# Expression profiles of key genes involved in rice gall midge interactions reveal diversity in resistance pathways

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**Resistance against the Asian rice gall midge, *Orseolia oryzae* (Wood-Mason), is conferred by any one of the 11 major genes identified so far. Resistance conferred by these genes may or may not involve a hypersensitive reaction (HR). Three of these gall midge resistance genes have been already cloned. In this study, expression of 20 key genes, identified based on our earlier studies, in 12 rice genotypes was profiled through quantitative real time (RT)-PCR at two time points. Results highlighted diversity in resistance pathways either involving reactive oxygen species (ROS)-hypersensitive reaction (HR) salicylic acid-mediated systemic acquired resistance or strong suppression of ROS-HR at initial stage itself. Some of the susceptible test genotypes derived from crosses between gall midge resistant and susceptible parents expressed trace activity of these genes even during compatible interaction.**

**Keywords:** HR+ type resistance, HR– type resistance, HR, ROS, SAR.

THE Asian rice gall midge (*Orseolia oryzae* Wood-Mason) is one of the major pests that affects rice yield in India causing annual yield loss around 0.8% (Rs 330 crores/US$ 80 million)[1]. Development and cultivation of resistant rice varieties is the main approach against this pest. High level of resistance is conferred by some genes which have been reported in over 300 germplasm accessions belonging to the primary gene pool[1]. Over 100 resistant rice cultivars have been released for cultivation, and as a response to extensive cultivation of these resistant rice varieties, virulent populations of gall midge, known as biotypes, have rapidly evolved to overcome host resistance[2]. Two distinct phenotypes have been noted among the gall midge resistant rice genotypes. In type 1, the host initiates defense response with localized tissue necrosis, a typical hypersensitive reaction (HR); at the maggot feeding site within 48 h after infestation (HAI) which results in maggot mortality. This is referred to as HR+ type resistance. In type 2, the host plant activates its defense without any HR but still results in maggot mortality during the same period. This resistance is referred as HR– type. On the other hand, susceptibility is manifested through production of tubular leaf sheath gall called silver shoot that encircles the feeding maggot which allows it to complete its life cycle within the gall and emerge as an adult. This process renders the tiller sterile and no panicle is formed leading to yield loss.

So far, 11 gall midge resistance (R) genes have been characterized in rice and designated as *Gm1* through *Gm11* (ref. 3). Of these, only *gm3* is recessive[4] and the rest are dominant. Two of the genes, *Gm1* and *Gm8* confer HR– type resistance while all the others confer HR+ type resistance[1]. Eight of the R genes have been tagged and mapped and closely linked markers have been identified for their introgression. Candidate genes for *gm3* (ref. 5), *Gm4* (ref. 6) and *Gm8* (ref. 7) have been identified and validated and functional markers for these have been developed. Two of these belong to nucleotide-binding site leucine-rich repeat (NBS-LRR) family of R genes. Thus far, seven gall midge biotypes have been defined[2] based on the reaction of rice lines carrying different R genes. Though virulence genes in the insect have not been characterized, genetics of virulence suggest involvement of single recessive gene[8]. Plant resistance and the pest virulence are known to display gene-for-gene relationship[9]. Genome-wide gene expression profiling through either microarray or suppressive subtraction hybridization (SSH) library analysis has identified pathway genes involved in rice-gall midge interactions and revealed diversity in resistance pathways. The resistance in rice variety Suraksha harbouring *Gm11* and expressing HR+ type resistance is similar to resistance against pathogens mediated through salicylic acid (SA) pathway[10]. Another rice variety, Kavya, with *Gm1* gene providing HR– type of resistance displayed a novel yet not clearly understood pathway of constitutive resistance[11]. The rice variety, Aganni, with *Gm8* shows another HR– type of resistance. It is a variant of HR+ with possible suppression of HR through activation of ROS quenching genes[12]. Resistance in rice line RP2068, carrying *gm3*, also confers HR+ type resistance. It involved in up-regulation of lipid peroxidation

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genes and accumulation of gamma amino butyric acid (GABA) – a neurotransmitter and insect anti-feedant[13]. Though role of some of these pathway genes in genotype specific interaction has been studied, their probable involvement in wider context of rice-gall midge interaction has not been investigated.

