

A novel mechanism of gall midge resistance in the rice variety Kavya revealed by microarray analysis

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Abstract The Asian rice gall midge [*Orseolia oryzae* (Wood-Mason)] is an important rice pest causing an annual average yield loss of about US \$80 million in India. Rice varieties possess several discrete resistance (R) genes conferring resistance against the pest in two distinct ways, i.e., with (HR+ type) or without (HR– type) the expression of hypersensitive reaction (HR). The aim of the present work is to understand the molecular basis of compatible and incompatible (HR– type) rice gall midge interactions between the rice variety Kavya and the two gall midge biotypes: the virulent GMB4M and the avirulent GMB1 using transcriptional microarray gene expression analysis. A large number of differentially expressed genes (602 genes in incompatible interaction and 1,330 genes in compatible interaction with at least twofold changes, p value <0.05) was obtained from the microarray analysis that could be grouped into six clusters based on their induction during both or either of the interactions. MapMan software was used for functional characterization of these genes into 13 categories (BINs). Real-time polymerase chain reaction validation of 26 genes selected through the analysis revealed four genes viz. NADPH

oxidase, *AtrbohF*, cinnamoyl-CoA reductase, and von Willebrand factor type A domain containing protein coding genes to be significantly upregulated during the incompatible interaction. But most of the signature genes related to HR+ type resistance like salicylic acid pathway-related genes and disease resistance protein coding genes were downregulated. On the other hand, during the compatible interaction, genes related to primary metabolism and nutrient transport were upregulated and genes for defense and signaling were downregulated. We propose a hypothesis that HR– type of resistance in the rice variety Kavya against gall midge could be due to the constitutive expression of an R gene and a case of extreme resistance which is devoid of cell death. Compatible interaction, however, modulated a large number of differentially expressed transcripts to reprogram cell organization, cell remodeling, and relocation of nutrients through transport to support insect growth.

Keywords Nonhypersensitive resistance · Biotypes · Insect–plant interaction · Microarray · RT-PCR

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Introduction

The Asian rice gall midge, *Orseolia oryzae* (Wood-Mason), is an important rice pest. This insect is essentially a wet-season pest and can cause severe damage under favorable weather with high humidity and moderate temperatures, resulting in an estimated annual yield loss amounting US\$ 80 million in India (Bentur et al. 2003) or about US\$ 500 million in Asia (Herdt 1991). The genetics of rice gall midge interaction is well-characterized. Eleven resistance (R) genes (designated *Gm1* through *Gm11*) have been identified, so far, from different rice varieties (Himabindu et al. 2010). Seven distinct gall midge biotypes, differing in their virulence against these R genes have been reported (Bentur

et al. 2003; Vijaya Lakshmi et al. 2006). The interaction between the plant–insect is on gene-for-gene basis and defense response is triggered by initial feeding of the maggot within 48 h. Two distinct interactions are noted when gall midge attempts to feed on a rice plant. In compatible interaction, the rice variety is susceptible and the virulent insect establishes in the plant and a gall is formed. The insect is able to complete its development and emerge from the plant as an adult fly. In incompatible interaction, the rice variety is resistant and the avirulent insect fails to establish and is killed within the plant. Incompatibility is manifested in two distinct ways, i.e., with or without the expression of hypersensitive reaction (HR; HR+ or HR– type; Bentur and Kalode 1996). The HR+ type resistance response of the plant manifests in tissue necrosis at the feeding site and the insect mortality. In contrast, insect mortality in HR– type response is without tissue necrosis. Rice varieties carrying gall midge resistance genes such as *Gm1*, *Gm8*, and few other uncharacterized genes show HR– type response, while those carrying other known *R* genes exhibit HR+ type of response. Efforts are being made to identify and clone some of the gall midge resistance genes from the rice plant. However, the molecular basis of rice gall midge interactions is still to be deciphered. The rice variety Kavya, carrying *Gm1* gene, is resistant to four out of seven gall midge biotypes found in India and shows HR– type of resistance (Biradar et al. 2004). Development and cultivation of the gall midge-resistant rice varieties has been the main approach to mitigate the pest inflicted production loss. However, cultivation of resistant rice varieties with a single resistance gene has often led to evolution of new virulent biotypes (Vijaya Lakshmi et al. 2006). Pyramiding of two distinct and effective genes in a single cultivar is the strategy being adopted to counter this phenomenon.

Studies carried out so far on molecular basis of insect–plant interactions have shown induction of resistance through a complex network of signal perception, transduction, and induction of a cascade of downstream genes involved in the secondary metabolism and defense. Plant defense against insect herbivores has been generally reported to be distinct from that noted against plant pathogens (Gatehouse 2002). Response of plants against leaf-feeding insects has been found, akin to wounding, to be associated with jasmonic acid pathway while defense against plant sap sucking insects like aphids and the sedentary gall forming insects has been found associated with salicylic acid pathway (Walling 2000; Gatehouse 2002). Expression of HR as a manifestation of plant resistance is widely documented against pathogenic fungi (Alexander et al. 1993), bacteria (Ponciano et al. 2006), and viruses (Pfitzner and Goodman 1987). Involvement of HR in insect–plant interaction is also not uncommon (Grover 1995). Genotypic variations in HR expression have also been

reported in resistant germplasm (Bentur and Kalode 1996; Hoglund et al. 2005). Thus, plant resistance to pathogen or to a specific group of insects need not always to be HR mediated. HR-independent resistance (HR– type) has been reported in many plant–pest interactions. These include barley resistance against all races of powdery mildew, *Erysiphe graminis* sp *hordei*, conferred by the *mlo* gene (Freialdenhoven et al. 1994; Knogge 1996), the potato *Rx* gene-mediated resistance to potato virus X (Kohm et al. 1993) and wheat resistance to black rust pathogen *Puccinia triticina* conferred by *Lr34/Yr18* genes (Hulbert et al. 2007). The phenotype resulting from the *dnd1* mutation in *Arabidopsis* indicated that cell death was separate and independent of the resistance mechanisms leading to suppression of the pathogen. The phenotype of the *dnd1* mutant of *Arabidopsis* was loss of HR without loss of disease resistance (Yu et al. 1998). Also consistent with the distinction of HR and disease resistance were the effects of humidity and temperature that could inhibit the HR response without compromising resistance (Baulcombe et al. 1994; Hammond-Kosack et al. 1996). The cell death response may be to reinforce or stimulate the induction of defenses (Dangl et al. 1996; Alvarez et al. 1998) by activation of local and systemic acquired resistance (Staskawicz et al. 1995; Ryals et al. 1996).

Earlier, to gain insight into the molecular basis of gall midge resistance in rice, we have analyzed differentially expressed genes in the rice variety Suraksha with *Gm11* gene expressing HR+ type resistance using suppression subtractive hybridization library (Rawat et al. 2010; Rawat 2012). In continuation, we have now examined rice gall midge interactions in the rice variety Kavya adopting a transcriptome profiling strategy using microarray analysis. We observed that the signature genes typically involved in HR+ type resistance were downregulated in this incompatible interaction. Thus, this study reports a different mechanism of resistance. This study also covered compatible interaction which involved upregulation of the genes related to nutrient transport and metabolism.

Material and methods

Plant and insect material

The experimental material consisted of the rice (*Oryza sativa* L.) variety Kavya grown under greenhouse conditions at the Directorate of Rice Research, Hyderabad, India and the two gall midge biotypes (GMB1 and GMB4M) maintained on the appropriate differential rice cultivars and in physical isolation from each other, as described in detail by Vijaya Lakshmi et al. (2006) and Himabindu (2009). GMB1 is

avirulent while the GMB4M is virulent on Kavya. Uninfested Kavya plants were used as the control.

Experimental treatments and RNA extraction

The Kavya seedlings infested with GMB4M or GMB1 represented tissue samples for compatible or incompatible interactions, respectively. Seedlings under the same conditions but without infestation were used as uninfested control. Fifteen-day-old seedlings of the test cultivars were exposed to newly emerged female and male adult insects (25 female and 10 male adults on each tray) according to the standard procedure (Himabindu 2009; Rawat et al. 2010). Each experiment contained three independent sets of seedlings that were treated in the same way and at different times in different chambers as biological replicates. Larval hatching time was monitored with additional infested plants under the same growth chamber. Tissue samples were collected at four time points (24, 48, 96, and 120 h after larval hatching) from 20 plants and pooled for each replication. Only those plants with 10–15 larvae per plant were chosen for tissue collection. The time chosen for sample collection was based on the pest biology, 24 h is critical to determine whether a larva can live or die on a specific plant. Only the tissue at the feeding site (about 1.5–2 cm of the meristematic stem tissue) was collected for RNA extraction.

