTs) is a measure of the abundance of the corresponding cDNA in non-normalized libraries. In other words, information on randomly picked cDNAs represents the relative expression levels of genes in a particular library. In this study, of the 347 cDNAs examined, the sequences of 244 clones were assembled in 50 clusters, resulting of a redundancy of 63.7%.

For unique cDNA fragments, cDNA comparisons through GenBank non-redundant databases using BLAST revealed that 87 clones of 153 cDNAs (89.5%) showed a high degree of sequence similarity with genes previously reported in other organisms. The remaining 10.5% (16 unique sequences from 153 clones) did not meet the established match criteria (*E*-value cut-off at 10^{-5}). The 16 cDNAs without a significant match were successfully aligned against the draft sequences of *O. sativa*, demonstrating that they originated from plant transcripts (data not shown). The BLASTX or BLASTN accession numbers, putative identities, species, scores, E values, and expression ratio of the database-matched clones are listed in Table 2.

The putative functions of the 179 ESTs were categorized by MIPS functional categorization by aligning them with Arabidopsis proteins, at an E-value of less than 10^{-5}, and compared with those of planthopper-infested *O. sativa* ESTs (Table 2, Fig. 3). High portions of singletons were grouped as either unclassified proteins (49.7%) or as not-determined (8.4%). About one-half of the *omii* clones appeared to be known genes related to one of the categories of subcellular localization (27.7%), metabolism (14.8%), or energy (14.2%). Other genes related to protein fate (12.9%), cell rescue and defense (9.7%), protein synthesis (7.1%), or cellular transport/transport mechanism (6.5%) were expressed at twice the level of their counterparts in the normal EST library of *O. minuta* (5.3%, 4.6%, 3.3%, and 3.9%, respectively) (Cho et al. 2004). The proportions of cell fate (6.5%), cellular communication/signal transduction mechanism (5.8%), and control of cellular organization (5.8%) genes were also doubled in the subtracted library—2.9%, 3.8%, and 2.9%, respectively, in the normal EST library. The categories related to transcription (4.5%) and cell cycle and DNA processing (3.9%) were at relatively low percentages (5.5% and 3.6% in the normal EST of *O. minuta*, respectively). ESTs associated with the defense mechanism in *omii* were present at a threefold higher percentage (9.7%) than those of planthopper-infested *O. sativa* (3.4%), which could be secondhand evidence for the high resistant character of *O. minuta* against vermin. The twofold-shifted levels of metabolism-related transcripts (14.8%) in the *omii* library are known to play an essential role in the resistance against pathogen attack. The defense mechanisms to various environmental stresses are accompanied by an enhancement of the metabolic pathway and by signal transduction to effect resistance gene expression (Fig. 4). Redundancy-check analysis revealed that the ten most abundant transcripts occupied 41.5% of the *omii* library. The most redundant sequence (9.9%), resistance-related non-coding RNA (nc1), has been reported to be the inducible RNA in planthopper-infested *O. sativa* L. spp *indica* by Chinese investigators working on the EST project, thereby providing supporting evidence for the successful construction of our subtracted library.

Successful resistance mechanisms to different stresses involve cell rescue, defense, and signal transduction and operate via enhanced metabolic pathways (Scheideler et al. 2002). Our redundancy-check analysis in *omii* also showed that metabolism-related genes, such those coding for the alpha 1,4-glucan phosphorylase L isozyme (8.6%) and the H isozyme (6.4%), were enriched (Table 3). Although our results do not include the expression patterns

Table 2 *omii* ESTs, functional categorizations, and expression ratio of the microarray analysis. Similarities with probabilities of fewer than 10^{-4} were regarded as being significant