Plants have diverse defense mechanisms to defend themselves against animals ranging from microbes to mammals. One such mechanism is specifically induced against insects and is triggered by a range of elicitors/effectors from oral secretions[14] or by secretary salivary gland proteins as in case of rice-gall midge[15] and wheat-Hessian fly interactions[16]. Once triggered, resistance manifests at several levels: expression of HR at feeding site in certain instances, followed by a signalling process within and across the affected area involving either SA or jasmonic acid (JA) and finally induction of systemic acquired resistance (SAR) that involves synthesis, transport and accumulation of a range of antimicrobial/antibiotic substances. These include secondary metabolites such as phenolics, terpenoids, flavonoids, other phytoalexins and/or pathogenesis related proteins such as protease inhibitors. As plants are constantly under attack from a diverse range of pathogenic microbes and herbivores, their genome has evolved the capacity to harbour a large number of key R genes of different classes and families. Thus it is hypothesized that few or several of the downstream pathways evoked by the key R genes are likely to be common. This hypothesis has not been tested elaborately in any crop plant with different R genes targeting the same species of pest. Our study aims to test this hypothesis and was carried out using rice genotypes with different R genes associated with both HR+ and HR– type resistance against gall midge. In addition, compatible interaction between the insect and closely related rice genotypes lacking the R gene was also noted. Our results suggest diversity within and among these two types of interactions.

## Materials and methods

### *Plant material*

Twelve rice genotypes were selected for the study (Table 1). Five of these were the originally reported donor lines carrying gall midge resistance genes *Gm1*, *gm3*, *Gm4*, *Gm8* and *Gm11* (ref. 3). TN1 with no gall midge resistance gene was included as control. In addition, three R genes introgressed and three S lines (without R gene) derived as recurrent inbred lines (RILs) from the crosses involving the donor line and TN1 in advanced generation ($>F_8$) were also included in the study to note gene expression under different genetic backgrounds. Pre-germinated seeds of the test genotypes were sown in four plastic trays (60 × 30 × 30 cm) filled up to 8 cm deep with fertilizer-enriched soil. Each tray had two rows of 20 plants each of the 12 test genotypes along with the standard susceptible (TN1) and resistant (Suraksha) lines.

### *Infestation with gall midge and tissue sample collection*

When the test plants were 15 days old, three trays were exposed to 25 female and 10 male adult gall midge biotype 1 (GMB1) in a nylon mesh cage. A fourth tray, not exposed to insects, served as the control. Two days after insect release, the exposed and control trays were transferred to the humidity chamber (>90% RH) for egg incubation and left undisturbed for another two days. Plants were regularly observed for egg hatching and tissue samples were collected at two time points 24 and 120 HAI according to Rawat *et al.*[11]. Basal part of the plant, 2 cm above soil, was cut, dissected under binocular microscope to remove live/dead maggots before placing it in RNA later (Qiagen, Germany) and stored at –20°C.

### *Tissue processing and RT-PCR expression profiling*

On the basis of data obtained from studies involving both SSH library[17,10] and microarray analyses[11], 20 genes involved in rice-gall midge interaction were selected for profiling (Table 2). RT-PCR was conducted with RNA isolated from plants from three independent infestations at two time points (24 and 120 HAI). Total RNA was treated with RNase-free DNase I (Qiagen, Germany) to remove DNA contamination from RNA. Three micrograms of RNA was used for first-strand cDNA synthesis using the SuperScript III RT (Invitrogen, USA) following the manufacturer's guidelines. RT-PCR was performed using applied biosystems 7500 RT-PCR system with the SYBR green chemistry (Applied Biosystems, USA) according to the manufacturer's instructions. Gene-specific primers for RT-PCR were designed using Primer Express Software (Applied Biosystems). Rice ubiquitin gene, *OsUbq* (GenBank accession no. AK059694) was used as the endogenous control. RT-PCR was carried out in a fixed volume of 20 µl containing 10 µl 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 (trays) in two 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 sec and annealing and extension at 60°C for 1 min. A melt curve analysis was carried out to determine the specificity of the reaction. Quantity of each mRNA was calculated according to Rawat *et al.*[11]. Fold value ≥2.0 was considered significant. The data from different PCR runs or cDNA samples were compared by using the mean of CT values of the three

**Table 1.** Details of the rice genotypes used in this study

| Genotype | Parentage | R gene | Type of resistance | Resistance against gall midge biotype (GMB) |
|---|---|---|---|---|
| RP2068-18-3-5 | Swarnadhan/Velluthacheera | *gm3* | HR+ | 1, 2, 3, 4, 4M |
| Abhaya | CR 157-392/OR 57-21 | *Gm4* | HR+ | 1, 2, 3, 4, 4M |
| 482R* | TN1 × Abhaya | *Gm4* | HR+ | NT |
| 489S* | TN1 × Abhaya | No gene | Susceptible | None |
| MR1523 | IR8 × Ptb21 | *Gm11* | HR+ | 1, 2, 3, 4 |
| 267R$^{+}$ | TN1 × MR1523 | *Gm11* | HR+ | NT |
| 65S$^{+}$ | TN1 × MR1523 | No gene | Susceptible | None |
| Kavya (WGL-48684) | (WGA-27120 × WGL-17672) × (Mahsuri × Surekha) | *Gm1* | HR– | 1, 3, 5, 6 |
| Aganni | Landrace from Kerala | *Gm8* | HR– | 1, 2, 3, 4, 4M |
| 74R$^{\dagger}$ | TN1 × Aganni | *Gm8* | HR– | NT |
| 34S$^{\dagger}$ | TN1 × Aganni | No gene | Susceptible | None |
| TN1 | Dwarf Chow-wu-gen × Tsai-Yuan-Chunj | No gene | Susceptible | None |

*R and S lines were identical at 321 out of 349 SSR loci tested, $^{+}$R and S lines were identical at 467 out of 470 SSR loci tested, $^{\dagger}$R and S lines were identical at 340 out of 362 SSR loci tested, NT not tested.