A portion of the seedlings in each experiment was kept for phenotypic confirmation later. Basal part of the plants, above the root, were cut and dissected to remove live and dead maggots and meristematic tissue from the feeding site was collected and immersed in RNALater (Ambion, USA). Total RNA was extracted from meristematic stem tissues by using TRIzol (Invitrogen, USA) according to the procedure provided by the manufacturer. The RNA samples were further purified with DNAase (Qiagen, GmbH, Germany) through an RNeasy Plant Mini kit according to the manufacturer's instruction (Qiagen, GmbH, Germany).

Microarray analyses

Transcriptome analysis was performed using Affymetrix GeneChip Rice Genome Arrays (Affymetrix), and three biological replicates were analyzed. RNA quality was verified using an Agilent Bioanalyzer (Agilent Technologies) and spectrophotometric analysis (NanoDrop ND-1000; NanoDrop Technologies) to determine concentration and the A260/A280 and A260/A230 ratios. Preparation of labeled copy RNA from 5 µg of total RNA, target hybridization, as well as washing, staining, and scanning of the arrays were carried out by Ocimum Biosolutions (Hyderabad, India) as described in the Affymetrix GeneChip Expression Analysis Technical Manual (http://media.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf).

Microarray normalization and data analysis

The hybridization data was analyzed using GeneChip Operating Software (GCOS 1.4) (http://media.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf). The scanned images were examined for any visible defects. Satisfactory images were analyzed to generate raw data saved as CEL files using the default settings of GCOS 1.4 from Affymetrix. A global scaling factor of 500, a normalization value of 1, and default parameter settings for the rice genome array were used. The detection calls (present, absent, or marginal) for the probe sets were made by GCOS. CEL files generated by GCOS were imported into Avadis 4.3 (Strand Life Sciences, India) for further analysis. Raw intensity data were initially normalized using the robust multichip average algorithm allowing gene expression levels with statistical significance ($p < 0.05$) above background level were recorded as present. For calculating the expression index of probe sets, we used the perfect match/mismatch model and setup “absent” by truncating the low-expression values to the tenth percentile of the probe set signals. The expression values were \log_2 transformed after calculating the expression index. The signal log ratio is the change in the expression level of a transcript between the control and experimental samples, and \log_2 (ratio) ≥ 1 (twofold change) or \log_2 (ratio) ≤ -1 (0.5-fold change). A linear model approach has been used for identifying differentially expressed genes for a specific threshold fold change (FC) and an adjusted p value. During analysis, the log fold change was set to 1 (FC=2) and the adjusted p value was set 0.05. The analysis was performed using “Limma” software from R-package (<http://www.r-project.org>). The locus information corresponding to the Affymetrix probe set IDs was obtained from the National Science Foundation rice nucleotide array project (<https://www.ricearray.org>) through the rice genome server of The Institute for Genome Research (TIGR; version 5.0). Functional analyses of the differentially expressed genes were done with the MapMan software based on the Wilcoxon Rank Sum Test.

Data submission and accession number

The microarray data has been deposited in NCBI's Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo>) and is accessible through GEO series accession number GSE22538.

Functional annotation and metabolic pathway analysis using MapMan software

Functional annotation and metabolic pathway analysis were performed using MapMan (Thimm et al. 2004; Usadel et al. 2005). MapMan was also used to identify the functional

categories associated with a set of sequences (e.g., differentially expressed) and to find the metabolic pathways or other cellular functions up- or downregulated in microarray experiments. Functional classification in the mapping file (Osa_affy_150909) that structures the rice gene from an Affymetrix into distinct metabolic and cellular processes from the MapMan program was used. Differentially expressed rice genes were functionally annotated by performing Basic Local Alignment Search Tool alignment against the TIGR database. MapMan software was employed to show the differences in gene expression in different cellular and metabolic process. Ratios were expressed in a \log_2 scale for importing into the software and changes in expression were displayed via a false color code.

Real-time PCR validation

The microarray results were validated via real-time polymerase chain reaction (PCR) with two types of samples: the same RNA samples that were used for microarray analysis and newly prepared RNA samples from independent infestations. Real-time PCR conducted with RNA samples prepared from independent infestations with two time points (24 and 120 h after gall midge infestation). Three micrograms of RNA was used for first-strand cDNA synthesis using the SuperScript[®] III RT enzyme (Invitrogen, USA) following the manufacturer's guidelines. Real-time PCR was performed using Applied Biosystems 7500 real-time PCR System with the SYBR green chemistry (Applied Biosystems, USA) according to the manufacturer's instructions.

Twenty-six genes having greater than twofold change when compared to control were further validated using quantitative PCR (qRT). Gene-specific primers for real-time PCR were designed using Primer Express[®] Software (Applied Biosystems; Table 1). Rice ubiquitin gene, *OsUbiq* (GenBank accession no. AK059694) was used as the endogenous control. Total real-time PCR volume of 20 μ l contained 10 μ l SYBR Green I PCR Master Mix (Applied Biosystems, USA), 500 nM each of forward and reverse primers and 20 ng of the cDNA samples. To calculate mean relative expression levels, cDNAs from three independent biological samples in three technical replications each were used. PCR was initiated with a pre-incubation at 50°C for 2 min and denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. The real-time PCR efficiencies for the reactions were >95%. A melting curve analysis was performed to determine the specificity of the reaction. Fluorescence data produced sigmoid-shaped amplification plots when plotted against the number of cycles. After normalization, quantity of each mRNA was calculated from

the threshold points located in the log-linear range. The data were analyzed using the 7500 Sequence Detection Software (Applied Biosystems, USA) with default baseline and threshold.

Results

Gene expression profiling through microarray analysis

Of the 51,279 probe sets contained in the Affymetrix Rice GeneChip, 50,382 hybridization signals were detected in our study. Total number of probe sets for analysis was reduced to 24,150 transcripts after removing probe sets with ambiguous signals and those that were not called "present" in at least two replicates. Among these transcripts, 602 and 1,330 genes recorded at least twofold changes in expression levels at a p value <0.05 in paired t tests (either up or down) between uninfested control vs. GMB1-infested plant samples representing incompatible interaction (ESM Table 1, S1) or between uninfested control vs. GMB4M-infested samples representing compatible interaction (ESM Table 1, S2), respectively. Thus, number of genes that were either up- or downregulated during compatible interaction was about two times greater than that during incompatible interaction. We also identified 236 upregulated and 224 downregulated genes common to both the interactions. However, only 553 and 44 genes showed upregulation only in compatible or only in incompatible interactions, respectively. In contrast, 317 and 98 genes were specifically downregulated only during these interactions, respectively (Fig. 1). Further, these up- or downregulated genes obtained from the microarray data were grouped into six expression clusters using Euclidean distance as distance measure and complete linkage algorithm (Fig. 2). Genes were analyzed for their putative function using rice database (rapdb.dna.affrc.go.jp). Out of 236 commonly upregulated genes in both the interactions, cluster I contained 126 genes with specific function and remaining 110 genes were from hypothetical, expressed protein, unknown function, or transposons class. Genes in this cluster were those involved in various cellular processes including metabolism, transport, and regulation of gene expression and/or signal transduction. Similarly, out of 224 commonly downregulated genes, cluster II contained 110 genes that were mostly related to oxidative burst and pathogenesis-related proteins, phenylalanine ammonia lyase, cytochrome P450—the signature genes known to play important role in HR+ type resistance. Out of 553 upregulated genes in compatible interaction, cluster III contained 385 genes that were mostly related to stress response, including various heat shock proteins, primary metabolism and nutrient transporters and those involved in regulation and/or signal transduction. Out of 317 downregulated genes

