

Deep and Comparative Transcriptome Analysis of Rice Plants Infested by the Beet Armyworm (*Spodoptera exigua*) and Water Weevil (*Lissorhoptrus oryzophilus*)

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Abstract The beet armyworm (*Spodoptera exigua*) and the rice water weevil (*Lissorhoptrus oryzophilus*) are two important insect pests in rice production. To identify insect-responsive genes in rice, we performed a deep transcriptome analysis of Nipponbare rice leaves infested with both beet armyworm and water weevil using massively parallel signature sequencing (MPSS). Many antisense, alternative, and novel transcripts were commonly and specifically induced and suppressed in the infested tissue. Key genes involved in the defense metabolic pathways such as salicylic acid and jasmonic acid biosynthesis pathways

were up-regulated in the infested leaves. To validate the MPSS results, we analyzed the transcriptome of the rice leaves infested with water weevils using Solexa's sequencing-by-synthesis (SBS) method. The MPSS and SBS data were highly correlated (Pearson's correlation coefficient=0.85), and 83% of genes had similar gene expression in both libraries. Our comprehensive and in-depth survey of the insect-infested libraries provides a rich genomic resource for further analyzing the function of key regulatory genes involved in insect resistance in rice.

Keywords Beet armyworm · Water weevil · MPSS · SBS · Transcriptome analysis

R. C. Venu and M. Sheshu Madhav contributed equally to the project.

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Introduction

Herbivorous insects are responsible for destroying one fifth of the world's total annual crop production. Plants have evolved several layers of defense mechanisms against herbivorous insects (Mello and Silva-Filho 2002; Korth 2003). Understanding the molecular basis of these host mechanisms to insect attack is essential for effective control of insect damage in crop production. In the last decade, extensive research has revealed the expression pattern of defense-related genes in the infested plants by using different gene expression profiling technologies such as microarrays. Microarray-based genome-wide transcriptomic analyses have been performed in several plant species, including *Arabidopsis thaliana* (De Vos et al. 2005; Reymond et al. 2000, 2004; Stotz et al. 2000), bean (Arimura et al. 2000), *Nicotiana attenuata* (Voelckel et al.

2004), *Populus trichocarpa* × *Populus deltoides* (hybrid poplar; Major and Constabel 2006; Ralph et al. 2006), *Picea sitchensis* (Sitka spruce; Ralph et al. 2006), *Medicago truncatula* (Leitner et al. 2005), and rice (Yuan et al. 2008). Although many commonly induced or suppressed defense-related genes were identified in the plants infested with phloem-feeding or chewing insects in comparison with mechanical wounding, there were considerable differences in the transcriptomic response of infested plants to different insects (Zheng and Dicke 2008). For example, a similar number of differentially expressed genes (~200) were identified in *Arabidopsis* plants damaged by cell-content feeding thrips (*Frankliniella occidentalis*) and chewing-biting caterpillars (*Pieris rapae*), but the gene sets of those identified genes that responded to the two insects were quite different (De Vos et al. 2005). Interestingly, *Arabidopsis* plants showed a different defense response to insects with a similar feeding mode, such as aphids (*Myzus persicae*) and whiteflies (*Bemisia tabaci*) (Kempema et al. 2007). Moreover, transcriptomic changes in different cultivars after attack by the same insect were different (Broekgaarden et al. 2007). These results demonstrate the complexity of the defense mechanisms in plants after insect attack.

Plant defense responses to herbivory and wounding are often mediated by jasmonic acid (JA), salicylic acid (SA), and ethylene (ET; Walling 2000; Leon et al. 2001; Ryan 2000). For example, DNA microarray studies indicate that the JA pathway has a dominant role in regulating global changes in gene expression in response to both mechanical wounding and herbivory (De Vos et al. 2005; Devoto et al. 2005; Major and Constabel 2006; Ralph et al. 2006; Reymond et al. 2000, 2004). Emerging evidence indicates that phloem-feeding insects actively suppress jasmonate-based defenses (Thompson and Goggin 2006; Zarate et al. 2007). The role of SA in host resistance is less important with chewing insects than with phloem-feeding insects like aphids and brown plant hoppers, which induce SA-dependent responses (Zhang et al. 2004). ET also affects the expression of defensive proteins and secondary metabolites (Harfouche et al. 2006; Hudgins and Franceschi 2004; Winz and Baldwin. 2001). Relative to JA, however, ET production during herbivore attack is considered to play a minor role in the active defense response (von Dahl and Baldwin 2007). Transcript profiles elicited by phloem-feeding insects are markedly different from those induced by herbivorous insects from other feeding guilds and are generally associated with the activation of SA-responsive genes and weak expression of JA-responsive genes (De Vos et al. 2005; Gao et al. 2007; Kempema et al. 2007, Thompson and Goggin 2006).

In this study, we aimed to understand the common and specific transcriptional responses of rice plants to two important insect pests: the beet armyworm and the rice

water weevil. The beet armyworm feeds by chewing, and the rice water weevil feeds by scraping. The beet armyworm occurs throughout the USA east of the Rocky Mountains and can be a sporadic pest on rice plants in the southeastern USA. The rice water weevil, which is a much more serious threat to rice production than the beet armyworm, is distributed throughout North and South America. In addition, this species is now found in many Asian countries, including Korea, Japan, and China, where it is considered one of the most important invasive insect pests on rice.

To profile the transcripts expressed in the rice plants 24 h after infestation by armyworm and water weevil, we used massively parallel signature sequencing (MPSS) and sequencing-by-synthesis (SBS) technologies. We identified many up- or down-regulated genes that were commonly or specifically expressed in the rice plants infested by armyworm and water weevil. Some of these genes belong to different metabolic pathways involved in the production of SA, JA, ET, and other secondary metabolites. Our results provide the first comprehensive view of the transcriptome changes after insect infestation in rice plants based on two high-throughput sequencing methods. The identified candidate genes are excellent starting materials for further elucidating the function of important genes involved in the rice and insect interactions.