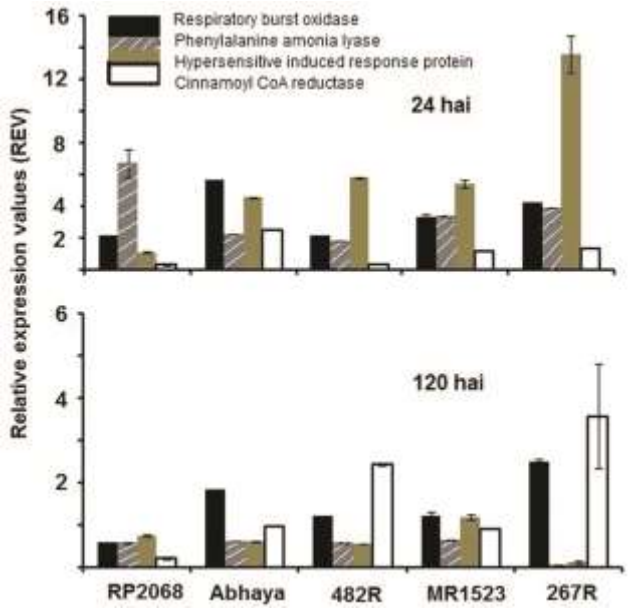


**Figure 1.** Relative gene expression induced during incompatible interaction between rice genotypes with different R genes conferring HR+ type resistance and an avirulent gall midge biotype at 24 and 120 hours after infestation (HAI). Error bars represent mean ± SE.

biological replicates that was normalized to the mean of CT values of the endogenous gene. The relative standard curve method was used for the quantification of mRNA levels and displayed as relative expression values (REV). Expression ratios were calculated using the $2^{\Delta\Delta Ct}$ method[18]. The data were analysed using the Bio-Rad CFX Manager 3.1 Software (Bio-Rad, USA) with default baseline and threshold. Relative transcription levels are presented graphically. Results are presented as mean ± SE of relative expression in comparison with corresponding uninfested control sample. Mean values higher than 2-folds were compared through paired *t* test at $P < 0.05$ level using Microsoft Excel program (Supplementary Table 1).

## Results

### *Genes shortlisted from SSH and microarray data*

Based on our earlier reports, 20 genes were shortlisted (Table 2)[10,11,17]. These genes are related to defense (Bin 20), cell organization (Bin 31), secondary metabolism (Bin 16), hormone signalling (Bin 17) and those involved in regulation of transcription, primary metabolism, hormone signalling, transport, etc.

### *Genes induced in HR+ defense pathways*

Out of the 20 genes, 3 genes related to defense and secondary metabolism, viz. respiratory burst oxidase (LOC_Os05g38980), phenylalanine ammonia lyase 1 (PAL1) (LOC_Os04g43800.1) and hypersensitive-induced response protein (Hirp) (LOC_Os10g32700) were up-regulated in 4 of the 5 genotypes with HR+ type resistance at 24 HAI (Figure 1 and Supplementary Table 1). Thus these three genes can be considered to have an early response leading to HR expression. In two of the introgressed lines 482R and 267R carrying *Gm4* or *Gm11* gene respectively, Cinnamoyl CoA reductase (CCR) (LOC_Os09g25150) was seen significantly up-regulated at 120 HAI as compared to levels (<2 fold) at 24 HAI.

### *Gene induced in HR– defense pathway*

Only one gene related to defense, i.e. von Willebrand factor type A domain (LOC_Os11g45990), was up-regulated in all the three genotypes with HR– resistance at 120 HAI (Figure 2 and Supplementary Table 1). However, in Kavya the expression levels of this gene were high at both 24 and 120 HAI. In addition, CCR (LOC_Os09g25150) was also up-regulated in two of the test lines, except Aganni, at both the time points.