Table 1 Sequence information of the primers used for real-time PCR analysis

Sample no	Gene name	Probe ID	Primer sequences (5'–3')	T _m (°C)	Product size (bps)
1	Aldose 1-epimerase	Os.35113.1.S1_at	F: GGACCCACATGGCTTTGC R: ACTGGAAATGGTGGTGGATCA	59	66
2	Auxin response factor	Os.8374.1.S1_at	F:CATCCGAGTACGAGCTGAATCC R:TATCAGACAGGCAGGCGTACAG	62	123
3	Cytochrome P450 protein	Os.9691.1.S1_at	F:CAGCGTGCCAAGACCCATA R:CCATCGAGGCAACCATCCT	62	55
4	Transmembrane amino acid transporter	Os.47836.1.A1_at	F:TACGACGTCCGCTCATTG R:TTGTAGTCCTTCTGCTTGTTCCG	57	65
5	CCT/B-box zinc finger protein	Os.16422.1.S1_s_at	F:GCCAAGTTCTGGCTCTCCTT R:TGTCCATGTAGAACCCTACGC	60	64
6	von Willebrand factor type A domain	Os.48711.1.A1_at	F:AGTTTGTATCAGGAAGCTTGCT R:GCTATATTCCTTGACGGGTCCAT	59	80
7	Aquaporin protein	Os.12345.1.S1_s_at	F:GCCACCCTCTCTTCGCTT R:GCCACCATTGGTCAATTG	62	69
8	hsp20/alpha crystallin family protein 1	Os.7537.1.S1_at	F:CCTCCCCGGCGTCAA R:GGTGCCACTTGTCTGTTCTTGT	62	113
9	hsp20/alpha crystallin family protein 2	Os.4775.1.S1_at	F:ACGGTGGCAAGAAGAGCAT R:CTTCTTGGCCTGCTGGTTC	60	59
10	PAZ domain containing protein	Os.40026.1.S1_at	F:CATTGGTGAGGGACTTGAGTGTT R:GCGAAAGGCCATCTGTGT	60	71
11	THION2—plant thionin family protein	Os.8655.1.S1_at	F:CAAAGAGTTGTTGCCATCCA R:CGCGAAACGGCATGAGTTAT	59	59
12	Peptidyl-prolyl cis-trans isomerase	Os.15219.1.S1_at	F:CGCCACGATCCCTCACAT R:TGGTAGCCGAATCGCAACTT	62	70
13	Cinnamoyl-CoA reductase	Os.9685.1.S1_a_at	F:ACATCCTCGGCAAGCTCTTC R:TTCACCTCGTCAGAGCACCTT	61	61
14	Respiratory burst oxidase	Os.53380.1.S1_at	F:TGGGTCTCCAACACTTACGAAA R:CGTTGTCGTCTGGCTGAATTC	58	62
15	GDSL-like lipase/acylhydrolase	Os.54869.1.S1_at	F:CCATCACTGACACCGGGAAT R:GATGCCATACGGGAGATGCT	60	80
16	Glycosyl hydrolases family 16	Os.11465.1.S1_at	F:GGACGACACGCCGATCAG R:CACAGGCTGGAGTAGAGCTTCA	62	93
17	Hypersensitive-induced response protein	Os.32630.1.S1_at	F:TTGTTACGGTTGTTGCATCCA R:GATTTTGGGTTGCTCAGCTTGT	57	85
18	<i>OsMADS18</i> —MADS-box family gene	Os.4573.1.S1_at	F:CGGGAGGAGCAAAATGGA R:GAGCCGTCACGGTGTGGT	60	58
19	PPR repeat containing protein	Os.8261.1.S1_at	F:TCGCCAAGTACGGGACATTT R:GCAGATAGGCAGTGGGTGATG	59	90
20	Annexin	Os.46749.1.S1_x_at	F:GTCGCACCACGATGAACTTAGTC R:CCGCTGTTCTTCGCATCATA	60	75
21	Cytokinin dehydrogenase precursor	Os.46895.1.S1_at	F:GGCCCTTGATGGCATTGTAG R:ACCACCCACATCAGCATAAGAA	58	100
22	Ent-Kaurene synthase chloroplast precursor	Os.16233.1.S1_a_at	F:AGGATATCCCAGGCGAGGTT R:ACGCGGTAAACTTGCTTTCC	59	62
23	Flavonol synthase/flavanone 3-hydroxylase	Os.28435.4.S1_at	F:CAGGGCCTGCAGATCAAGAA R:GTGTGTCGCCAATGTTAACGA	59	85
24	Lipoxygenase	Os.13008.1.S1_at	F:GCCGGAACAAGGACAGGAA R:TCGGAGAAGGGCTTCATCAG	62	79
25	Xylanase inhibitor	Os.53616.1.S1_at	F:CTTCGAGCGCATCCACATC R:TCTCCACACCGTCCACTTGTC	62	88
26	<i>OsPRI0α</i>	Os.5031.1.S1_at	F:ACCATCTACACCATGAAGCTTAAC R:GTATTCCTCTTCATCTTAGGCGTA	60	334

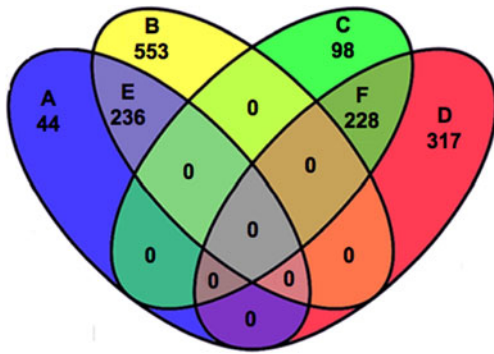


Fig. 1 Venn diagram representing common and specifically up- and downregulated genes in the rice variety Kavaya during compatible (after infestation with the rice gall midge biotype, GMB4M) and incompatible (after infestation with the rice gall midge biotype, GMB1) interactions. Numbers represent genes upregulated in incompatible (A), compatible (B), common to both (E) interactions and genes downregulated in incompatible (C), compatible (E), common to both (rice gall) interactions

of compatible interaction, cluster IV contained 156 genes which were mostly related to disease resistance proteins, Myb transcription factor, and AP2 domain containing proteins. Out of 44 upregulated genes of incompatible interaction, cluster V had 29 genes pertaining to respiratory burst protein, late embryonic abundant protein, NADPH oxidase, *AtrohF*, cinnamoyl CoA reductase, and TINY ethylene responsive transcription factor. Out of 98 downregulated genes of incompatible interaction, cluster VI contained 49 genes those were related to hypersensitive-related proteins,

flavonol synthase, thionin, OsWAK transcription factors, and transport-related genes.

Differentially responsive genes

The differentially expressed candidates were evaluated for their biological relevance through Gene Ontology (<http://www.geneontology.org/>; ESM Table 2) and KEGG Pathway analysis (<http://www.genome.jp>; ESM Table 3). Fisher's exact test was carried out to determine the significance of the GO term. We further analyzed the genes from each cluster that appear to be the major targets of differential regulation during compatible and incompatible interactions using MapMan software (Thimm et al. 2004; Usadel et al. 2005) with *O. sativa* mapping file. Mapping file functionally classified all the genes of Affymetrix rice chip in 36 major categories (BINs) and several subBINs. Out of 36 major BINs, 13 BINs involved in these interactions are described in detail.

Expression of genes common to both the interactions

Two functional categories "Transcription factors" (BIN 27) and "Development" (BIN 33) were found to be commonly upregulated (ESM Table 1, S3). However, compatible interaction induced larger number of genes compared to incompatible interaction. The main type of transcription factors commonly induced in both the interactions were AP2/