Clone ID	Accession no.[a]	Putative identity	Species	Score	E-value	Functional category[b]	Microarray data[c]		
							PH6H[d]	PH1D[e]	PH3D[f]
omii-012	CD026346	At3g19170/MVI11_8	*Arabidopsis thaliana*	283	1.00E-75	06 14 40	1.32	1.77	1.30
omii-014	CD026348	HMG1 protein	*Zea mays*	109	2.00E-23	04 14 40	0.62	6.43	1.55
omii-016	CD026349	Granule binding starch synthase II precursor	*Oryza sativa*	178	4.00E-44	01 02 06 30 40	1.17	1.41	1.54
omii-017	CD026350	Putative Serine/threonine Kinase	*Oryza sativa*	180	1.00E-44	10	1.12	0.74	2.03
omii-024	CD026353	Structural polyprotein	*Rhopalosiphum padi* virus	123	1.00E-27	99	0.71	3.03	1.54
omii-027	CD026354	Resistance-related non-coding RNA (nc1)	*Oryza sativa*	985	0	ND[g]	16.74	2.53	4.08
omii-029	CD026355	Translation factor Sui1 homolog	*Oryza sativa*	65	6.00E-10	05 40	1.76	1.79	3.49
omii-036	CD026356	Putative transketolase	*Oryza sativa*	84	1.00E-15	01 02 30 40	1.01	2.08	3.07
omii-050	CD026359	Putative sesquiterpee cyclase 1	*Oryza sativa*	547	2.00E-51	ND	1.25	1.25	1.97
omii-052	CD026360	Putative senescence-associated protein	*Pisum sativum*	63	2.00E-09	14 25	3.56		
omii-054	CD026361	NTCP23-like cysteine proteinase	*Nicotiana tabacum*	81	8.00E-15	ND	2.90	2.48	3.38
omii-084	CD026367	Unknown protein	*Oryza sativa*	58	7.00E-08	99	0.87	1.71	1.85
omii-089	CD026368	Putative brown planthopper-inducible protein BpHi008A	*Oryza sativa*	73.2	2.00E-12	99	1.28	1.02	1.06
omii-097	CD026370	Hypothetical protein	*Arabidopsis thaliana*	238	2.00E-66	99	1.45	1.92	1.38
omii-116	CD026373	Peroxisomal fatty acid beta-oxidation multifunctional protein	*Oryza sativa*	245	4.00E-64	01 02	1.53	1.78	3.82
omii-117	CD026374	Unknown protein	*Oryza sativa*	292	3.00E-78	99	1.06	1.95	1.37
omii-125	CD026376	Cysteine proteinase 1 precursor	*Oryza sativa*	64	8.00E-10	02 06 08 11 40	2.03	1.75	4.05
omii-127	CV133208	Ribulose 1,5-bisphosphate carboxylase	*Oryza sativa*	54.3	8.00E-07	01 02	1.03	0.90	1.56
omii-128	CD026377	Glucose-6-phosphate/phosphate translocator	*Oryza sativa*	114	5.00E-25	08 40 67	0.43	1.86	0.69
omii-150	CD026381	Alpha 1,4-glucan phosphorylase H isozyme	*Oryza sativa*	474	1.00E-133	01 02 40	4.74	1.98	2.11
omii-163	CD026383	Cathepsin B cysteine proteinase	*Triticum aestivum*	60	2.00E-08	06	3.44	2.92	4.25
omii-166	CD026384	MADS box transcription factor	*Oryza sativa*	192	4.00E-48	04 25 40	1.29	1.26	2.00
omii-174	CD026386	Putative protein	*Arabidopsis thaliana*	123	1.00E-27	01 02 25 40	0.87	1.21	1.99
omii-176	CD026387	Nicotianamine aminotransferase A	*Oryza sativa*	361	5.00E-99	01 10 14 20	1.20	3.15	3.59
omii-185	CD026388	Cysteine proteinase inhibitor-1	*Oryza sativa*	94	7.00E-19	06	1.59	1.52	1.74
omii-189	CD026391	Putative cell death suppressor protein	*Oryza sativa*	513	2.00E-96	11	1.80	1.67	4.22
omii-206	CD026395	Unknown protein	*Oryza sativa*	94	7.00E-19	01 04 08 10 11 13 40 67	1.63	1.68	2.50
omii-214	CD026396	Vacuolar targeting receptor	*Oryza sativa*	372	1.00E-102	06 08	1.10	1.38	2.04
omii-217	CD026398	4-alpha-glucanotransferase	*Oryza sativa*	370	1.00E-101	ND	2.14	1.99	2.25
omii-222	CD026399	Unknown protein	*Pisum sativum*	77	1.00E-13	99	0.46	1.47	0.80