**Table 2.** Shortlisted genes from the reported data and sequence information of the primers used for real time PCR validation

| Gene name | Locus ID | Primer sequences | Product size (bp) |
|---|---|---|---|
| SAM dependent carboxy methyltransferase | LOC_Os02g48770 | F1: TTGCCGGGCTCTTTCTACAC<br>R1: GCTTGTCAGGCACCTTGGA | 100 |
| Ent-Kaurene synthase (EKS) | LOC_Os04g09900 | F1: AGGATATCCCAGGCGAGGTT<br>R1: ACGCGGTAAACTTGCTTTCC | 62 |
| Isoflavin reductase | LOC_Os12g16410 | F1: TGCCTGGCAAACTTCGAGAT<br>R1: ACTCATCCATGCGGGTGTACT | 85 |
| Peptidyl prolyl *cis*/*trans* isomerase | LOC_Os08g44520 | F1: CGCCACGATCCCTCACAT<br>R1: TGGTAGCCGAATCGCAACTT | 70 |
| OsPR10$\alpha$ (PR10a) | LOC_Os12g36830 | F1: ACCATCTACACCATGAAGCTTAAC<br>R1:GTATTCCTCTTCATCTTAGGCGTA | 334 |
| Riboflavin synthase | LOC_Os02g36340 | F1: ACTCCCAACGGAATCCATCA<br>R1: CCGCAACACATTTCAAACCA | 62 |
| NAC domain containing protein | LOC_Os08g10080 | F1: AAGGAGGACTGGGTGCTATGC<br>R1: TCTTCAGATGATGGGCTTGGA | 71 |
| ABC transporter | LOC_Os04g38570 | F1: CGAATGCAATGGAGAGGAAGAC<br>R1: ACCGCATATAACCCAGTTCCAA | 67 |
| Respiratory burst oxidase (RbO) | LOC_Os05g38980 | F1: TGGGTCTCCAACACTTACGAAA<br>R1: CGTTGTCGTCTGGCTGAATTC | 62 |
| von Willebrand factor type A domain | LOC_Os11g45990 | F1: AGTTTGTCATCAGGAAGCTTGCT<br>R1: GCTATATTCCTTGACGGGTCCAT | 80 |
| Hypersensitive-induced response protein (Hirp) | LOC_Os10g32700 | F1: TTGTTACGGTTGTTGCATCCA<br>R1: GATTTTGGGTTGCTCAGCTTGT | 85 |
| Cinnamoyl-CoA reductase (CCR) | LOC_Os09g25150 | F1: ACATCCTCGGCAAGCTCTTC<br>R1: TTCACCTCGTCAGAGCACCTT | 61 |
| Flavonol synthase/flavanone 3-hydroxylase | LOC_Os10g40934 | F1: CAGGGCCTGCAGATCAAGAA<br>R1: GTGTGTCGCCAATGTTAACGA | 85 |
| Glyceraldehyde-3-phosphate dehydrogenase | LOC_Os06g45590 | F1: CAAGGCTGGAATTGGCTTAAGTT<br>R1: GTAGCCCCACTCGTTGTCGTA | 70 |
| TCTP | LOC_Os11g43900 | F1: GATGGCGGCTTGGTGTTT<br>R1: CAGCCCATGAGAGAAGTAAAGGA | 75 |
| NADPH oxidase | LOC_Os01g53294 | F1: GAAAGAGGAAAAGCCGAAAAGG<br>R1: CCCACCGGATTACCGAAAC | 69 |
| Chitin inducible gibberallin | LOC_Os07g36170 | F1: CCAATCCAACGTCTAGGTGCTT<br>R1: TTTGTGCCAGAGTTTCCATGTCTA | 68 |
| Pyruvate dehydrogenase E1 component | LOC_Os06g13720 | F1: ATCGAAAAGCCCCGCATACT<br>R1: TGACTGCAAGAACATCCATACCA | 80 |
| WD domain containing protein | LOC_Os12g42150 | F1: CAGCGTGCCAAGACCCATA<br>R1: CCATCGAGGCAACCATCCT | 90 |
| Phenylalnine ammonia lyase 1 | LOC_Os04g43800 | F1: CCAACCCTGTGACCAACCAT<br>R1: CGAGATGAGGCCGAGAGAGT | 74 |

### *Genes induced in both HR+ and HR– defense pathways*

Out of the 20 genes tested in 8 genotypes – 3 with HR– resistance and the rest with HR+ resistance – 4 genes displayed induced activity in both the groups (Figure 3, Supplementary Table 1). *OsPR10*$\alpha$ (LOC_Os12g36830) was up-regulated at 24 HAI in two genotypes of each group carrying *Gm11* or *Gm8* genes. As indicated above, CCR displayed induction in three test rice lines representing both the groups at 24 HAI and in four test lines at 120 HAI. Third gene NADPH oxidase (LOC_Os01g53294) was induced over two-fold in three genotypes harbouring *Gm3* or *Gm8*. The von Willebrand factor type A

domain protein coding gene was involved in two lines carrying *Gm11* (HR+) gene at 24 HAI besides three lines carrying *Gm1* or *Gm8* (HR–) genes at 120 HAI.

### *Genes induced only in a specific genotype*

The gene coding for Ent-Kaurene synthase-EKS (LOC_Os04g09900) was induced only in Abhaya at 24 HAI (Figure 4, Supplementary Table 1); WD domain protein gene (LOC_Os12g42150) in Kavya at 120 HAI and Flavonol synthase/flavanone 3-hydroxylase (LOC_Os10g40934) in Aganni and 74R at 24 HAI.