Results

Library characteristics and sequence matching analysis

About 1.0 to 1.2 million individual 17-base signatures were obtained in the four MPSS libraries (PLA, PLW, PLC, and NLD, Table 1). These signatures were processed with reliability and significance filters as described by Meyers et al. (2004a). A total of 46,904 distinct 17-base signatures were obtained from the MPSS libraries (Table 1 and Fig. 1). To compare the expression levels across the libraries, we normalized the frequency of signatures in each library to one million (transcripts per million or TPM). Figure 1 shows the number of pooled distinct signatures from the four MPSS libraries separated by reliability and significance filters and further grouped based on their match in the Nipponbare genomic sequence. When all the unique, reliable, and significant signatures (≥ 4 TPM) from the four libraries were clustered, a total of 37,532 unique signatures were obtained. Clustering of reliable significant signatures led to identification of 26,282 unique signatures that had only one hit in the genome (hits=1). A total of 5,358 reliable significant signatures matched the genome more than once (hits>1). About 5% of the signatures were significant unreliable, and 30% of them had genome

Table 1 Statistics of Insect-Infested and Control Rice MPSS and SBS Libraries

Technology	MPSS				SBS
	Beet armyworm-infested plants (PLA)	Water weevil-infested plants (PLW)	Mechanical wounded plants (PLC)	Unwounded control plants (NLD)	Water weevil-infested plants (SPLW)
Signature category					
Total sequenced	1,150,869	1,012,170	1,213,577	1,254,824	3,051,005
Distinct	21,365	20,282	18,202	20,791	99,837
Reliable	18,311	18,593	17,048	18,659	64,332
Unreliable	3,054	1,689	1,154	2,132	35,505
Significant	15,326	16,673	14,259	14,901	33,123
Nonsignificant	6,039	3,609	3,943	5,890	66,714
Reliable significant	14,480	15,912	13,779	14,395	31,955
Reliable nonsignificant	3,831	2,681	3,269	4,264	32,377
Unreliable significant	846	761	480	506	1,168
Unreliable nonsignificant	2,208	928	674	1,626	34,337
Distinct genes expressed ^a	7,941	8,871	7,738	8,146	13,964
1–100 TPM	19,370	17,865	16,147	18,132	96,570
101–1,000 TPM	1,826	2,251	1,874	2,497	3,002
1,001–10,000 TPM	160	160	168	153	254
>10,000 TPM	9	6	13	9	11

^a Genome-matched reliable significant signatures

matches (Fig. 1). Distinct reliable significant signatures in PLA (14,480), PLW (15,912), PLC (13,779), and NLD (14,395) were identified. Thirteen transcripts with expression level >10,000 TPM were expressed in the PLC library (Table 1).

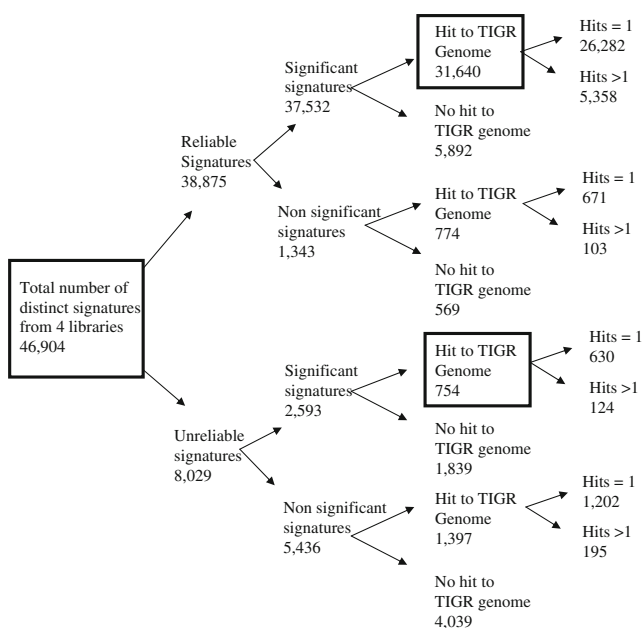


Fig. 1 Filter results for four MPSS libraries. A total of 46,904 distinct 17-base expressed signatures from four MPSS libraries were processed according to three filters—“significance,” “reliability,” and “genomic match”—as described by Meyers et al. (2004a).

All the reliable experimental signatures were matched to the rice genomic sequence to determine the precise location of expressed sense and antisense transcripts in the rice genome. About 87–90% of the signatures matched to the japonica (Nipponbare) genomic sequence (Table S2 of the Electronic Supplementary Material). Also, 80–83% of the signatures matched to rice annotated genes, of which nearly 73% and 15% signatures belonged to sense and antisense transcripts, respectively. Among them, about 5% represented both sense and antisense signatures. About 86% of the signatures matched to the existing ESTs at the TIGR database. In addition, based on the precise location/matching of the experimental signatures on the annotated genes, the number of sense (classes 1, 2, 5, and 7) and antisense (classes 3 and 6) signatures were identified in PLA (11,182 and 2,025), PLW (11,858 and 2,008), PLC (10,779 and 1,925), NLD (8,935 and 1,432), and SPLW (13,813 and 3,378; Table 2).

Expression pattern of antisense, alternative, and novel transcripts, and transcripts of transcription factors (TFs) after insect infestation

About 63–68% of the reliable signatures in the two libraries generated from infested plants matched the Knowledge-Based *Oryza* Molecular Biological Encyclopedia (KOME) full-length (FL) cDNAs. Among the matched signatures, about 57–60% were sense signatures and 10% were antisense (Table 2, Table S2 of the Electronic Supplementary Material), and about 2% matched both sense and antisense

Table 2 Classification of the Reliable MPSS Signatures from PLA, PLW, and PLC Libraries Based on their Location on the Annotated Genes

Signature category ^b	PLA		PLW		PLC		NLD		SPLW	
	Total signatures	Grouped by gene ^a	Total signatures	Grouped by gene ^a	Total signatures	Grouped by gene ^a	Total signatures	Grouped by gene ^a	Total signatures	Grouped by gene ^a
Class 1 (exon, sense strand)	5,335	4,707	5,861	5,161	5,206	4,534	5,758	5,090	5,868	5,250
Class 2 (500 bp 3'-UTR)	5,263	4,673	5,314	4,746	5,020	4,483	4,825	4,368	11,490	9,053
Class 3 (exon, antisense strand)	1,862	1,635	1,836	1,628	1,771	1,559	1,471	1,336	3,634	3,044
Class 4 (un-annotated region)	825	0	872	0	819	0	857	0	2,858	0
Class 5 (intron, sense strand)	404	383	498	473	412	390	485	452	2,095	1,781
Class 6 (Intron, antisense strand)	163	160	172	165	154	149	113	110	425	398
Class 7 (span splice site, sense strand)	180	178	185	183	141	139	173	171	337	328
Classes 1, 2, 5, 7 (Sense signatures)	11,182	8,786	11,858	9,284	10,779	8,407	11,241	8,935	19,790	13,813
Classes 3, 6 (antisense signatures)	2,025	1,769	2,008	1,773	1,925	1,691	1,584	1,432	4,059	3,378
Total	21,365	9,469	20,282	10,013	18,202	9,097	20,791	9,558	26,707	14,301

^a Grouped by gene including transposons

^b See Meyers et al. 2004a for class definitions

strands of the same full-length cDNA. Expression of the antisense transcripts was confirmed by matching the significant reliable signatures from each library with the rice antisense full-length cDNAs in the KOME database. The total number of genes with antisense transcripts was 1,769 in PLA, 1,773 in PLW, 1,691 in PLC, and 1,432 in NLD (Table 2), suggesting a significant induction of antisense gene expression in the insect-infested and wounded leaves. The specifically induced or suppressed genes with antisense in the PLA and PLW libraries were identified by comparison with the two control libraries (PLC and NLD). Forty antisense genes in PLA were ≥ 5 -fold induced, and none was suppressed relative to PLC and NLD, respectively (Table 3). Similarly, 44 and 3 genes were ≥ 5 -fold induced and suppressed in PLW, respectively, when compared to the two controls (Table 3). The identities of the genes encoding antisense transcripts with ≥ 5 -fold induction or suppression are listed in Table S3 of the Electronic Supplementary Material.