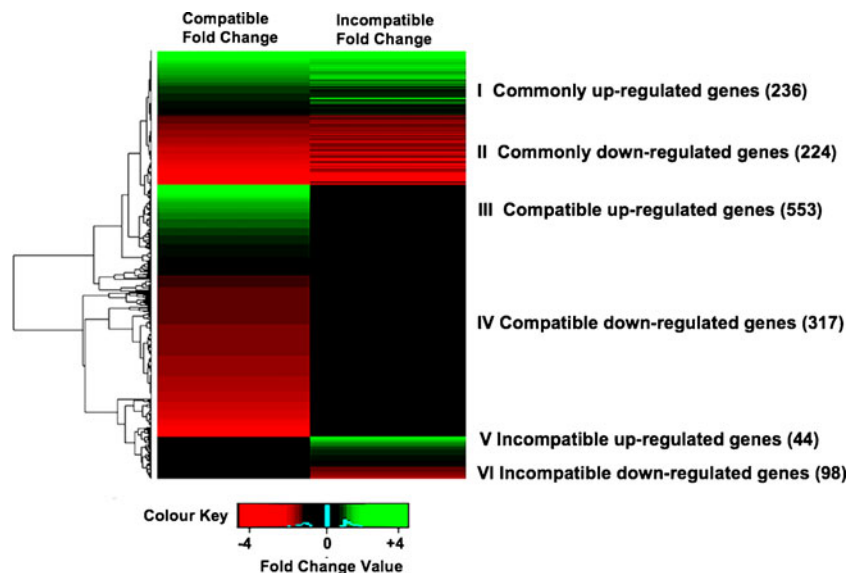


Fig. 2 Cluster display of differentially expressed genes during compatible and incompatible interactions of the rice variety Kavaya with gall midge biotypes GMB1 or GMB4M. Differentially expressed genes were grouped into six clusters: cluster I (upregulated) and cluster II (downregulated) in both the interactions; cluster III (upregulated) and cluster IV (downregulated) specific to the compatible interaction and,

cluster V (upregulated) and cluster VI (downregulated) specific to the incompatible interaction. Numbers correspond to differentially expressed genes in comparison with uninfested control. Genes were clustered using Euclidean distance and complete linkage algorithm. The cutoff values for grouping of up- and downregulated genes were kept at ≥ 4 - or ≤ 4 -folds, respectively

EREBP, MADS box transcription factor family, basic helix–loop–helix family, and Homeobox transcription factor family. But GRAS transcription factor family, WRKY domain transcription factor family, growth-regulating factor and histone deacetylase 2b in this BIN were induced only in compatible interaction. In “Development BIN” (BIN 33) genes coding for nodulin-like protein 5NG4, protein RCN1, protein MTN3 and MARD1 were commonly upregulated. However, genes of this BIN coding for embryogenesis-associated protein EMB8 in compatible interaction and late embryogenesis abundant protein D-34 in incompatible interaction were specifically upregulated. One gene encoding senescence associated protein DH was found to be commonly downregulated in both the interactions.

Five functional categories such as secondary metabolism (BIN 16), hormone signaling (BIN 17), defense-related genes (BIN 20), redox (BIN 21), and signal transduction (BIN 30) were found to be commonly downregulated in both the interactions (ESM Table 1, S4). In secondary metabolism (BIN 16), phenylpropanoids, chalcones, dihydroflavonols were prominent. However, few genes such as flavonol synthase/flavanone-3-hydroxylase and dihydroflavonol-4-reductase were downregulated only in incompatible interaction while isoflavone reductase was downregulated only in compatible interaction. Plant hormone signaling-related pathway genes (BIN 17) such as jasmonate synthesis-related gene lipooxygenase, gibberelin synthesis-related genes ent kaurene synthase, salicylic synthesis-related benzoate carboxyl methyltransferase, and phenylalanine ammonia lyase were commonly downregulated in both the interactions. Significantly, most of the genes in defense-related category (BIN 20) were found to be commonly downregulated in the interactions like those encoding for pathogenesis-related proteins (*PR1 β* and *PR10 α*), xylanase inhibitors, germin-like protein subfamily 1, hypersensitive-induced response protein. However, few genes of this BIN such as those coding for nucleotide-binding site–leucine-rich repeats (NBS–LRR) disease-resistance protein, von Willebrand factor type A domain containing protein and NADPH oxidase were upregulated in both the interactions. Genes of redox BIN (BIN 21) like catalase and L-ascorbate oxidase and L-ascorbate peroxidases were commonly downregulated. Genes related to signal transduction (BIN 30) such as receptor kinase DUF26, wall associated kinase, Ca⁺² signaling receptors were commonly downregulated. But few genes encoding for leucine-rich repeat III and G-protein receptor of the BIN were upregulated in both the interactions.

Expression of genes specific to the compatible interaction

Out of 13 BINs, genes present in the six BINs such as cell wall metabolism (BIN 10), primary metabolism (BIN 13), DNA synthesis and chromatin remodeling

(BIN 28), protein synthesis and turnover (BIN 29), cell organization and division (BIN 31), and transporters (BIN 34) were upregulated in compatible interaction (ESM Table 1, S5). However, none of the BINs was found to be downregulated. In BIN 10, genes related to cell wall degradation like pectate lyases and polygalacturonases and cell wall modification-related genes were upregulated significantly in compatible interaction. Large number primary metabolism (BIN 13) genes involved in glycolysis, TCA cycle, nucleotide, and lipid metabolism were upregulated in the compatible interaction. Genes such as replication factor, superfamily II DNA helicase, DNA glycosylase, DNA repair protein RAD51, and exonuclease of DNA synthesis and chromatin remodeling BIN (BIN 28) were upregulated during this interaction. In protein synthesis and turnover BIN (BIN 29) genes of different sub-BINs like protein activation, protein synthesis, protein targeting, post-translational modification, and protein degradation were upregulated in compatible interaction. In cell organization and division BIN (BIN 31), genes related to cell division such as microtubule-associated protein MAP65-1a, meiotic recombination protein DMC1, and genes related to cell cycle such as cyclin-A2, CYCD6, cyclin delta-3, and cyclin-dependent protein kinase were upregulated. A large number of genes from transporters category (BIN 34) such as amino acids transporters, metabolite transporters, peptides and oligopeptides, ABC transporters, auxin hydrogen symporter, antiporter/drug transporter, and phosphatidylinositol transporter were upregulated in compatible interaction. Further, defense-related (BIN 20) abiotic stress responsive genes (BIN 20.2) such as heat shock proteins and chaperones were also upregulated.

Expression of genes specific to the incompatible interaction

Genes specifically modulated in incompatible interaction, were relatively few in number (44 upregulated and 98 downregulated). Among the upregulated genes, biotic stress and respiratory burst (BIN 20.1.1) genes, *AtrbohF*, cytochrome P450 protein encoding genes, respiratory burst oxidase and cinnamoyl-CoA reductase were present (ESM Table 1, S6). Protein synthesis and degradation (BIN 29)-related genes such as cysteine proteinase 2 and serine carboxypeptidase 1 were also upregulated. From primary metabolism category (BIN 11), phospholipase D beta 1, lipid-binding protein, and aldose 1-epimerase of lipid metabolism were upregulated. BINs for secondary metabolism (BIN 16), salicylic acid (SA) hormone signaling (BIN 17), and defense- (BIN 20) and transport-related (BIN 34) genes were noticeably downregulated in the incompatible interaction.

Validation of selected transcripts through real-time PCR

Microarray results of 26 differentially expressed genes from both the interaction were validated using real-time PCR (Tables 1 and 2). The real-time PCR results were generally in agreement with microarray data as shown in ESM Fig. S1. Correlation value was 0.25 and 0.22 between microarray data and real-time PCR data at 24 and 120 h time point, respectively, which were significant at $p \leq 0.05$.

Genes modulated at 24 h after infestation

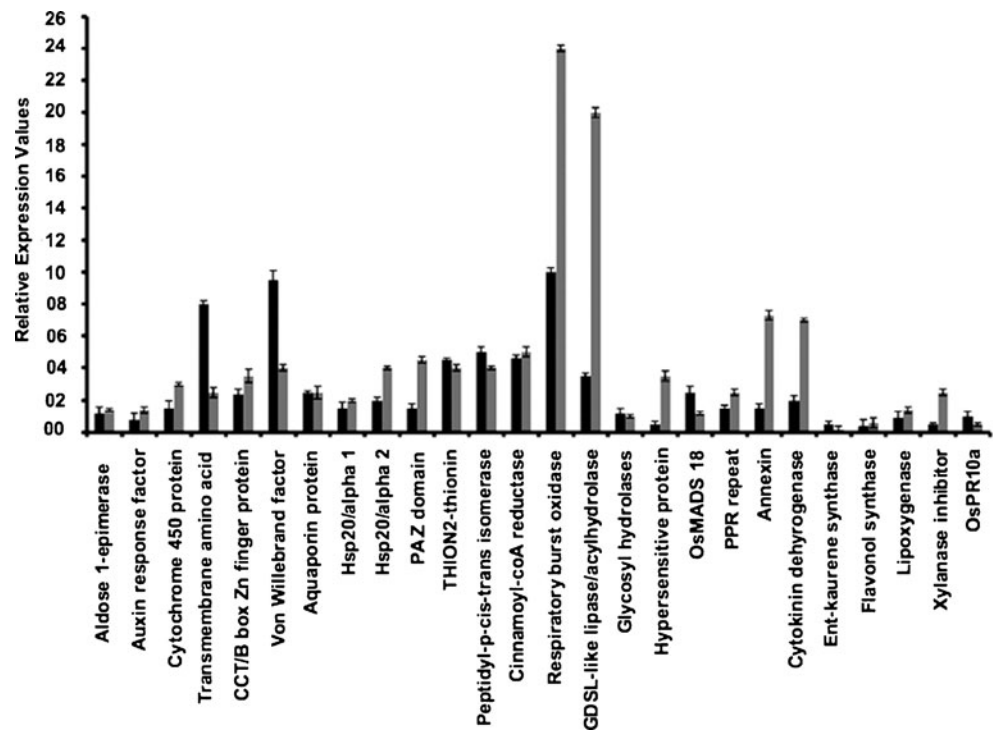
Out of 26 genes, 10 genes in compatible interaction and four genes in incompatible interaction were upregulated with high fold values (Fig. 3). Out of 10 upregulated genes in compatible interaction, six genes were from BIN 20 viz., xylanase inhibitor, PAZ domain containing protein, respiratory burst oxidase, cytochrome P450 protein, hypersensitive-induced