### *Genes induced during compatible interaction*

Four susceptible lines, including the check variety, TN1 (without any gall midge resistance gene), were included in the study to determine involvement of genes during compatible interaction. Only two genes pertaining to primary metabolism were observed to be induced on being challenged with GMB1 (Supplementary Table 1). Glyceraldehyde-3-phosphate dehydrogenase (LOC_Os06g45590) showed higher expression in three lines at 24 HAI while pyruvate dehydrogenase E1 component (LOC_Os06g13720) was induced in all the four lines at 120 HAI. However, it was observed that these genes were also induced during incompatible interaction in RP2068, 267R, 74R and MR1523 at least at one of the time points. TCTP, earlier suggested to be a susceptibility gene, was found induced in TN1 and, also in 482R at 24 HAI.

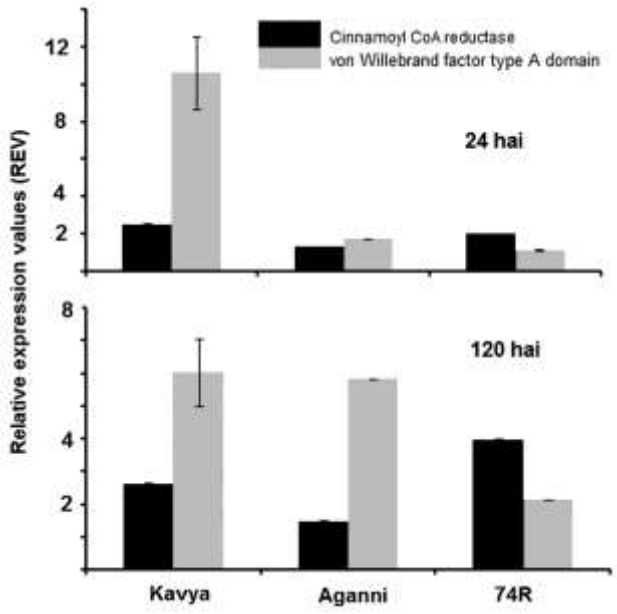


**Figure 2.** Relative gene expression induced during incompatible interaction between rice genotypes with different R genes conferring HR– type resistance and an avirulent gall midge biotype at 24 and 120 HAI. Error bars represent mean ± SE.

### *Genes induced during both compatible and incompatible interactions*

Besides the two above-mentioned genes related to primary metabolism, seven genes mentioned above under resistance pathways were also observed to be induced either in

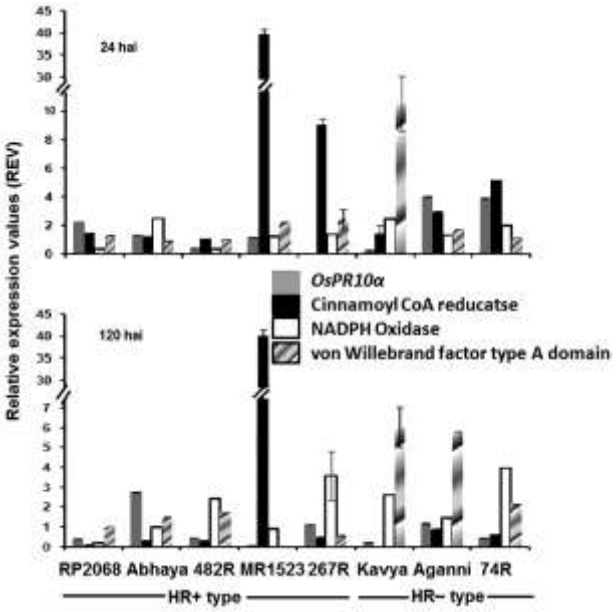


**Figure 3.** Relative gene expression induced during incompatible interaction among rice genotypes conferring both HR+ and HR– type resistance and an avirulent gall midge biotype at 24 and 120 HAI. Error bars represent mean ± SE.

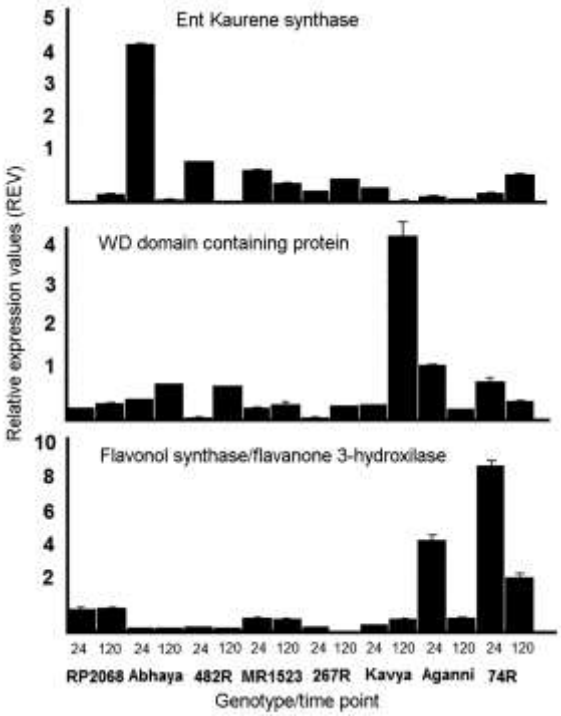


**Figure 4.** Relative gene expression induced during incompatible interaction in any one of the genotypes or genotypes carrying the same R gene and an avirulent gall midge biotype at 24 and 120 HAI. Error bars represent mean ± SE.

