Role of pathogenesis-related genes in rice–gall midge interactions

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A sequence encoding putative pathogenesis-related protein *OsPR10α* (GenBank accession no. GQ487633) was identified from suppression subtractive hybridization cDNA library of rice cultivar Suraksha, after infestation with the Asian rice gall midge (*Orseolia oryzae*) involving incompatible interaction with hypersensitive reaction (HR). Full-length cloning (RACE) and homology search proved its identity as root-specific *OsPR10α* (*RsOsPR10α*). The expression of *RsOsPR10α* was analysed at different time intervals to understand its role in the resistance mechanism in rice against the pest. Real-time PCR detected significant upregulation of the gene, 40.4 and 23.5-fold at 24 and 120 h after infestation respectively, with the avirulent gall midge biotype 1 (GMB1) in Suraksha. Such upregulation was not observed after infestation with the virulent biotype (GMB4M). Significantly, *RsOsPR10α* expression was not upregulated in the cultivar Kavya, exhibiting resistance without HR against GMB1 or in the susceptible cultivar TN1 with either of the biotypes. Similar expression analysis of two other *PR* genes, *OsPR1α* and *OsPR2,* did not reveal significant changes in the transcript levels. Further, sequence analysis of full-length *RsOsPR10α* and its promoter region from the three cultivars revealed mutations in Kavya at four putative *cis*-acting elements, viz. PAL A box binding site, GT-1 binding site, bZIP binding site and GATA binding site. These results suggest that *RsOsPR10α* plays an important role in the events leading to gall midge resistance with HR. They also demonstrate that rice–gall midge interactions share similarities with the reported rice–pathogen interactions rather than with in rice–insect interactions. To the best of our knowledge, there are no reports implicating *PR10α* gene in plant resistance against insects.

Keywords: Biotype, extreme resistance, gene expression, plant–insect interaction, pathogenesis-related genes.

Plants have evolved efficient defence mechanisms to protect themselves from pathogen infection and insect attack, through a complex network of signal perception, transduction and induction of a cascade of downstream genes. Specific recognition of pathogens by plants often induces a hypersensitive reaction (HR), which is characterized by rapid, localized cell death at the site of infection that inhibits the spread of invading pathogens[1]. HR as a manifestation of plant resistance is widely documented against pathogenic fungi[2], bacteria[3], viruses[4] and nematodes[5]. Insects with piercing/sucking feeding mode or with a sedentary feeding stage evoke plant responses that are, in many ways, similar to those by pathogens[6]. However, plant resistance to pathogens and to a specified group of insects need not always be HR-mediated. HR-independent resistance has been reported in potato against PVX virus[7], against gall midge in rice[8] and in *Salix viminalis* against gall midge *Dasineura marginemtorquens*[9].

Pathogenesis-related (PR) proteins have been widely implicated in HR-mediated resistance in plants against pathogens[10]. PR protein families have been grouped into 17 different classes, primarily on the basis of their amino acid sequences[11]. Three highly homologous *OsPR10* (*RPR10*) genes – *OsPR10α* (*PBZ1*), *OsPR10b* and *OsPR10c* have been identified. *OsPR10α* encodes the protein PBZ1, while *OsPR10c* appeared to be a nonfunctional pseudogene[12]. Further, *OsPR10α* is reported to be induced by pathogen infection and signalling molecules such as salicylic acid, jasmonic acid[12], abscisic acid[13] and cytokinins like kinetin[14]. Two novel transcripts of the gene, jasmonic acid-induced *JIOsPR10α*[15] and root-specific abiotic stress and pathogen-induced *RsOsPR10α*[16] have been reported. However, induction of *PR10α* in the plant upon insect attack has not been reported for any species.