About 10–12% of the expressed genes showed alternative splicing when compared with the TIGR alternative-splice-form clusters. Among them, the genes with alternative transcripts that were induced or suppressed specifically in PLA and PLW relative to the two controls were identified (Table 3). A total of 223 and 40 specifically induced genes (≥ 5 -fold induction) produced alternative transcripts in PLA and PLW, respectively. Some of the pathogen defense-related genes, such as those encoding metallothionein-like protein type 2 (TC334871, TC323823, TC320546, TC311902, TC319184), nonspecific lipid transfer protein (TC327106), aspartic proteinase precursor (TC300646), BTH-induced protein phosphatase 1 (TC300268), thioredoxin (TC304211), calmodulin (TC338165), catalase (TC342436), Rho-GTPase-activating protein (TC355505), cysteine proteinase inhibitor 2 (TC315144), small GTP-binding protein (TC335268), and stress-related protein (TC341761), generated alternative transcripts (Table S3 of the Electronic Supplementary Material).

Table 3 Specifically Induced or Suppressed (Fivefold or More) Antisense Genes (with KOME Antisense Transcripts Support), Alternative Transcripts (with TIGR Alternative Transcripts Support)

Library	Antisense genes		Alternative transcripts		Transcription factor genes	
	Induced	Suppressed	Induced	Suppressed	Induced	Suppressed
PLA	40	0	223	15	137	94
PLW	44	3	40	71	166	80

and Genes Encoding Transcription Factors in Insect-Infested Plants Compared to Wound and Unwound Plants

The novel transcripts that matched the genome sequence but were not present in the KOME FL-cDNAs and TIGR-EST databases, and the novel genes that matched the genome sequence but were not present in the TIGR ESTs, KOME FL-cDNAs, and TIGR annotated rice genes, were searched in both PLA and PLW. About 1,000 novel transcripts and 1,200–1,300 novel genes were identified (Table S3 of the Electronic Supplementary Material).

The TF genes that were induced or suppressed in PLA or PLW compared to PLC and NLD are given in the Table S4 of the Electronic Supplementary Material. Some of the important stress-related transcription factor genes encoding LIM domain-containing protein (Os06g13030), heat shock protein (Os03g63750), zinc finger domain protein (Os03g55540), homeobox-leucine zipper protein (Os10g39030), and Myb-related transcription factor (Os01g09280) were highly induced in both PLA and PLW relative to PLC and NLD. However, some of the NAC domain-containing transcription factor genes (Os11g08210 and Os02g36880) were suppressed in both PLA and PLW libraries compared to PLC and NLD.

Promoter analysis of the genes responsive to insect infestation

Promoter analysis revealed the presence of many conserved *cis* motifs in the upstream regions of the up-regulated genes. In the 12 highly induced genes (≥ 50 -fold) in both PLA or PLW, 17 types of *cis* motifs were identified (Table 4 and Table S4 of the Electronic Supplementary Material). These motifs were highly represented in the promoters of the plus or minus strand of the 12 defense-related genes. The precise locations of the known *cis* elements in the promoter regions of all 12 genes are listed in the Table S4 of the Electronic Supplementary Material.

Identification of genes in the defense-related metabolic pathways

A network map of defense-related metabolic pathways was generated based on the biochemical pathways reported at the Gramene website (<http://www.gramene.org/>; Fig. 2). The important metabolic pathways responsible for the production of secondary metabolites including SA, JA, ET, and other hormones were integrated based on the genes identified in the four MPSS libraries. Genes that were at least 5-fold up- or down-regulated in PLA and PLW (relative to PLC and NLD) and that were involved in the production of these defense molecules are presented. Many genes involved in the biosynthesis of JA, like lipoxygenases (Os12g37290, Os08g39850, Os04g37430) and 12-oxophytodienolate reductase (Os06g11240), were up-regulated in both PLA and PLW libraries (Fig. 2). The key gene encoding phenylalanine ammonia-lyase (Os04g43760), which catalyzes the biosyn-

thesis of SA through L-phenylalanine, was up-regulated in both PLA (36-fold) and PLW (44-fold). In contrast, the gene encoding isochorismate synthase 1 (Os09g19734), which produces SA through chorismate, was down-regulated in both PLA (81-fold) and PLW (72-fold). However, many of the genes belonging to ET biosynthesis were down-regulated in both PLA and PLW, such as those encoding 1-aminocyclopropane-1-carboxylate synthase (Os01g55540, 58-fold), centromere/kinetochore protein zw10 (Os11g34310, 5-fold), tyrosine aminotransferase (Os11g42510, 14-fold), tyrosine transaminases (Os10g25140, 14-fold, Os09g28050, 6-fold), and 1-aminocyclopropane-1-carboxylate oxidase (Os09g27820, 25-fold). A large group of genes involved in brassinosteroid production, cytokinin production 7-*N*-glucoside biosynthesis, and phenylpropanoid biosynthesis were also highly expressed.

Genes commonly expressed in both beet armyworm- and water weevil-infested plants but not in wounded or untreated control plants

A total of 878 transcripts (653 genes) were 5-fold or more up-regulated and 371 transcripts (340 genes) were 5-fold or more down-regulated in both PLA and PLW, relative to those in the two control libraries (Fig. 3; Table S5 of the Electronic Supplementary Material). Among them, the known defense genes with 5-fold induction and commonly or specifically present in the two libraries from insect-infested rice are listed in Table 5. Among the defense genes, we observed the up-regulation of the genes encoding Bowman-Birk protease inhibitors (Os01g60730, Os01g04050), lipoxygenase (Os12g37260), nucleic acid binding protein (Os03g07370), terpene synthase 8 (Os04g27790), OsWRKY78—superfamily of rice TFs having WRKY and zinc finger domains (Os07g39480), metallothionein-like protein type 2 (Os01g05650), RING-H2 finger protein (Os01g60730), cysteine-rich receptor-like protein kinase (Os07g43560), and 4-coumarate-CoA ligase (Os01g67530; Table 5). Other genes belonging to secondary metabolite production were also up-regulated, including those encoding squalene monooxygenase (Os03g12910), tyrosine decarboxylase gene (Os07g25590), phenylalanine ammonia-lyase gene (Os04g4376), and *N*-acylethanolamine amide hydrolase (Os11g06900; Table 5; Table S5 of the Electronic Supplementary Material). Some of the genes involved in the protein degradation pathway were up-regulated in the plants infested with either pest but not in the wounded and untreated plants (Table 5; Table S3 of the Electronic Supplementary Material); these genes included 26S protease regulatory subunit 7 (Os06g09290), brix domain-containing proteins (Os01g33030), hexose carrier protein HEX6 (Os10g41190), tab2 protein (Os02g39740), F-box domain-containing proteins (Os09g32870, Os08g09760,