one or two of the compatible interactions involving introgressed lines. For instance, *OsPR10α* was found induced (>2 fold) in 65S (a line derived from MR1523) and 34S (a line derived from Aganni) during compatible interaction (Figure 5, Supplementary Table 1). Likewise, respiratory burst oxidase and NAC domain containing protein genes were up-regulated in 34S at 120 HAI, while NADPH oxidase and von Willebrand factor type A protein genes were upregulated in 489S (a line derived Abhaya) at 120 HAI; chitin inducible gibberellin in 65S from 24 HAI and SAM dependent carboxy methyltransferase gene in 34S at 24 HAI (Supplementary Table 1).

## Discussion

Several studies have focused on plant pathogen/insect interactions to unravel diverse defense pathways[19]. Plant defense against pathogenic microorganisms and herbivores is a well established two-tier system[16]. Innate immunity or basal defense is based on the plant receptors that recognize pathogen/microbe associated molecular patterns (PAMP/MAMPs), or damage/herbivore associated molecular patterns (DAMP/HAMPs). Receptors on plant cell surface recognize these patterns and mount pattern triggered immunity (PTI) which is broad based and race nonspecific. However, a range of invaders have evolved elicitors or effectors that neutralize PTI and successfully colonize the plant host. Second tier of defense of plants is mediated through several classes of resistance (R) genes that detect the specific effectors from a strain of pathogen and mount effector triggered immunity (ETI). This defense is rapid and race-specific. It is also generally accompanied by localized tissue necrosis at the invading site called hypersensitive reaction (HR) and subsequent induction of systemic acquired resistance (SAR). A suite of genes and pathways is regularly implicated in defense against biotrophic pathogens and gall forming/phloem feeding insects that involve reactive oxygen species (ROS)-mediated HR and SA-mediated signalling. Phenylpropanoid pathway is the main route for SA synthesis. A range of pathogenesis related (PR) protein-coding genes are also reported to be involved. But more specifically, defense against necrotrophic pathogens and leaf feeding herbivores involves JA-mediated signalling pathway that is often antagonistic to SA-mediated pathways[20]. Even more significantly, every case of plant–pest interaction studied has identified some unique genes. It is thus evident that several variant forms of defense pathways are utilized by different plant genotypes against the same pathogen or its races.

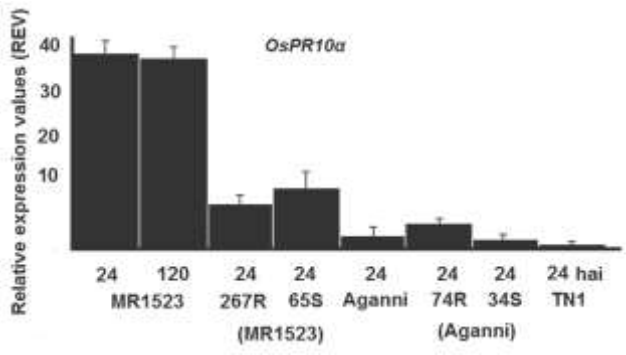


**Figure 5.** Relative expression of *OsPR10α* gene (LOC_Os12g36830) during both incompatible and compatible interactions in rice genotypes carrying *Gm11* or *Gm8* gene and their introgressed susceptible lines lacking the gene. The susceptible check TN1 with no R gene is also included. Error bars represent mean ± SE.