response protein, and hsp20 alpha 2 proteins; the other four genes were coding for GDSL lipase (BIN 13), cytokinin dehydrogenase (BIN 17), PPR repeat containing protein (BIN 27), and annexin (BIN 31). The four genes upregulated in the incompatible interaction were von Willebrand factor A containing protein (BIN 20, 9.5-fold), MADS transcription factor (BIN 27, 2.5-fold), peptidyl prolyl cis/trans isomerase (BIN 31, fivefold), and transmembrane amino acid transporter (BIN 34, eightfold; Fig. 3). Four genes upregulated in both the interactions with similar fold values were for cinnamoyl-CoA reductase (BIN 20), thionin (BIN 20), CCT/B-box Zn finger protein (BIN 27) and aquaporin protein (BIN 34). Four genes were found to be downregulated in both the interactions such as flavonol synthase/flavanone 3-hydroxylase (BIN 16), entkaurene synthase (BIN 17), *OsPRI0α* (BIN 20) and Lipoxygenase (BIN 29). The remaining four genes such as Aldose-1-epimerase (BIN 13), glycosyl hydrolase 16 (BIN 13), hsp20 alpha 1 protein (BIN 20), and auxin response factor

Table 2 Relative expression values (Fold values) for the selected ESTs at 24 h and 120 h in the rice variety Kavya upon infestation by GMB1 and GMB4M when compared with respective uninfested controls

Sample no	Gene name	Probe ID	Fold values after GMB1 infestation		Fold values after GMB4M infestation	
			24 h	120 h	24 h	120 h
1	Aldose 1-epimerase	Os.35113.1.S1_at	1.2	1.2	1.4	1.4
2	Auxin response factor	Os.8374.1.S1_at	0.8	0.4	1.4	1.5
3	Cytochrome P450 protein	Os.9691.1.S1_at	1.5	0.5	3.0	1.4
4	Transmembrane amino acid transporter	Os.47836.1.A1_at	8.0	5.0	2.5	18.0
5	CCT/B-box zinc finger protein	Os.16422.1.S1_s_at	2.4	1.2	3.5	2.5
6	von Willebrand factor type A domain	Os.48711.1.A1_at	9.5	5.5	4.0	5.0
7	Aquaporin protein	Os.12345.1.S1_s_at	2.5	0.2	2.5	0.3
8	hsp20/alpha crystallin family protein 1	Os.7537.1.S1_at	1.5	0.4	2.0	0.4
9	hsp20/alpha crystallin family protein 2	Os.4775.1.S1_at	2.0	0.2	4.0	0.2
10	PAZ domain containing protein	Os.40026.1.S1_at	1.5	0.2	4.5	0.3
11	THION2—plant thionin family protein	Os.8655.1.S1_at	4.5	4.5	4.0	8.5
12	Peptidyl-prolyl cis-trans isomerase	Os.15219.1.S1_at	5.0	1.7	4.0	0.5
13	Cinnamoyl-CoA reductase	Os.9685.1.S1_a_at	4.6	7.0	5.0	3.0
14	Respiratory burst oxidase	Os.53380.1.S1_at	10.0	0.5	24.0	0.4
15	GDSL-like lipase/acylhydrolase	Os.54869.1.S1_at	3.5	1.5	20.0	2.0
16	Glycosyl hydrolases family 16	Os.11465.1.S1_at	1.2	2.0	1.0	3.5
17	Hypersensitive-induced response protein	Os.32630.1.S1_at	0.5	0.1	3.5	0.2
18	<i>OsMADS18</i> —MADS-box family gene	Os.4573.1.S1_at	2.5	5.0	1.2	15.0
19	PPR repeat containing protein	Os.8261.1.S1_at	1.5	0.5	2.5	2.5
20	Annexin	Os.46749.1.S1_x_at	1.5	1.0	7.3	2.5
21	Cytokinin dehydrogenase precursor	Os.46895.1.S1_at	2.0	1.0	7.0	2.5
22	Ent-kaurene synthase chloroplast precursor	Os.16233.1.S1_a_at	0.5	0.1	0.2	0.2
23	Flavonol synthase/flavanone 3-hydroxylase	Os.28435.4.S1_at	0.4	0.2	0.6	0.2
24	Lipoxygenase	Os.13008.1.S1_at	0.9	0.2	1.4	0.2
25	Xylanase inhibitor	Os.53616.1.S1_at	0.5	0.2	2.5	0.3
26	<i>OsPRI0α</i>	Os.5031.1.S1_at	1.0	1.2	0.5	1.0

Fig. 3 Relative expression profiles of 26 genes in the rice variety Kavya at 24 h after gall midge infestation as revealed by real-time PCR. Error bars mean±SD. Dark bars incompatible (Kavya-GMB1), light bars compatible (Kavya-GMB4M) interaction



(BIN 27) were not significantly modulated in either of the interactions.

Genes modulated at 120 h after infestation

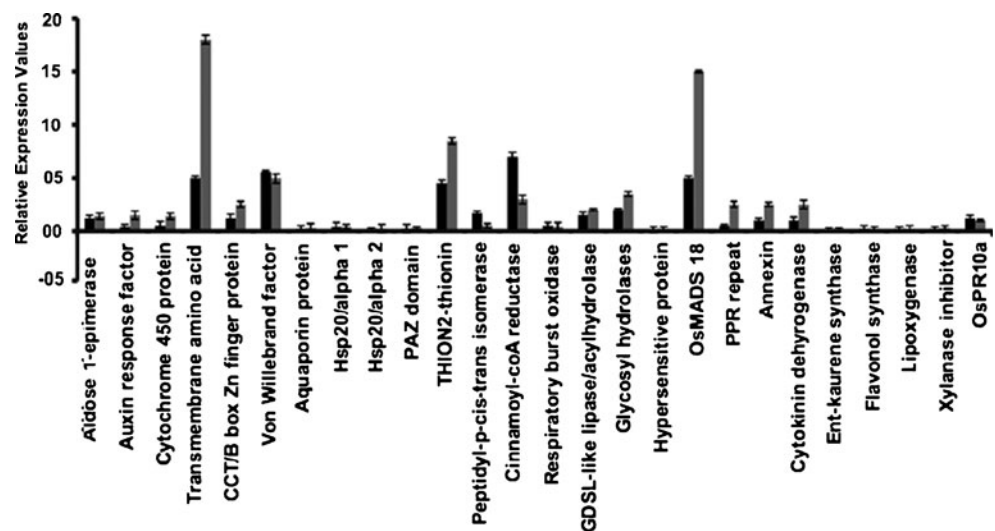
At 120 h after gall midge infestation, 15 genes were down-regulated in both the interactions (Fig. 4). Nine genes were upregulated in compatible interaction. Among these, three genes such as transmembrane amino acid transporter (18-fold, BIN 34), MADS transcription factor (15-fold, BIN 27) and thionin (8.5-fold, BIN 20) had very high fold values while the remaining six genes were induced with 2- to 2.5-fold value in compatible interaction. Two genes coding for

von Willebrand factor A containing protein (BIN 20, 5.5-fold) and cinnamoyl-CoA reductase (BIN 20, sevenfold) were upregulated in incompatible interaction.