Table 4 Conserved *cis* Motifs in the Promoters of Beet Armyworm and Water Weevil-Induced Defense-Related Genes

GeneID	Os01g03320	Os01g04050	Os01g05650	Os01g09280	Os01g53810	Os01g72420	Os01g73450	Os02g24190	Os02g32200	Os03g07370	Os06g13030	Os07g53110
PLA	60	246	65	99	52	82	67	79	50	176	325	124
PLW	172	181	145	60	79	53	53	52	64	89	114	83
PLC	0	0	2	0	2	0	0	0	0	0	0	0
NLA	0	0	0	0	0	0	0	0	9	0	0	0
ARRIAT	(+)	(+)	(+)	(+)	(+)(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
BIHD1OS	(-)	(+)	(+)(-)	(+)	(+)(-)	(+)	(+)	(-)	(+)	(+)	(+)	(+)
CAATBOX1	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
CACTFPPCA1	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
DOFCOREZM	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
EBOXBNNAPA	(+)	(+)	(+)	(+)(-)	(+)(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
GATABOX	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)(-)
GTICONSENSUS	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)(-)
GTIGMSCAM4	(+)(-)	(+)	(+)(-)	(-)	(+)	(+)	(-)	(-)	(+)	(+)	(+)(-)	(+)(-)
GTGANTG10	(+)(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)(-)	(+)
IBOXCORE	(+)	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(+)(-)	(+)	(+)	(-)
MYCCONSENSUSAT	(+)(-)	(+)(-)	(+)(-)	(+)(-)	(+)(-)	(+)(-)	(+)(-)	(+)(-)	(+)(-)	(+)(-)	(+)(-)	(+)(-)
POLLEN1LELAT52	(+)(-)	(-)	(+)(-)	(+)	(+)(-)	(+)	(+)(-)	(+)(-)	(-)	(+)	(+)(-)	(+)(-)
ROOTMOTIFTAPOX1	(+)(-)	(+)(-)	(+)(-)	(+)(-)	(+)(-)	(-)	(+)(-)	(+)(-)	(+)(-)	(+)(-)	(+)(-)	(-)
TATABOX5	(+)(-)	(+)	(-)	(+)(-)	(+)	(-)	(+)	(+)	(+)(-)	(-)	(-)	(+)
WBOXNTERF3	(+)(-)	(-)	(+)	(-)	(+)(-)	(+)	(+)(-)	(+)(-)	(+)(-)	(+)	(-)	(+)(-)
WRKY71OS	(+)(-)	(+)(-)	(+)(-)	(-)	(+)(-)	(+)(-)	(+)(-)	(+)(-)	(+)(-)	(+)(-)	(-)	(+)(-)

Plus strand (+); Minus strand (-); Conserved *cis* elements present in all the 12 defense-related gene promoters are shown; Os01g03320 (Bowman-Birk-type bran trypsin inhibitor precursor; GATCTATTCGTCTATCG); Os01g04050 (Bowman-Birk-type wound-induced proteinase inhibitor WIP1 precursor; GATCTGTGTGATATACA); Os01g05650 (metallothionein-like protein type 2; GATCCAGTTACAAGTGA); Os01g09280 (myb-related transcription activator; GATCAATAAGGCTGATG); Os01g53810 (transferrin receptor-like dimerization domain-containing protein, GATCACTACACGATTCC); Os01g72420 (C2 domain-containing protein; GATCTCTTCTTGCAATD); Os01g73450 (uridylyate kinase; GATCTCTAGAGTTTTTA); Os02g24190 (cyclin-dependent protein kinase; GATCAITTTGTGTGGGA); Os02g32200 (thioesterase family protein; GATCACAAATGCTTCA); Os03g07370 (endonuclease/nucleic acid binding protein; GATCCGTATTAAGGCA); Os06g13030 (LIM domain-containing protein; GATCCAGCAGAACCTCA); Os07g53110 (calcium-dependent protein kinase, isoform 2; GATCCATCGAC TATGTT)