The Asian rice gall midge [*Orseolia oryzae* (Wood-Mason)] is an important insect pest of rice causing an annual average yield loss of about US$ 80 million in India[17]. Upon infestation, most cultivars of rice produce leaf-sheath galls called silver shoots that render the tillers sterile. However, rice cultivars differ in their response to insect attack. Infestation of gall midge in the susceptible cultivars leads to compatible interaction resulting in gall development and completion of the insect life cycle

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Table 1. Rice cultivars used in the study and their reaction against the two gall midge biotypes

Cultivar	Parentage	Source of resistance	*R* gene	Reaction against gall midge biotype*		Resistance reaction
				GMB1	GMB4M	
TN1	Chow-wu-gen/Tsai-yuvan-chung	None	None	S	S	None
Suraksha	Sasyasree/CR57-MR1523	Ptb21	*Gm11*	R	S	HR-dependent
Kavya	WGL27120/WGL17620// Mahsuri/Surekha	Eswarakora	*Gm1*	R	S	HR-independent

S, Susceptible; R, Resistant; HR, Hypersensitive reaction (*source: Vijaya Lakshmi *et al.*[19])

within the plant. Whereas in the resistant cultivars infestation leads to incompatible interaction resulting in insect mortality within 48 h after hatching. The incompatible interaction manifests itself mainly as antibiosis in two distinct ways, i.e. with or without the expression of HR. Although HR is considered to be an important component of plant defence, a causal relationship between HR and gall midge resistance has not been established.

In our previous study (unpublished), a cDNA fragment (303 bp) was isolated from a suppression subtractive hybridization (SSH) cDNA library from the meristematic stem tissue (tissue at the insect feeding site) of the rice cultivar Suraksha infested with the avirulent gall midge (incompatible hypersensitive interaction), which showed homology to the rice *RsOsPR10α* gene reported earlier by Hashimoto *et al.*[16]. In the present study we have cloned the full-length gene using 3′ rapid amplification of cDNA ends (3′RACE) and examined the gene expression in response to infestation with virulent and avirulent gall midge biotypes in three rice cultivars to gain insight into differential expression. The promoter region of all the three cultivars was isolated to characterize different alleles. In addition, the expression profiles of *PR1α* and *PR2* were also analysed in relation to gall midge infestation.

Materials and methods

Plant material and insect biotypes

The experimental material consisted of three different rice (*Oryza sativa* L.) cultivars (TN1, Suraksha and Kavya) grown under greenhouse conditions[18] at the Directorate of Rice Research, Hyderabad, India and two gall midge biotypes (GMB1 and GMB4M) maintained on the appropriate differential rice cultivars and in physical isolation from others, as described in detail by Vijaya Lakshmi *et al.*[19]. These cultivars and insect biotypes represented both compatible and incompatible interactions with or without HR (Table 1).

Tissue preparation and RNA isolation

Fifteen-day-old seedlings of the test cultivars were exposed to newly emerged female and male adult insects (50 each) according to the standard procedure[18]. Plants were regularly observed for egg-hatching and sampled at 24 and 120 h after egg-hatching. The meristematic tissue from the feeding site of the insect was dissected and collected in RNALater® (Ambion, USA). Total RNA was isolated using Trizol® (Invitrogen, USA) according to the manufacturer's instructions, its quality checked in denaturing gel and quantified using NanoDrop® 2000C (JH Bio Innovations). RNA samples were treated with RNAase-free DNAase using the DNA-free kit (Ambion Inc., Austin, TX) prior to cDNA synthesis.

Isolation of a gall midge-induced cDNA clone

The SSH cDNA library was constructed using RNA from meristematic stem tissue of rice cultivar Suraksha challenged with GMB1 as the tester and RNA from uninfested tissue as the driver (unpublished data). Based on the sequencing result of the subtractive library, a cDNA clone (GenBank accession no. GQ487633), which showed high level of similarity to *Oryza sativa PR10α* (GenBank accession no. AB127580) was identified. The sequences obtained was compared against the sequences in the NCBI GenBank database using the online BLASTN and BLASTP programs (www.ncbi.nlm.nih.gov).