omii-239	CD026403	Starch-associated protein R1	*Solanum tuberosum*	302	3.00E-83	01	1.61	1.79	1.74
omii-240	CD026404	Unknown protein; protein id: At2g20920	*Arabidopsis thaliana*	170	2.00E-41	ND	0.64	5.82	1.52
omii-243	CD026405	Hypothetical protein	*Oryza sativa*	110	2.00E-23	10 40	1.37	1.32	1.57
omii-264	CD026409	Peptidyl-prolyl *cis-trans* isomerase, chloroplast precursor	*Oryza sativa*	54	1.00E-06	14	3.61	1.79	5.43
omii-267	CD026411	Phosphoglucomutase	*Oryza sativa*	286	1.00E-76	01 02 40	2.08	1.55	1.64
omii-273	CD026413	Ion protease homolog	*Oryza sativa*	101	9.00E-21	03 04 06 40	2.74	2.22	1.17
omii-279	CD026414	Phosphoribulokinase precursor	*Oryza sativa*	191	5.00E-48	ND	1.32	1.21	1.61
omii-286	CD026418	Oryzain gamma chain precursor	*Oryza sativa*	160	6.00E-39	02 06 08 10 11 40	2.69	2.57	3.53
omii-291	CD026420	Alpha-1,4-fucosyltransferase	*Lycopersicon esculentum*	276	1.00E-73	ND	1.33	1.31	1.38
omii-295	CD026422	Hydrolase-like protein	*Oryza sativa*	240	6.00E-63	99	1.59	2.63	1.53
omii-298	CD026512	Putative plastidic general dicarboxylate transporter	*Oryza sativa*	91	1.00E-17	20	1.13	2.63	2.29
omii-299	CD026423	Jacalin homolog	*Hordeum vulgare*	151	5.00E-36	11	1.46	1.79	
omii-324	CD026427	Hypothetical protein	*Oryza sativa*	67	2.00E-10	ND	1.16	1.25	2.71
omii-336	CD026428	Magnesium chelatase	*Oryza sativa*	230	1.00E-59	01 03 40	1.43	2.10	1.30
omii-340	CD026430	Unknown protein	*Oryza sativa*	156	2.00E-37	99	1.37	1.46	1.77
omii-351	CD026432	Translation initiation factor 5A	*Oryza sativa*	136	2.00E-31	01 05 40	1.83	1.85	3.05
omii-360	CD026434	Translationally controlled tumor protein homolog (TCTP)	*Oryza sativa*	108	5.00E-23	05 40	1.69	2.22	4.88
omii-363	CD026435	Putative mitochondrial processing peptidase	*Arabidopsis thaliana*	209	2.00E-53	02 06 14 40	3.22	2.14	3.02
omii-376	CD026438	Nicotianamine aminotransferase A	*Oryza sativa*	118	5.00E-26	01 14 20	2.94	2.91	3.34
omii-393	CD026442	Putative PKCq-interacting protein	*Oryza sativa*	57	1.00E-07	02 03 06 11 67	1.30	2.92	1.76
omii-414	CD026445	Cysteine proteinase AALP	*Zea mays*	51	9.00E-06	02 06 08 11 40	1.39	2.06	1.58
omii-467	CD026450	Carboxyl terminal protease-like protein	*Arabidopsis thaliana*	160	6.00E-39	06 30	4.51	1.45	3.19
omii-468	CD026451	MATE efflux family protein	*Arabidopsis thaliana*	51	7.00E-06	99	1.14	5.81	1.45
omii-476	CD026453	Profilin 5	*Zea mays*	104	7.00E-22	14 30 40	1.99	2.00	2.91
omii-511	CD026457	Putative ADP-glucose pyrophosphorylase	*Oryza sativa*	255	3.00E-58	01 05 30 40	1.84	1.54	1.75
omii-528	CD026461	Similar to histone protein Hist2h3c1	*Sphenostylis stenocarpa*	143	1.00E-14	03 04 30 40	1.00	2.67	1.44
omii-532	CD026462	Metallothionein-like protein	*Oryza sativa*	139	3.00E-32	11	1.59	2.31	1.93
omii-541	CD026464	Putative 32.7-kDa jasmonate-induced protein	*Hordeum vulgare*	151	8.00E-36	ND	1.26	1.11	4.60
omii-571	CD026468	Class III chitinase	*Sphenostylis stenocarpa*	48	6.00E-05	01 02 03 05 10 11 14 40	2.50	1.65	2.41
omii-573	CD026469	Starch-associated protein R1	*Solanum tuberosum*	214	4.00E-55	01	3.72	1.69	2.33
omii-584	CD026472	Unknown protein	*Arabidopsis thaliana*	90	2.00E-17	ND	1.41	1.25	1.93