Discussion

Plants have developed diverse mechanisms of biotic stress resistance mediated through either jasmonic acid or SA pathways and with or without expression of HR. Most of but not all, *R*-gene-triggered resistance is accompanied with HR. Insect resistance with HR-like symptoms have also been observed in cases such as wheat (*Triticum aestivum*)

Fig. 4 Relative expression profiles of 26 genes in the rice variety Kavya at 120 h after gall midge infestation as revealed by real-time PCR. Error bars mean±SD. Dark bars incompatible (Kavya-GMB1), light bars compatible (Kavya-GMB4M) interaction



resistance to the Hessian fly (*Mayetiola destructor*; Grover 1995), peach (*Prunus persica*) resistance to the aphid *Myzus persicae* (Sauge et al. 1998), rice (*O. sativa*) against the gall midge *O. oryzae* (Bentur et al. 2003), willow (*Salix viminalis*) tree varieties against the gall midge (*Dasineura marginemtorquens*; Hoglund et al. 2005) and bean (*Phaseolus vulgaris*) resistance to the bean-pod weevil (*Apion godmani*; Garza et al. 2001). Cell death that occurs during HR+ type resistance is reported to be controlled by the oxidative burst (Atkinson et al. 1996; Heath 2000), production of NO (Delledonne et al. 2002), and the interaction between some of these different signaling pathways (Delledonne et al. 2002). Despite the observation that several resistance responses (RR) are accompanied by HR, the signal transduction requirement for the RR and HR can be different depending on the host–pathogen combination (Greenberg and Yao 2004). Host cell death occurs in most of the cases, but not in all the reported interactions between plant and pathogens. Non-cell death or HR– type resistance are rare (Yu et al. 1998), though. However, the rice gall midge interactions involve both HR+ and HR– types of resistance.

The present study revealed unique features of HR– type of resistance in the rice variety Kavya against the gall midge at the molecular level. HR– type of resistance as studied in potato carrying *Rx* gene against potato virus X revealed that resistance conferred by *Rx* gene against potato virus X is likely due to the involvement of NBS–LRR protein (Bendahmane et al. 1999) as noted for other *R* genes that conferred HR+ type of resistance. Upregulation of phenylpropanoid pathway and most of the typical *R* gene-mediated pathways were also observed in microarray study conducted on wheat expressing HR– type resistance conferred by *Lr34/Yr18* gene against the rust *P. triticum* (Hulbert et al. 2007; Bolton et al. 2008). In both the cases, defense-related genes were also induced including those encoding various pathogenesis-related (PR) proteins. The response conditioned by these genes is reminiscent of some components of HR+ type resistance but without the accompanying localized cell death. Such type of phenotype was also observed in *Salix*–*Dasineura* interaction which clearly suggested that hypersensitivity and symptomless resistance (HR– type) are two different mechanisms (Hoglund et al. 2005).

In contrast, our microarray studies revealed downregulation of secondary metabolism (BIN 16), hormone signaling (BIN 17) mainly SA pathway (BIN 17.8) and defense-related genes (BIN 20) and these results have been further validated in real-time PCR studies involving HR– type resistance in the rice variety Kavya against GMB1. Thus, HR– type resistance in the rice variety Kavya did not merely suppress cell death but also suppressed other defense-related genes as in case of compatible interaction. This is a novel and unique observation for any plant–pest interaction reported in the literature. It is important to note that SA-

mediated pathway, phenylpropanoid pathway, secondary metabolism, and defense-related genes such as PAL, NBS–LRR, and *OsPR10α* were induced in rice variety Suraksha expressing HR+ type of resistance against GMB4 biotype of gall midge (Rawat et al. 2010). We have also documented that *OsPR10α* was upregulated only in HR+ type resistance in Suraksha possessing *Gm11* gene but not in HR– type resistance in the rice variety Kavya with gall midge resistance gene *Gm1* (Rawat et al. 2010).

Based on the above information, we propose a hypothesis to understand the HR– type resistance mechanism in Kavya against rice gall midge. The HR– type of resistance could be due to the expression of a “constitutive R gene” and could be a result of “extreme resistance” which does not require invoking of a cell death mechanism. The base of this hypothesis is explained based on our microarray and real-time PCR results.

In the present study, we found evidence for strong transcriptional changes in the compatible interaction and a weaker response in the incompatible interaction as 870 and 142 transcripts, respectively, were specifically modulated while 460 transcripts were commonly induced (Fig. 1, ESM Table S1) in these interactions. Thus, the transcriptome of the rice variety Kavya responded weakly to the avirulent biotype of gall midge. This weak transcriptome response is consistent with recent findings in tomato (*Solanum lycopersicum*) and powdery mildew fungus interaction (Li et al. 2006; Fung et al. 2008). This suggests that transcripts involved in incompatible interaction could be constitutively expressing at high or low levels in this variety and only weakly modulated in response to the gall midge attack. Thus, *Gm1* conferred resistance in the rice variety Kavya could be constitutive, unlike *Gm11* conferred resistance in Suraksha which was distinctly induced upon pest infestation.

Further, it is also hypothesized that, in some host–pathogen interactions, a phenomenon called “extreme resistance” induces sufficient *R* protein signaling to arrest the pathogen without cell death (Bendahmane et al. 1999). Thus, some RR are extremely robust to arrest the pathogen without cell death. Torres et al. (2002) considered HR as the function of a weak or strong *R* gene. The weak *R* gene initiates cell death by triggering molecules related to hypersensitivity followed by *R* signaling. The response of the strong *R* gene, despite less production or depletion of ROS, was sufficient enough to trigger the *R* signaling. This concept is in line with findings of Bendahmane et al. (1999), demonstrating that HR occurrence and magnitude can be correlated directly to *R* and *Avr* protein levels. We suggest that *Gm1* conferred resistance that could be a mode of “extreme resistance” which is able to kill the insect without requiring cell death in the host as *R* proteins constitutively expressed (due to *Gm1* resistance gene) are sufficient to kill the insect without requiring HR expression.

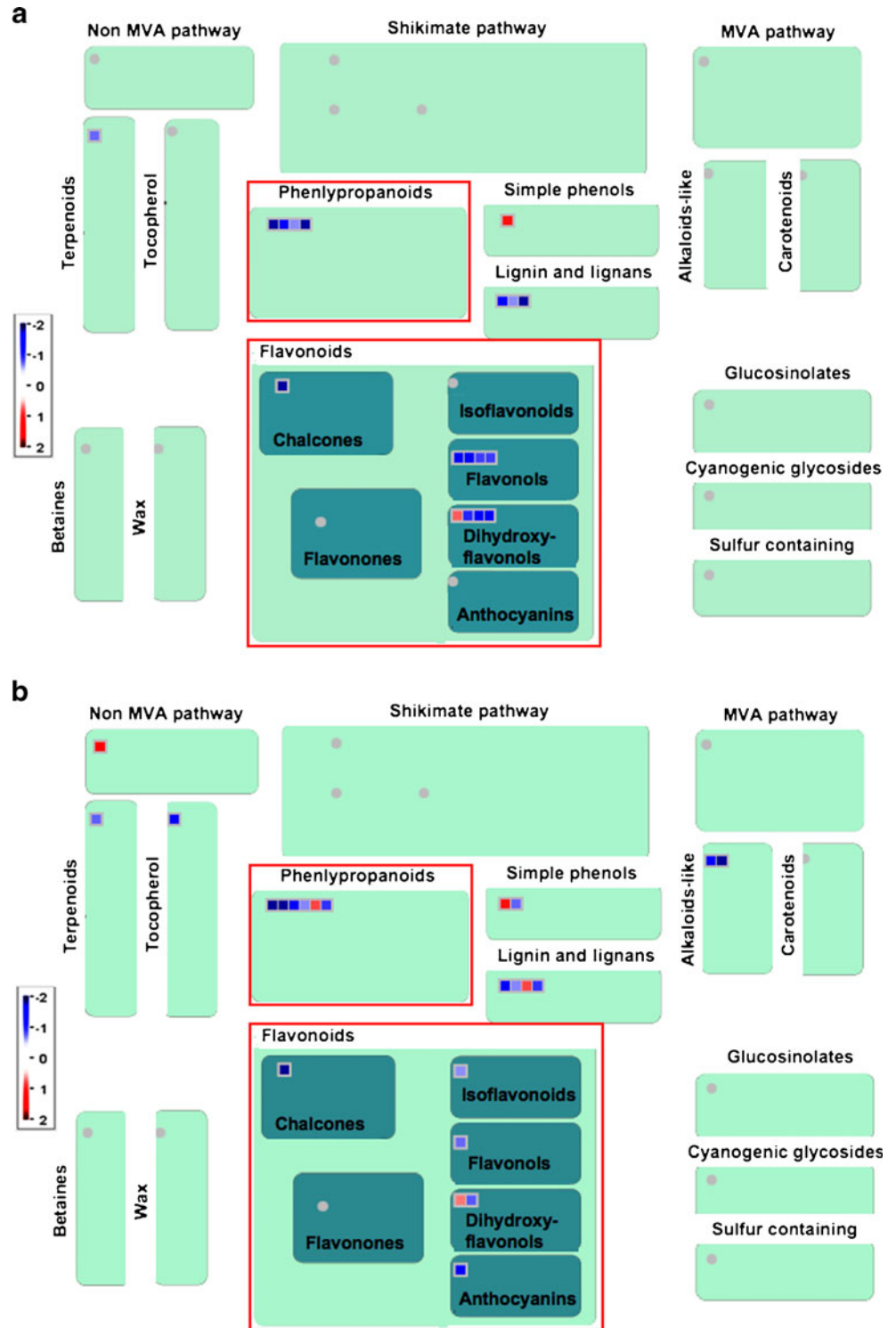
In our microarray study, four genes were significantly upregulated in incompatible interaction and these were NADPH