Our earlier studies[10–13,17] on rice-gall midge interactions have clearly brought out such diversity in defense against the pest in rice genotypes carrying different R genes. Gall midge biotype specific expression was also observed in some genotypes. General similarity of rice resistance to gall midge with that to pathogens was brought out in MR1523 rice carrying *Gm11* gene[10]. Such similarity has also been pointed out for the hemipterans[21] and gall flies[22]. Three candidate gall midge resistance genes *gm3* (ref. 5), *Gm4* (ref. 6) and *Gm8* (ref. 7) have been identified, cloned and functionally validated. Two of these, *gm3* and *Gm4*, belong to NBS-LRR family. Two groups within the family, i.e. toll/interleukin receptor–nucleotide binding site-leucine rich repeat (TNL) and non-TNL/coiled coil – nucleotide binding site-leucine rich repeat (CNL) with toll/interleukin receptor (TIR)/coiled coil (CC)/leucine zipper (LZ), nucleotide binding site (NBS), APAF-1, R protein and CED-4 (ARC) and leucine rich repeat (LRR) domains are reported to confer pest resistance in crop plants including rice[23]. Much of the variability is observed in the LRR domain that accounts for the response against various effectors produced by the invading pest/race/biotype. Both the candidate genes *gm3* (ref. 5) and *Gm4* (ref. 6) were observed to be induced following infestation with avirulent gall midge biotypes. This suggested a quick feedback from the NBS-LRR proteins, already present in the cell, to the nucleus for recruiting more protein following infestation. Also, the gene gets induced only in the genotype having the resistance-specific allele. NBS-LRR proteins act in concert with other known proteins. Rice hypersensitive induced reaction protein 1 (OsHIR1) has been identified as interacting partner of OsLRR1 which helps the protein localize in plasma membrane[24]. It is plausible that HIR proteins help operate calcium channel in the plasma membrane. Likewise heat shock proteins (hsp) also act as trans-partners with NBS-LRR proteins[25]. One of the HSP genes (LOC_Os12g32986) was induced in Aganni and its introgressed line 74R at 120 hai in our earlier study[12]. One of the two HSP20/alpha crystalline family protein genes was also up-regulated by 2-folds in Kavya during incompatible interaction compared to 4-fold increase during compatible interaction[10].

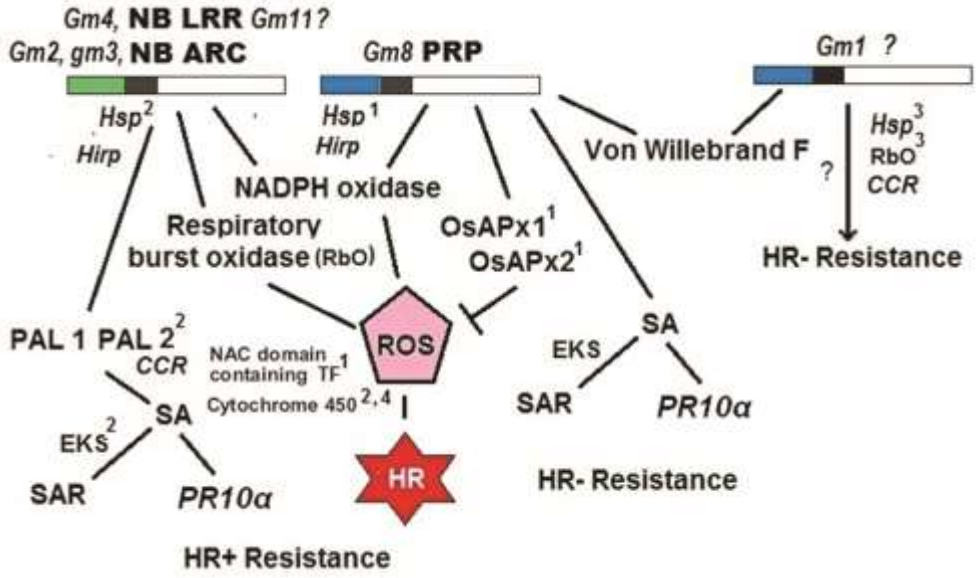


**Figure 6.** Schematic view of the diverse resistance pathways triggered by different R genes identified in rice against the Asian rice gall midge. CCR, Cinnamoyl-CoA reductase; EKS, Ent-Kaurene synthase; Hirp, Hypersensitive induced response protein; HR, Hypersensitive reaction; Hsp, Heat shock protein; NB ARC, Nucleotide binding (site) ARC (APAF-1, R protein and CED-4) domain; NB LRR, Nucleotide binding (site) leucine rich repeat; OsAPx, Cytosolic ascorbate peroxidase; PAL, Phenylalanine ammonia lyase; PRP, Proline rich protein; RbO, Respiratory burst oxidase; SA, Salicylic acid; ROS, Reactive oxygen species; SAR, Systemic acquired resistance; TF, Transcription factor [Ref. 1-12; 2-11, 3-17, 4-10].

Tissue necrosis or HR is reported to be the first R gene-mediated visible resistance response of plants controlled by the oxidative burst[26,27]. HR is considered a strategy by which plants restrict pathogen spread by killing infected and surrounding healthy cells. However, there also exist reports indicating failure of HR to contain spread of pathogens in certain cases[28]. The response itself is preceded by recognition of pest attack by R gene through elicitors/effectors and resulting influx of ions such as $Ca^{2+}$. These in turn lead to oxidative burst by producing reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, hydroxyl radicals, nitrous oxide, etc. While role of different members of this family may be defined by the genotype/species of plant and the pest, other members like singleton oxygen in HR induction are involved in a specific rice–gall midge interaction[13]. These compounds affect the cell membrane function by inducing lipid peroxidation[13]. One of the genes encoding NADPH oxidase involved in ROS production recorded induction in three of the genotypes tested at 24 HAI and another genotype at 120 HAI. But there was no HR expression. This could be due to concomitant over expression of two ROS quenching genes – cytosolic ascorbate peroxidases *OsApx1* and *OsApx2* – as noted in our earlier study[12]. Also, in the rice variety Kavya respiratory burst oxidase (*RbO*) was induced by 10-fold during incompatible interaction as compared to 24-fold during compatible interaction at 24 HAI, while the levels quickly fell below 2-fold at 120 HAI. Despite this no HR was observed. Further, oxidative burst was also noted when plants were exposed to leaf feeding insects[19] but without expression of HR. Thus, it is evident that generation of ROS need not always result in HR.