Table 2 (continued)

Clone ID	Accession no.[a]	Putative identity	Species	Score	E-value	Functional category[b]	Microarray data[c]		
							PH6H[d]	PH1D[e]	PH3D[f]
omii-587	CD026473	Cysteine proteinase 1 precursor	*Oryza sativa*	69	3.00E-11	14 20	1.29	1.25	1.64
omii-594	CD026476	Ribosomal Protein, Small subunit (17.2-kDa) (rps-15)	*Caenorhabditis elegans*	56	3.00E-07	05 40	1.43	1.50	1.92
omii-598	CD026478	Putative activator-like transposable element	*Oryza sativa*	98	1.00E-19	29	0.45	1.73	0.71
omii-600	CD026479	Serine carboxypeptidase-like protein	*Oryza sativa*	65	5.00E-10	06 40	4.25	2.94	22.60
omii-604	CD026480	Photosystem I chain psaN	*Oryza sativa*	192	4.00E-46	99	1.41	1.78	0.90
omii-605	CV133211	14-3-3 protein GF14 nu (GRF7) identical to 14-3-3 protein	*Oryza sativa*	145	6.00E-34	10 11 20 30	0.82	0.45	3.32
omii-612	CD026481	Putative thioredoxin peroxidase	*Oryza sativa*	224	4.00E-58	04 11 13 40	1.33	3.77	2.02
omii-623	CD026484	Carboxyl terminal protease-like protein	*Arabidopsis thaliana*	166	1.00E-40	06 30	8.63	1.59	7.72
omii-637	CD026486	Putative phosphorylase	*Oryza sativa*	951	1.00E-57	01 02 40	1.13	1.93	1.08
omii-701	CD026490	GTP-binding protein typA	*Oryza sativa*	292	3.00E-78	05 10 40	1.53	1.75	2.06
omii-712	CD026492	Phosphatase 2A regulatory A subunit	*Lolium perenne*	123	2.00E-27	03 04 05 06 08 14 40	1.81	1.12	1.32
omii-749	CD026499	Ribosomal protein S16	*Oryza sativa*	137	6.00E-32	05 40	0.49	1.55	0.65
omii-835	CD026503	Phytochelatin synthetase-like protein 2	*Sorghum bicolor*	247	7.00E-65	11	1.65	1.83	1.31
omii-836	CD026504	Alpha 1,4-glucan phosphorylase L isozyme	*Oryza sativa*	285	2.00E-76	01 02 40	4.61	2.06	3.53
omii-843	CD026505	Inorganic pyrophosphatase	*Oryza sativa*	119	2.00E-26	01 02	5.58	2.38	2.52
omii-855	CD026506	Chaperone protein dnaJ-like	*Oryza sativa*	243	1.00E-63	99	2.20	2.08	1.61
omii-931	CD026510	Chloroplast drought-induced stress protein, 34 kDa	*Oryza sativa*	169	2.00E-41	11	1.32	1.28	2.93
omii-932	CD026511	Auxin-regulated protein	*Oryza sativa*	220	8.00E-57	99	1.36	1.25	1.65