oxidase, *AtrbohF*, von Willebrand domain containing protein (vWA), and cinnamoyl CoA reductase (ESM Table S3, S6). The NADPH oxidase complex is composed of *RbohF/RbohD* genes and is also known as the respiratory burst oxidase (RBO). Several recent reports demonstrate that members of the Rboh family mediate the production of apoplastic ROS during the defense responses, as well as in response to abiotic

stress and developmental cues (Torres et al. 2005; Torres et al. 2006). ROS produced by *AtrbohD* and *AtrbohF* are negative regulators of the unrestricted cell death in lesion mimic mutant *lsd1* of *Arabidopsis* (Torres et al. 2005).

vWA domain containing protein and the cinnamoyl-CoA reductase were the only other genes which have shown relatively higher fold of transcript upregulation in incompatible

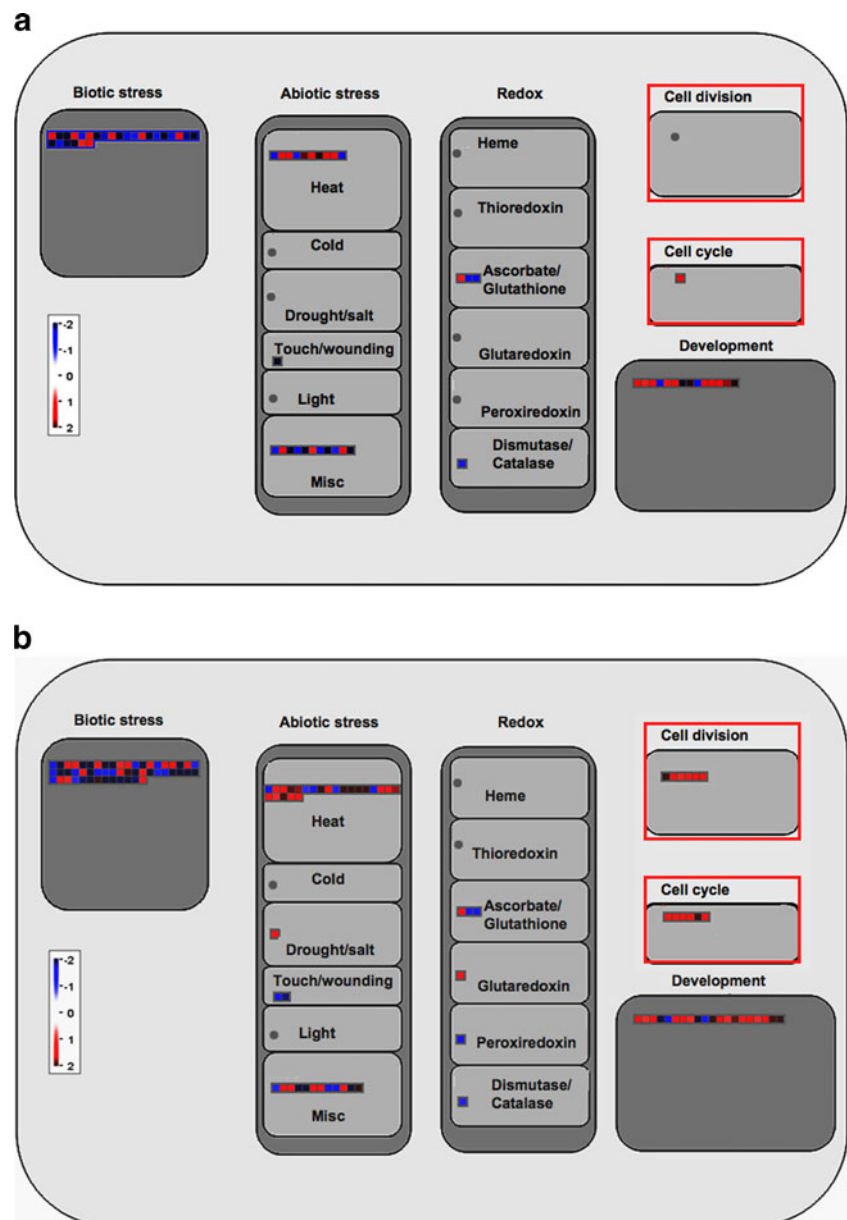
Fig. 5 MapMan-based visualization of the transcripts involved in “secondary metabolism” in the rice variety Kavya after infestation with gall midge biotypes (a) GMB1 and (b) GMB4M. Functional subBINs (*small squares*) shown in red or blue indicate their upregulation or downregulation, respectively. Red rectangles represent commonly downregulated pathways of phenylpropanoids and flavonoids. Color key \log_2 scale



interaction when compared with the compatible interaction at 120 h in real-time PCR validation. The vWA domain containing protein is a well-studied domain involved in cell adhesion, in extracellular matrix proteins, and in integrin receptors. Higher expression of vWA has been reported in resistance interaction between rice and parasitic plant *Striga hermonthica*, during the fourth and 11th day (~30-fold) of interaction through microarray analysis (Swarbrick et al. 2008). In the present study, a higher upregulation of vWA gene in incompatible interaction at both the time point suggests involvement of this gene in rice gall midge interaction. Cinnamoyl CoA reductase 1 (*OsCCR1*) is involved in lignin biosynthesis (Kawasaki et al. 2006). Lignin is polymerized through peroxidase activity of NADPH oxidase by using H₂O₂ in the cell wall and provides an undegradable mechanical barrier to most of the pathogens

(Kawasaki et al. 2006). CCR gene is also reported to be involved in production of monolignols which have antimicrobial activities (Keen and Littlefield 1979). In the absence of H₂O₂, the conversion of monolignol to lignin is reduced dramatically. H₂O₂, a molecule for ROS generation, was found to be depleted in HR⁻ type (symptomless) resistance in *Salix-Dasineura* interaction whereas levels of H₂O₂ were found to be more in HR⁺ genotypes (Hoglund et al. 2005). Therefore, we propose that low levels of H₂O₂ in HR⁻ type of resistance prevent the polymerization of monolignols to lignin. As a result, monolignols can get accumulated due to NADPH oxidase and CCR1 gene activities, in the incompatible interaction and this may prove to be detrimental for gall midge larva. However, the exact role of altered lignin biosynthesis pathway in HR⁻ type of resistance mechanism is still to be investigated.