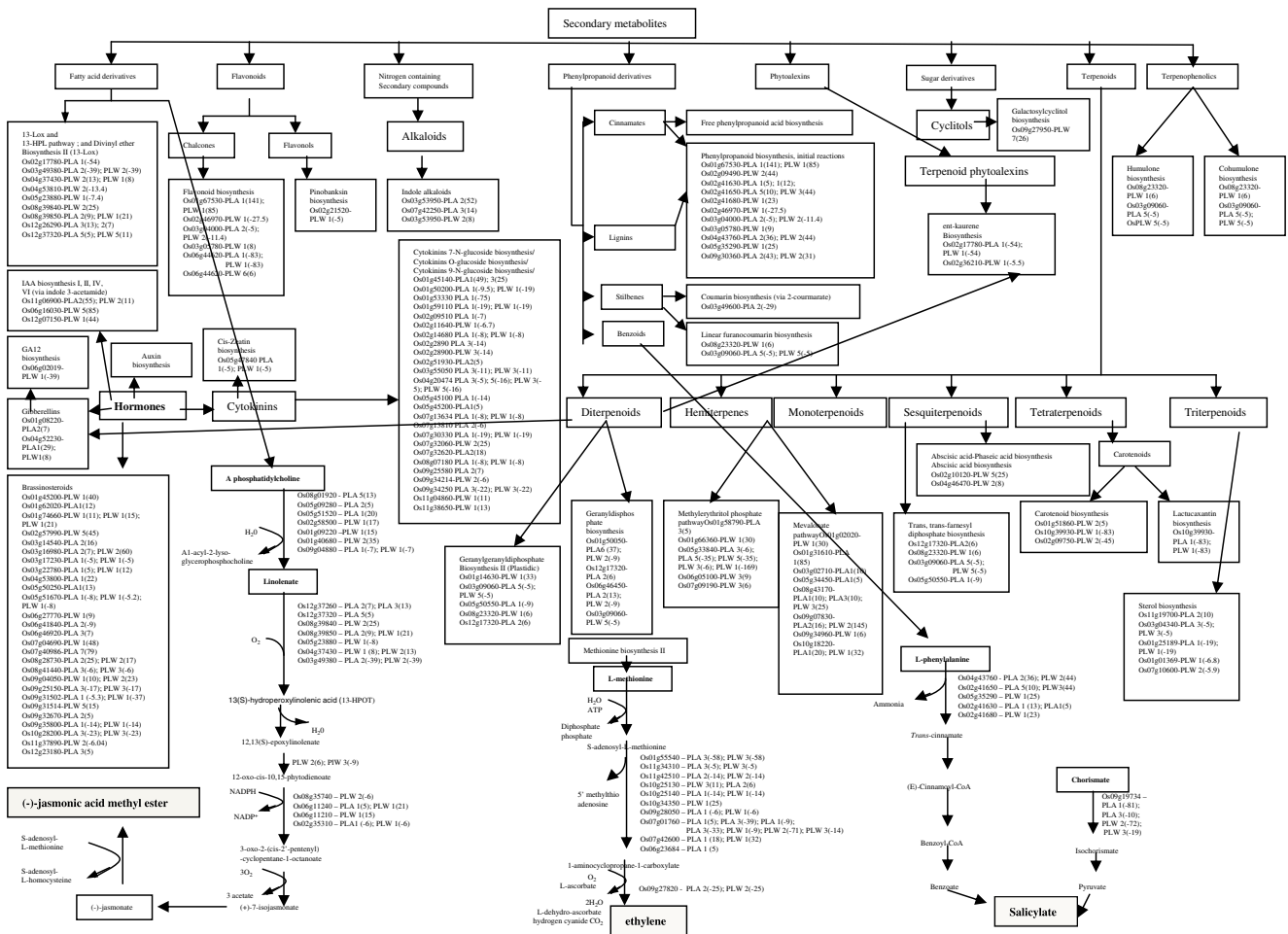


Fig. 2 Network of defense-related pathways showing the expression or suppression of key genes belonging to metabolism of secondary metabolites including salicylic acid, jasmonic acid, ethylene, and hormones. The genes that were up- or down-regulated 5-fold in PLA

or PLW are shown in parenthesis (*positive numbers* indicate up-regulated genes and *negative numbers* indicate down-regulated genes). The *number next to the library code* (PLA or PLW) shows the signature class as described by Meyers et al. (2004a).

Os11g32810, Os08g35960, Os11g07970), ubiquitin-conjugating enzyme E2N (Os01g48280), ubiquitin-conjugating enzyme E2S (Os06g45000), and ubiquitin ligase SINAT4 (Os03g24040). In addition, many genes involved in the

metabolism of cofactors and vitamins, carbohydrate metabolism, and energy metabolism were up-regulated in both kinds of insect-infested plants (Fig. S1 and Table S5 of the Electronic Supplementary Material).

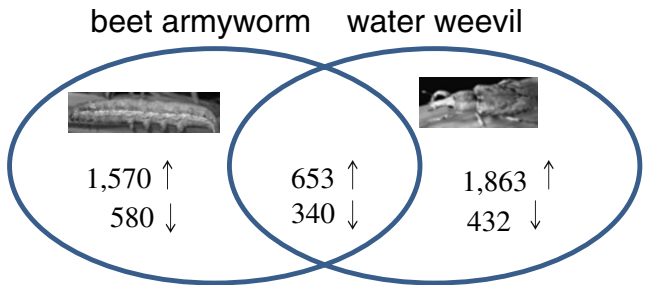


Fig. 3 Commonly and specifically induced and suppressed genes (5-fold relative to the controls) after beet armyworm and water weevil infestations. Commonly induced/suppressed genes were those induced/suppressed in both kinds of insect-infested plants while specifically induced/suppressed genes were those induced/suppressed in only one kind of insect-infested plant.

We also observed the induction of the NAD(P)H-dependent oxidoreductase gene (Os04g08550 and its six isoforms), which encodes a key enzyme involved in radical scavenging and the accumulation of reactive oxygen species. The induction seems to be specific to both insect infestations because expression of these genes did not increase in the mechanically damaged plants. The transcripts encoding several key JA biosynthetic enzymes like allene oxide synthase, allene oxide cyclase, and phospholipase D were up-regulated in rice after infestation by either insect (Fig. 2; Table 5; Table S5 of the Electronic Supplementary Material). Up-regulation was identified for several isoforms of the phenylalanine ammonia-lyase genes (Os04g43760, Os02g41650, Os05g35290, Os02g41630, and Os02g41680) involved in the SA biosynthesis pathway

Table 5 List of Defense-Related Genes Specifically and Commonly Induced in the Host After Beet Armyworm and Water Weevil Infestations

ID	Signature	PLA	PLW	PLC	NLD	Gene ID	Gene description
Genes commonly induced in both PLA and PLW							
1	GATCTGTGTGATATACA	246	181	0	0	Os01g04050	Bowman–Birk-type wound-induced proteinase inhibitor WIP1 precursor
2	GATCGATTTCATTGGG	206	119	11	0	Os05g31750	Annexin-like protein RJ4
3	GATCACAGTGTAGCGTG	178	526	2	0	Os12g37260	Lipoxygenase 2.1, chloroplast precursor
4	GATCCTGATTTAAGGCA	176	89	0	8	Os03g07370	Endonuclease/nucleic acid binding protein
5	GATCTGTAATTCGAGTT	146	218	0	0	Os07g43560	CRK10
6	GATCGTCGCGGAGGTGG	101	130	0	0	Os02g50770	Peroxidase 65 precursor
7	GATCGTGTGGTGGAGAG	82	132	2	0	Os04g27790	Terpene synthase 8
8	GATCATCAGAATTTGGT	70	102	18	3	Os07g39480	OsWRKY78—superfamily of rice TFs having WRKY and zinc finger domains
9	GATCCTGCCACTTGCCC	69	127	0	17	Os08g09860	FMN-dependent dehydrogenase family protein
10	GATCCAGTTACAAGTGA	65	145	2	0	Os01g05650	Metallothionein-like protein type 2
11	GATCATCCTCGCGGCGC	60	141	4	0	Os01g60730	RING-H2 finger protein ATL5A
12	GATCTATTCGCTATCG	60	172	0	0	Os01g03320	Bowman–Birk-type bran trypsin inhibitor precursor
Genes specifically induced in PLA							
13	GATCATGTAACTGTGG	231	0	0	0	Os07g40860	Vegetative cell wall protein gp1 precursor
14	GATCCATGGGCTGTACT	206	0	2	0	Os08g44020	Lyase
15	GATCAGTGGCAAGAAAC	174	0	0	0	Os12g44310	9,10-9,10 carotenoid cleavage dioxygenase 1
16	GATCTCTGCGCATGGTT	170	0	0	0	Os03g22810	Superoxide dismutase 1
17	GATCGACTTCTCCCATC	124	0	0	0	Os06g24990	Xylanase inhibitor protein 1 precursor
18	GATCGGCCACGACGACA	111	0	0	0	Os07g01660	Disease resistance response protein 206
19	GATCCGATGCTGTGTTG	103	25	12	0	Os08g04170	Zinc finger C-x8-C-x5-C-x3-H type family protein
20	GATCAACGAATTCAGCC	146	44	4	0	Os02g41860	Aquaporin PIP2.2
Genes specifically induced in PLW							
21	GATCTGCGATGAAGTGA	0	212	0	3	Os05g11320	Metallothionein-like protein type 3
22	GATCCACACAGTATAGC	0	195	0	0	Os06g16420	Amino acid transporter-like protein
23	GATCTCAGGGCGGAGGC	0	160	0	0	Os02g53420	Heat shock 70 kDa protein, mitochondrial precursor
24	GATCGAGCGCGGTTTCG	0	249	0	0	Os07g07320	Glutathione-S-transferase GSTU6
25	GATCAGCAGGATTAGGT	0	140	6	5	Os02g42690	Zinc finger, C3HC4-type family protein
26	GATCCTATGTTCAAAGA	9	147	0	0	Os02g40240	Leucine-rich repeat receptor protein kinase EXS precursor
27	GATCGCTCAATTTTTC	9	141	11	13	Os05g48970	C-terminal zinc finger
28	GATCATCTCGGCCGGGT	9	251	7	13	Os04g57880	DnaJ domain-containing protein
29	GATCTGTTTTGTTTGGT	2	108	14	10	Os06g03800	Ankyrin repeat domain-containing protein 28
30	GATCCCAAGTCGGCGT	11	110	4	0	Os02g46970	4-coumarate–CoA ligase 2