[a] GenBank accession number
[b] Functional categorization was carried out by alignment with the MIPS database. 01, Metabolism; 02, energy; 03, cell cycle and DNA processing; 04, transcription; 05, protein synthesis; 06, protein fate (folding, modification, and destination); 08, cellular transport and transport mechanisms; 10, cellular communication signal transduction mechanism; 11, cell rescue, defense, and virulence; 13, regulation of interaction with cellular environment; 14, cell fate; 20, systemic regulation of interaction with environment; 30, control of cellular organization; 40, subcellular localization; 67, transport facilitation; 98, classification not yet clear-cut; 99, unclassified proteins
[c] Median value of triplicates
[d] PH6H, 6 h post-insect infestation
[e] PH1D, 24 h post-insect infestation
[f] PH3D, 72 h post-insect infestation
[g] ND, not determined

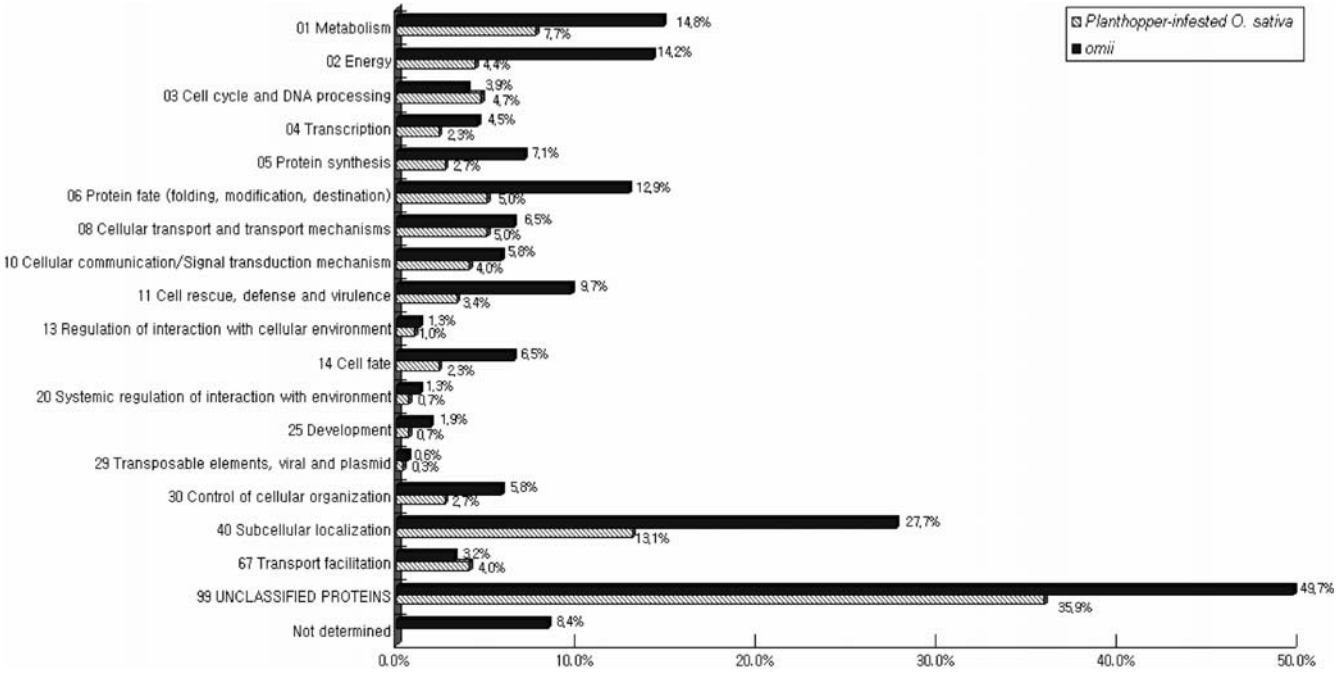


Fig. 4 Functional classification of ESTs obtained from the *omii* library and comparison with planthopper-infested *O. sativa*. ESTs were classified on the basis of their biological functions as determined by alignment with Arabidopsis protein sequences using an E-value cutoff of 10^{-5}. The *X-axis* indicates percentage (%) of ESTs matched with Arabidopsis protein DB