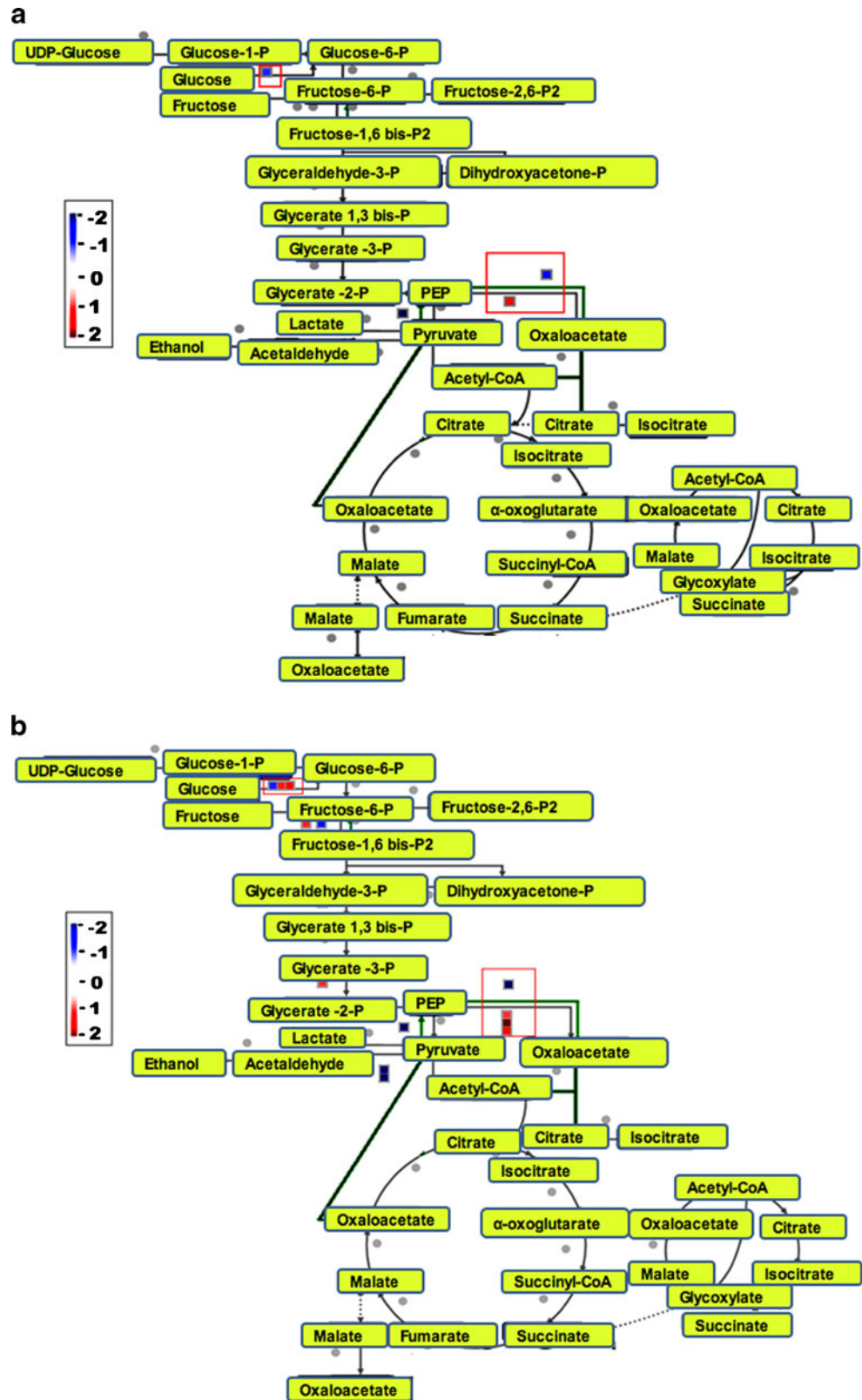
Fig. 6 MapMan-based visualization of the transcripts involved in “cellular response” in the rice variety Kavya after infestation with gall midge biotypes (a) GMB1 and (b) GMB4M. Functional subBINS (small squares) shown in red or blue indicate their upregulation or downregulation, respectively. Red rectangles represent differentially expressed pathways of cell division and cell cycle. Color key log₂ scale



The present study provides the first ever details of a novel molecular basis of relatively durable gall midge resistance of the HR- type conferred by *Gm1*, a gene that has been

widely deployed through breeding to develop gall midge-resistant rice varieties. Another important implication of the current study is that it provides a better understanding of the

Fig. 7 MapMan-based visualization of the transcripts involved in “glycolysis and TCA cycle” in the rice variety Kavya after infestation with gall midge biotypes (a) GMB1 and (b) GMB4M. Functional subBINs (small squares) shown in red or blue indicate their upregulation or downregulation, respectively. Red rectangles represent differentially expressed genes of glycolysis pathway. Color key \log_2 scale



mechanism of resistance conferred by *Gm1* and its suitability in gene pyramiding along with HR⁺ type resistance conferred by genes such as *Gm11*. We suggest that HR⁻ type resistance in the rice variety Kavaya against GMB1 has a novel mechanism that could be explained by the proposed hypotheses. The hypothesis suggests that HR⁻ type resistance could be due to the involvement of a “constitutive *R* gene” as revealed by low profile of transcriptome induction by *Gm1* gene. This could also be a case of “extreme resistance mechanism” which is devoid of cell death. It is also noteworthy that resistance conferred by *Gm1* gene in the rice variety Kavaya is more durable than that conferred by other gall midge-resistant genes which exhibit inducible HR⁺ type resistance (Bentur et al. 2008). Nevertheless, this novel mechanism of resistance needs further characterization.

In the present study, transcriptome profiling of compatible interaction of Kavaya with GMB4M suggested that the virulent gall midge elicits a range of physiological and morphological changes in the rice plant. These changes are understandably brought about by the activation of susceptibility-related transcription regulators, which in turn upregulate expression of genes involved in the metabolism, cell organization, transport of nutrients while downregulating genes involved in defense. The virulent larva, thus orchestrates the plant genetic machinery so as to accommodate its growth. This generalized picture emerged through the present microarray study and real-time PCR validation. However, upregulation of stress-related genes such as respiratory burst oxidase (24-fold), thionin (8.5-fold), and heat shock proteins (fivefold) was observed at 24 h in compatible interaction. These results indicate that Kavaya mounted an elevated stress response during early hours of virulent gall midge infestation. This response subsides during the course, resulting in susceptibility. Similarly in the wheat–Hessian fly interaction, virulent larva is initially exposed to the stress response of the host during compatible interaction (Anderson and Harris 2006; Harris et al. 2006; Liu et al. 2010). Downregulation of the genes related to biotic stress defense pathway (BIN 20.1; Fig. 5a and b) including NB-ARC, PR genes, xylanase inhibitors and disease resistance proteins and genes related to secondary metabolism (BIN 16) especially phenylpropanoid pathway (BIN 16.2) genes such as PAL and anthranilate *N*-benzoyltransferase protein suggests that the virulent larvae efficiently suppressed the plant defense (Fig. 6a and b). The present study also provides evidence for increased and improved food quality that came from the strong upregulation of genes involved in primary metabolism (Fig. 7a and b) as also observed in the wheat–Hessian fly compatible interaction (Liu et al. 2007). Evidence for increased nutrient flow comes from the strong upregulation of genes that encode various transporters (BIN 34), e.g., sugar transporters, peptide transporter PTR2, a phosphate carrier protein, aquaporin, and amino acid transporter-like protein. Our study also revealed induction of transmembrane

amino acid transporter gene up to 18-fold during later hours of compatible interaction (as revealed by real-time PCR validation). Earlier studies on rice gall midge interaction reported increased concentrations of amino acids in the host that favored gall midge multiplication (Regupathy and Subramanian 1972) and probably hints at the role of amino acids in the susceptibility of plant. In this regard, transmembrane amino acid transporter may help virulent larva to get better access to food/nutrients. In contrast to the wheat–Hessian fly studies, genes related to DNA structure, synthesis and repair (BIN 28), and cell organization (BIN 31) such as cell division (BIN 31.2) and cell cycle (BIN 31.3) were significantly ($p < 0.05$) upregulated in the compatible interaction (Fig. 6a and b). Thus to support maggot survival, growth, and gall formation, the entire plant cell machinery is geared up to enhance/augment vegetative growth in the compatible interaction. Transcriptional reprogramming in compatible interaction of rice gall midge featured here suggested that susceptibility is not a default phenotype but is the result of coherent interplay of a large number of genes. Manipulating any of the key genes would result in resistance against the pest.

Further, to validate the hypothesis that *Gm1*-conferred resistance is due to constitutive expression of a *R* gene. We propose proteomics and metabolomics approach and induce mutation in the Kavaya variety aimed at loss of resistance which will help to provide a better insight into the novel mechanism of resistance.

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