(Fig. 2; Table S5 of the Electronic Supplementary Material). In addition, calmodulin (Os06g06160) and a calcium-binding protein were also induced in plants infested with either insect (Table S5 of the Electronic Supplementary Material).

Genes differentially expressed in beet armyworm- and water weevil-infested plants

A total of 1,666 transcripts (1,570 genes) were specifically up-regulated (i.e., up-regulated in one kind of insect-infested plant but not the other), and 587 transcripts (580 genes) were specifically down-regulated in PLA compared with the two

controls (Fig. 3 and Table S5 of the Electronic Supplementary Material). Similarly 2,033 transcripts (1,863) were specifically up-regulated, and 444 transcripts (432 genes) were specifically down-regulated in the PLW library (Fig. 3 and Table S5 of the Electronic Supplementary Material). The genes encoding transcription factors containing known domains such as MADS, PLATZ, RWP-RK, SET, and ZIM were highly up-regulated in the beet armyworm-infested plants (Table 5 and Tables S4 and S5 of the Electronic Supplementary Material), whereas the genes encoding transcription factors with ABI3VP1, ARF, ARID, AUX/IAA, SNF2, SBP, TCP, TUB, and WRKY domains were highly up-regulated in water weevil-infested plants (Table 5

and Tables S4 and S5 of the Electronic Supplementary Material). Defense- or metabolism-related genes encoding vegetative cell wall protein gp1 (Os07g40860), 9,10 carotenoid cleavage dioxygenase (Os12g44310), superoxide dismutase (Os03g22810), fungal xylanase inhibitor (Os06g24990), and aquaporin PIP2.2 (Os02g41860) were specifically up-regulated in the beet armyworm-infested plants (Table 5 and Table S5 of the Electronic Supplementary Material). Up-regulation of some defense- or metabolism-related genes also occurred in the water weevil-infested plants; these genes encoded metallothionein-like protein type 3 (Os05g11320), glutathione-*S*-transferase (Os07g07320), zinc finger, C3HC4-type family protein (Os02g42690), leucine-rich repeat receptor protein kinase (Os02g40240), ankyrin repeat domain-containing protein (Os06g3800), and 4-coumarate-CoA ligase 2 (Os02g46970; Table 5 and Table S5 of the Electronic Supplementary Material).

Validation using RT-PCR and SBS

The expression pattern of 14 genes randomly selected from the PLA and PLW libraries were further evaluated using RT-PCR (see the gene list in Table S1 of the Electronic Supplementary Material). Four up-regulated and three down-regulated genes in PLA or PLW compared to the two controls were analyzed through RT-PCR. About 65% of the genes (nine genes) showed a similar expression pattern in both RT-PCR and MPSS data (Fig. 4a). It is noteworthy that the gene encoding allene oxide synthase (a marker gene for JA synthesis) and the gene encoding phenylalanine ammonia-lyase (a marker gene for SA synthesis) showed up-regulation in plants infested with both insects compared to the controls (Fig. 4a).

The SBS library SPLW was constructed using the same RNA used to construct the PLW MPSS library. A total of

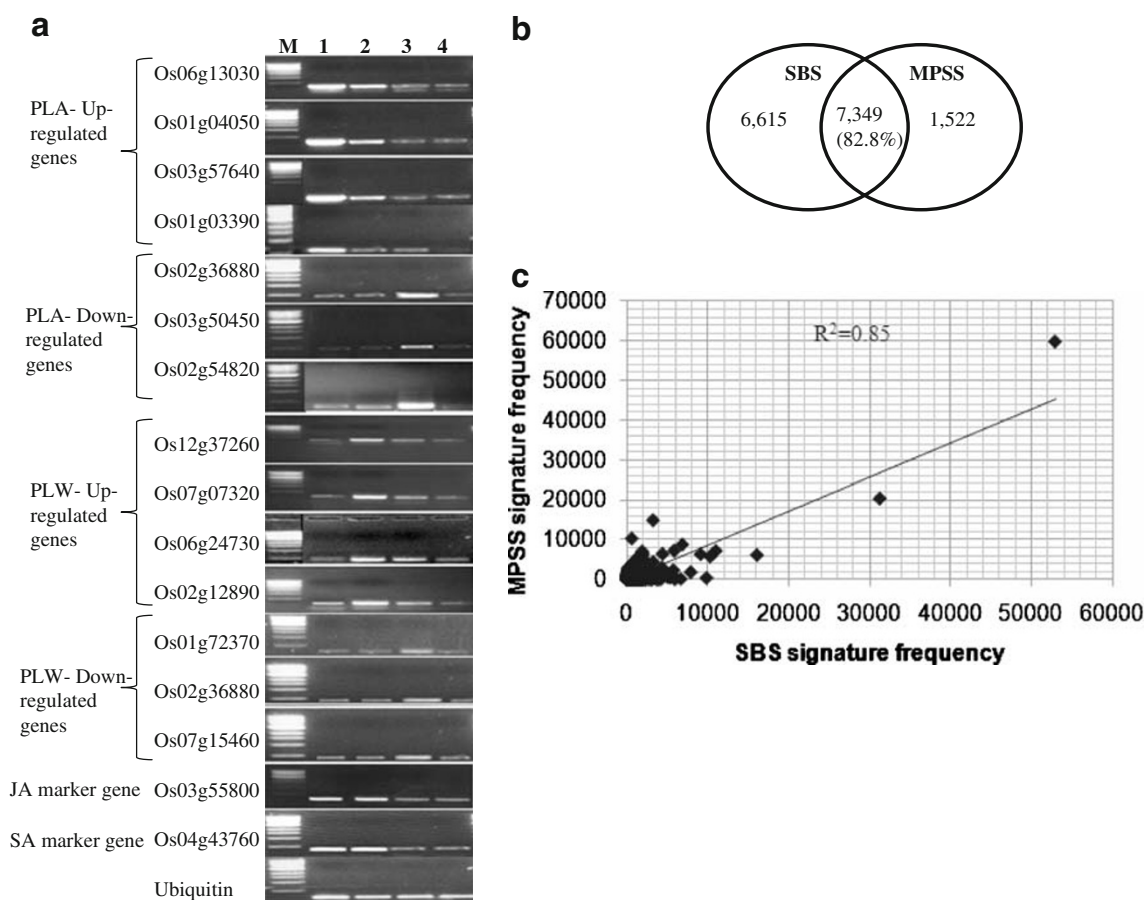


Fig. 4 Validation of MPSS data using RT-PCR and SBS analyses. **a** Validation of the MPSS tags identified in PLA and PLW using RT-PCR analysis. The RNA isolated from leaves of plants that were infested with beet armyworm (1), infested with water weevil (2), mechanically wounded (3), or unwounded (4) was used. M 1-kb size ladder. Ubiquitin gene was amplified as a loading control. **b** Commonly and specifically expressed genes in PLW (MPSS) and