Table 3 The most prevalent transcripts found in the *omii* library

blast accession no.[a]	Putative identity	Number of hits[b]	Percentage (%)
AY166969	Resistance-related non-coding RNA (nc1)	38	9.9
AAK15695	Alpha 1,4-glucan phosphorylase L isozyme	33	8.6
AAG45939	Alpha 1,4-glucan phosphorylase H isozyme	26	6.8
BAA82828	Starch branching enzyme rbe4	13	3.4
BAA14404	Oryzain gamma chain precursor	13	3.4
AAT85108	Metallothionein-like protein	13	3.4
XP_465161	Nicotianamine aminotransferase A	8	2.1
AAA84592	Ribulose 1,5-bisphosphate carboxylase	5	1.3
NP_920245	Phosphoglucomutase	5	1.3
AAA87042	Putative 32.7-kDa jasmonate-induced protein	5	1.3
	Sum of top ten redundant clones	159	41.5

[a] GenBank accession number of the most similar sequence as identified by BLASTX and BLASTN alignments
[b] Number of clones assigned to the same GenBank accession

of all of the transcripts of *O. minuta* induced under insect-infested conditions, we suggest that *O. minuta* copes with pathogen attack by increasing the level of its metabolism (glycolysis, Krebs cycle, and pentose phosphate pathway, as previously reported by Scheideler et al. 2002) and/or by expressing defense-related genes. Therefore, the metabolic pathway differences and the defense-related genes of stress-treated *O. minuta* will be studied in more detail in future investigations to develop stress-resistant rice cultivars. The results of the inferred functional categorizations proved that different kinds of genes, regardless of whether they were functionally identified or unknown, are involved in the mechanisms against biotic or abiotic stresses in *O. minuta*.

The resistant mechanism-related ESTs from the *omii* library

In the investigation reported here we have identified a number of defense-related genes reported in earlier studies—for example, metallothionein, cysteine proteinase inhibitor-1, chitinase, 14-3-3 protein, serine carboxypeptidase, sesquiterpene cyclase, and jacalin. Sesquiterpene cyclase (*omii-050*) plays an important role in the production of sesquiterpene, a type of phytoalexin, and is known to be induced following fungal infection and insect damage as a plant defense mechanism (Mandujano-Chavez et al. 2000; Shen et al. 2000; Ha et al. 2003). Many of our clones exhibited significant homology to metallothioneins, the expression patterns of which, especially that of type 1, are usually increased in senescent leaves and under conditions of biotic or abiotic stress (Potenza et al. 2001; Ma et al. 2003). Proteinase inhibitors (*omii-185*) are widely distributed in the plant kingdom and are known to be involved in defense systems against insect herbivores (Botella et al. 1996). Jacalin (*omii-299*) is involved in resistance to viruses containing NBS-LRR type resistance genes (Chisholm et al. 2000), and chitinase (*omii-571*) plays an important role against fungal infections by degrading the fungal cell wall (Bishop et al. 2000). Serine carboxypeptidase (*omii-600*) is known as the wound-inducible gene and functions in signal transduction components via the brassinosteroid pathway. The elevated peptidase activity that results from wound stress or from systemin or methyl jasmonate treatment has been studied (Li et al. 2001; Moura et al. 2000), while 14-3-3 protein (*omii-605*), the key component of signal transduction pathways, has been reported to have a possible regulatory role in many stress responses by binding with kinases (*omii-017*), phosphatases, and transcription factors (Ferl 1996; Sehnke et al. 2002). We also identified certain housekeeping genes, such as ribulose bisphosphate carboxylase (*omii-355*), in the subtracted library. The altered levels of these transcripts are known to play an essential role in counteracting pathogen attack (Scheideler et al. 2002). Undefined clones—i.e., "no match" and "no hit" sequences in the NCBI database—would represent unknown defense-related genes in wild rice. These clones could correspond to an untranslated region or to a sequence of a divergent coding region of a known gene that is too short to overlap with a known gene. Although the ESTs identified from this work represent only a small part of the total transcripts, valuable information on defense-related genes was obtained. In particular, a number of the unknown genes in the *omii* library will be selected for future study in order to further our understanding of the defense mechanisms of *O. minuta* against vermin infestation and, consequently, to establish a new strategy for molecular rice breeding.

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