It is now evident that ROS, generated during pathogen attack, is recognized by plant as a signal for triggering downstream defense responses. These involve induction of a large number of genes such as PAL and *PR* a common feature of plant–pathogen interactions also reported in plant–insect interactions, particularly the sap sucking and gall forming ones[16]. While PAL is the rate-limiting gene in the phenylpropanoid pathway, several copies of the gene are found in plant genome. In maize, 6 of the 20 PAL genes analysed showed that only *ZmPAL4* was induced after infection with root knot nematode[29]. Likewise, rice has 12 copies of PAL genes, only one of these (LOC_Os04g43800) was induced following gall midge infestation. In Suraksha, two PAL genes were induced following gall midge infestation[17].

Cinnamoyl CoA reductase (CCR) is the first enzyme in the mono lignol-specific branch in the lignin biosynthetic pathway. A member of the sorghum CCR gene family, *SbCCR2-2* was induced in response to aphid attack and thus, has been attributed a defense function[30]. The gene functions to strengthen cell wall and maintain tissue integrity. Being downstream of PAL with reference to lignin biosynthesis, the CCR gene was induced at 120 HAI in four of the genotypes in our study.

Obviously, HR is not the long term response or solution of the plant to the pest challenge. Hence HR is followed by induction of SAR mediated through SA

signalling. SAR involves production, distribution and accumulation of phytoalexins, antimetabolites, protease inhibitors or any of the members of the large family of PR proteins. Among the abundant PR protein encoding genes in rice, *PR10α* and *PR1* have been consistently implicated in defense against gall midge[31] and brown planthopper[32]. In the present study also, *PR10α* was found induced in four genotypes at 24 HAI. Phenomenal increase of expression of *PR10α* in rice line MR1523 has been consistent while its significance is still not clear. Another manifestation of SAR could be greater accumulation of gamma amino butyric acid (GABA), a neurotransmitter and an insect antifeedant, in rice genotype RP2068 following gall midge infestation[13].

Certain genes such as Ent-Kaurene synthase (EKS) in Abhaya, WD domain containing protein coding gene in Kavya and Flavonol synthase/flavanone 3-hydroxylase in Aganni and 74R (Figure 4) were specific to the genotype. Kaurene synthase like (KSL) family of genes in rice are involved in biosynthesis of diterpenoids[33] while *OsKSL2* is specifically involved in synthesis of ent-beyerene, an antimicrobial product, in roots. WD domain (tryptophan-aspartic acid-domain) proteins are represented as receptors for activated C kinases (RACK) in rice and maize[34] which form a complex with regulators of plant disease resistance. Kim *et al.*[35] observed isoflavone reductase like (*OsIRL*) rice gene conferring tolerance to ROS and suggested its role in ROS homeostasis. Induction of flavonol synthase is suggestive of production of flavonoids as antibiotics to establish systemic resistance. Overall, these specific genes most likely represent specialized accomplishment of SAR in the genotype. We reported the induction of von Willebrand factor type A domain protein coding gene in all three genotypes showing HR-type of resistance. Interestingly, this gene has not been reported as a part of the plant defense in any other plant–pest system.

Two genes, glyceraldehyde-3-phosphate dehydrogenase and pyruvate dehydrogenase E1, showed up-regulation during compatible interactions in three lines at 24 HAI or in all the four lines at 120 HAI. This is suggestive of up-regulation of genes related to primary metabolism and transport of nutrient to feed the growing maggots as reported earlier[17].

## Conclusion

In summary, our results highlight diversity in resistance pathways in rice against the gall midge triggered by different R genes (Figure 6). Though most of the rice gall midge resistance genes operate through the expression of HR, it is not a prerequisite. Suppression of HR could be accomplished via at least two different ways. While it is not clear if plants have any distinct advantage by adopting a HR– type of resistance (often referred to as extreme resistance), our earlier studies hint that HR– resistance (e.g. resistance conferred by *Gm1*) is more durable than that conferred by HR+ type (e.g. resistance conferred by *Gm2*)[36]. In addition, the present study also suggests that investigating the role(s) of *PR10α* and von Willebrand factor type A proteins in plant resistance to insects, in general, and rice resistance to gall midge, in particular, would yield valuable information with regard to plant–pest interactions.


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