SPLW (SBS). The distinct expressed genes identified in the SBS and MPSS libraries were used in the analysis. **c** Correlation of the gene expression patterns between the MPSS and SBS libraries. The genome-matched reliable and significant signatures from both MPSS and SBS libraries were subjected to Pearson's correlation analysis. Sixteen outliers that affected the correlation were removed based on regression analysis as described in Gowda et al. (2006).

three million signatures were obtained from the library, which is 3-fold greater than the number of reads in the PLW library (Table 1). The number of reliable significant signatures was about 2-fold greater in SPLW than in PLW (31,995 vs 15,912). Many of the low-copy signatures (1–100 TPM) were identified in the SBS library (96,570 in SPLW vs 17,865 in PLW). When the number of the annotated genes with reliable significant signatures was compared, the SBS library had about 57.4% more genes (13,964) than the MPSS library (8,871; Fig. 4b, Table 1), suggesting a much deeper coverage in the SBS library for transcriptome survey. Between the matched annotated genes in the two libraries, 7,349 (83%) genes were present in both libraries. The reliable significant signatures from SPLW and PLW libraries were compared using Pearson's correlation coefficient. A moderate correlation coefficient (0.65) was observed when MPSS and SBS expression data were compared without removal of any outlier transcripts. After removal of four outlier transcripts, the correlation coefficient was high (0.85; Fig. 4c; Table S6 of the Electronic Supplementary Material).

Discussion

With expected changes in climate and rice cropping systems, insect pests on rice are likely to become more epidemic and destructive in the future. Although insecticides are effective, undesirable environmental effects of insecticides and insect resistance to insecticides are becoming serious concerns in rice growing regions. It is clear that development of highly resistant cultivars is essential for sustainable rice production. However, the molecular basis of host resistance to insects in rice remains largely unknown. Yuan et al. (2008) identified 196 rice genes whose expression was significantly up-regulated by fall armyworm (*Spodoptera frugiperda*) caterpillars using a half-genome rice oligo microarray. The current study used two high-throughput sequencing techniques to provide the first large-scale and deep transcriptome analysis of rice plants infested with two insect pests. The deep-sequencing capacity of both techniques assured the collection of most transcripts in the rice tissues. Although MPSS and SBS are two different platforms, the transcriptomes generated by the two methods were highly correlated in our study. Many genes commonly or specifically induced or suppressed in the plants infested by the two insects have been identified. Novel genes were also obtained with antisense and alternative transcripts that are specifically expressed in the infested tissues. In addition, many highly and specifically expressed TF genes were found in the infested rice plants, and these genes may play important roles in regulating or coordinating insect-defense pathways or networks in rice.

Further elucidation of the function of these genes in host defense against insects will provide new insights into the molecular basis of insect resistance and novel genes for engineering insect-resistant rice.

We found that many genes involved in host-defense signaling pathways generate antisense transcripts after insect infestation. This kind of phenomenon was also observed in rice infected with the fungal pathogen *Magnaporthe oryza* (Gowda et al. 2007). However, the function of antisense genes in plant defense against pathogens and insects is unclear. In addition, we also observed alternative splicing in about 18% of the rice genes in the libraries of insect-infested rice and in about 14% of the rice genes in the library of uninfested rice. The importance of alternative splicing in the resistance to pathogens was found in tobacco and *Arabidopsis*; when the derivative of alternative splicing of the tobacco *N* gene and the *Arabidopsis* *RPS4* genes was silenced, the level of the *N*- and *RPS4*-mediated resistance was reduced or abolished (Jordan et al. 2002). In addition, R gene alternative splicing was dynamic during the defense response (Gassmann 2008). The function of both antisense and alternative transcripts identified in this study requires further detailed analysis in the defense response of plants to insect attack.

Terpenes are an important class of defense compounds that accumulate in plants after pathogen infection or arthropod-induced injury. Previous research has shown that Lepidopteran herbivory and oral factors induced transcripts encoding novel terpene synthases in *M. truncatula* (Gomez et al. 2005; Bede et al. 2006). Recently, Yuan et al. (2008) confirmed the induction of expression of seven of the 11 terpene synthase genes after fall armyworm infestation that was identified through the microarray experiments. Enzymes encoded by three TPS genes, Os02g02930, Os08g07100, and Os08g04500, were also biochemically characterized. In the current study, terpene synthase genes were induced in the host after both beet armyworm and water weevil infestations. In addition, we observed the induction of the Bowman–Birk family of proteinase inhibitors (BBPI) in both PLA and PLW libraries. BBPIs might contribute to plant defense against insect attack by inhibiting digestive enzymes of various insects. Transgenic plants expressing a BBPI gene had enhanced resistance to herbivory (Hilder et al. 1987). Genetic manipulation of the BBPI genes in transgenic rice may lead to new methods for insect control in rice production.

Various transcriptome analyses indicated that insect feeding elicits defense response in the host through SA-, JA-, and ET-regulated genes (Walling 2000; Moran et al. 2002; de Vos et al. 2007). The feeding of brown plant hoppers on rice up-regulates several genes involved in phenylpropanoid biosynthesis and genes required for sesquiterpene synthesis (Zhang et al. 2004; Cho et al.

2005). In tomato, aphid infestation up-regulates SA signaling (Li et al. 2006) while in *Arabidopsis* SA has been shown to have a neutral and negative effect on aphid and silver leaf whitefly growth, respectively (Pegadaraju et al. 2005; Zarate et al. 2007). Chewing insects largely induce JA because of the extensive damage caused by chewing (Howe 2004; Kessler and Baldwin 2002; Halitschke et al. 2003). In our study, many JA and SA biosynthetic genes were up-regulated in both PLA and PLW libraries, including the genes encoding phosphatidylcholine and linolenate 13(S)-hydroperoxylinolenic acid in the JA pathway and the genes encoding phenylalanine ammonia-lyase gene in the SA pathway. This up-regulation suggests an important role of both JA and SA in the response of rice to insect infestation. Endogenous ET has been shown to act as a cross-talk regulator with JA (Penninckx et al. 1998; Leon et al. 2001; Arimura et al. 2005, 2008). Enhanced production of ET has been reported in aphid-infested barley, which indicates active biosynthesis of this phytohormone in response to minimal wounding (Argandona et al. 2001). In the current study, however, the role of the ET-mediated signaling in insect-infested rice plants was unclear because the expression of the ACC synthase and ACC oxidase genes in the ET pathway was down-regulated. Nevertheless, the role of SA, JA, ET, and their cross-talks in the rice insect defense warrants further in-depth investigation.

MPSS has been used for whole genome transcription analysis in the last decade and has generated abundant data concerning expression in many organisms (Vega-Sanchez et al. 2007; Simon et al. 2009). Its complicated library construction procedure and high sequencing cost are two main limiting factors for the use in individual laboratories. As the cost of the next-generation sequencing methods has significantly decreased in the last few years, SBS sequencing has become a popular method for transcriptome analysis. To validate our MPSS results, we made and sequenced an SBS library using the same RNA sample that was used for the PLW library. Comparison analysis showed that about 83% of the genes were expressed in both MPSS and SBS libraries. Pearson's correlation analysis showed a high level of similarity (coefficient=0.85) in expression patterns of genes between these two platforms. However, SBS is a much better choice for transcriptome analysis because it costs 90% less than MPSS and generates 3-fold more transcripts. Furthermore, about 30% more transcripts have been found in the SBS library than in the MPSS library. Many of these additional signatures are low-copy transcripts, indicating that SBS is a powerful method for identifying rare transcripts. As the sequencing cost for SBS is further reduced in the future, SBS will likely become a routine transcriptomic analysis for many biological experiments.

Methods

Insect rearing, plant growth conditions, and insect infestations

Beet armyworm larvae were reared in the laboratory, and neonates were maintained on rice plants before third-instar larvae were used in the experiment. Rice water weevils were collected as adults from the field and maintained on rice plants in the greenhouse. Nipponbare rice plants (*Oryza sativa*) were grown in a greenhouse. When the plants were 6 weeks old, they were individually placed in 24 cages. Insects (100 army worms or 500 weevils per cage) were added to 12 of the cages (six cages for each kind of insect). When the insects were added to cages, the plants in six other cages were mechanically damaged with a hole punch; 2–5 mm were removed from leaf edges, and care was taken to avoid damaging the mid-vein. Leaves were damaged at intervals of approximately 4 cm along the leaf edge, and the treatment was repeated 30 min after the initial damage. The plants in the six remaining cages were untreated controls, i.e., they did not experience insect infestation or mechanical damage. All leaf tissue from all 24 cages was collected 24 h after the insects had been added to the cages and after the leaves had been initially wounded. Conditions during this 24-h period were the same as described earlier in this section.

RNA isolation and RT-PCR

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions. RT-PCR was performed as reported previously (Venu et al. 2007). PLA, PLW, PLC, and NLD refer to the libraries of plants infested with the beet armyworm, plants infested with the water weevil, mechanically wounded plants, and unwounded control plants, respectively (Table 1). Selected candidate genes that were up- or down-regulated in these four libraries were amplified by gene-specific primers, which are listed in Table S1 of the Electronic Supplementary Material.

MPSS and SBS library construction and bioinformatics

The total RNA isolated from beet armyworm-infested plants, water weevil-infested plants, mechanically wounded plants, and untreated control plants was used for the construction of MPSS libraries. In addition, the same RNA for the PLW library was used for the construction of the SBS (SPLW) library. The MPSS libraries were constructed and sequenced essentially as previously described (Brenner et al. 2000; Meyers et al. 2004a; b; Nobuta et al. 2007). The SBS library was constructed according to manufacturer's (Illumina) instructions with minor modifications. All data from the

MPSS and SBS libraries are deposited at our public websites: <http://mpss.udel.edu/rice/> and http://mpss.udel.edu/rice_sbs. The study used rice reference sequence (RefSeq) databases such as TIGR ESTs release version 17.0 (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=rice>), KOME FL-cDNA sequences (14, <http://cdna01.dna.affrc.go.jp/cDNA>), and release 5 of the TIGR pseudomolecules (23 January 2007) (ftp://ftp.tigr.org/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_5.0). The potential or “virtual” signatures were derived from the rice genome by extracting all occurrences of GATC plus the 14 nt sequence at the 3' terminus (16 nt in case of SBS). These signatures were used for matching analysis with the experimental MPSS or SBS signatures obtained in this study. All the virtual genomic signatures derived from the rice genome were assigned a “class” based on the position of the signature relative to annotated genes (Meyers et al. 2004a). Signatures that did not match to the genome corresponded to the “Class 0” signatures and those that matched the genome corresponded to Classes 1 to 7. The SAGEspy program (<http://www.osc.edu/research/bioinformatics/projects/sagespy/index.shtml>) was used to match the experimental MPSS signatures with the target rice databases to identify the sense, antisense, novel, and alternative transcripts from the MPSS libraries. Clustering analysis was done using in-house programs and Microsoft Access. The bioinformatics pipeline for the SBS data analysis was performed similar to MPSS data analysis with few modifications (Brenner et al. 2000; Meyers et al. 2004a; b; Nobuta et al. 2007). Classification of genes was done using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.jp/kegg/>).

Identification of antisense, alternative, and novel transcripts

To identify the antisense orientation of the MPSS signatures for the rice reference sequences, we converted all signatures into antisense orientation by a reverse complementation procedure. The antisense signatures from all the MPSS libraries were independently matched against the rice reference sequences. For validating identified antisense signatures from these MPSS libraries, we matched the antisense MPSS signatures against longer antisense rice FL-cDNAs available at the KOME database (Osato et al. 2003; <http://cdna01.dna.affrc.go.jp/cDNA/Analysis/antisenseweb/riceantisense.fasta>). If a single EST was represented by more than one MPSS signature, then all those signatures were considered as alternative splice/termination of the same gene. The alternative splice forms identified in the MPSS libraries were further confirmed by matching to the rice-alternative-splice-form clusters deposited at <http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/splnotes.pl?species=Rice>.

The reliable signatures that matched the rice genome but did not match rice gene expression databases like KOME FL-cDNAs and TIGR-EST databases were considered to be novel transcripts. Similarly, the genome-matched signatures were considered to be novel genes if they did not match the TIGR ESTs, KOME FL-cDNAs, and TIGR annotated rice genes.

Promoter analysis

To identify the targets/binding sites of insect-responsive transcription factors and the conserved *cis* elements among different up-regulated genes, we performed a promoter analysis of the genes commonly induced in both PLA and PLW. Regions 1.0 kb upstream of the expressed genes were extracted, and the *cis* elements within these DNA sequences were identified with the “PLACE Signal Scan Search” software (<http://www.dna.affrc.go.jp/htdocs/PLACE/>, Higo et al. 1999).

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