Rapid amplification of cDNA ends (RACE)

To obtain a full-length gene of partial cDNA clone, 3′RACE was performed using a pair of gene-specific primers (GSP1: 5′-CGGAGCGGGTGTGGAAGGTCTTCTCTGA-3′, GSP nested: 5′-CACCATGAAGCTCAACCCTGCT-GTG-3′). RACE was carried out using the GeneRacer® kit (Invitrogen, USA). Poly(A)$^+$RNAs extracted from meristematic tissue of Suraksha plants were challenged with GMB1. Reverse transcription was performed following the manufacturer's protocol.

The PCR product was gel-purified using QIAquick gel extraction kit (Qiagen, USA), cloned in PCR4-TOPO® cloning vector (Invitrogen, USA) and sequenced. Homology search was performed using online BLASTN and BLASTP programs. The consensus sequence of the full-length gene was obtained using MacVector version 10.6.0 (MacVector Inc., USA).

Table 2. Sequences of primers used in the study

S. no	Primer	Sequence (5′–3′)	Product size (bp)
1	*PR1α**	F: TCGTATGCTATGCTACGTGTTT R: CACTAAGCAAATACGGCTGACA	155
2	*PR2**	F: AAGATTGTTCTGAGAAGAGATCGATCGA R: GCTACGCGAAAATAGGTCTGGTAAACTT	202
3	*PR10α**	F: ACCATCTACACCATGAAGCTTAAC R: GTATTCCTCTTCATCTTAGGCGTA	334
4	*UBC**	F: CCGTTTGTAGAGCCATAATTGCA R: AGGTTGCCTGAGTCACAGTTAAGTG	98
5	*FLPR10α*	F: GCCCTACAGCAAATACACGTTC R: GTATTCCTCTTCATCTTAGGCGTA	1996
6	*GFP1*	CGGAGCGGGTGTGGAAGGTCTTCTCTGA	700
7	*GFP* nested	CACCATGAAGCTCAACCCTGCTGTG	537

S. no. 1–4*: Primers were used for real-time PCR.
S. no. 5: Primers were used for cloning the promoter of *RsOsPR10α*.
S. no. 6 and 7: Primers were used for 3′RACE to clone partial cDNA fragment.

Real-time PCR analysis

We have used 3 µg of RNA for first-strand cDNA synthesis with the SuperScript® III RT (Invitrogen, USA) following the manufacturer's guidelines. To analyse the response of *RsOsPR10α*, *OsPR1α* and *OsPR2* genes to gall midge infestation, 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.

Gene-specific primers for real-time PCR were designed using Primer Express® Software (Applied Biosystems, USA; Table 2). Rice ubiquitin gene *OsUBC* (GenBank accession no. AK059694) was used as the endogenous control. To calculate mean relative expression levels, cDNAs from three independent biological samples in three technical replications each were used.

Mean values from three biological replications were log-transformed to reduce the effects of outliners, mean-centred to equalize experimental averages and auto-scaled to equalize experimental standard deviation, as suggested by Willems *et al.*[20]. Relative transcription levels were presented graphically on log scale.

Promoter isolation

For isolation of the promoter region, genomic DNA of TN1, Suraksha and Kavya was isolated following the procedure of Zhang *et al.*[21]. The primers were designed to amplify the full-length genomic *RsOsPR10α* gene (FLPR10αF and FLPR10αR; Table 2), along with the 1183 bp upstream region based on the annotated whole genome sequence of rice (*O. sativa* subsp. *japonica*), available in the public domain. PCR was performed with the genomic DNA of TN1, Suraksha and Kavya for 35 cycles under the following conditions: initial denaturation of 94°C for 1 min, 35 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 2 min, followed by a final extension at 72°C for 3 min. Amplified products from the DNA of each cultivar were resolved on gel and specific products eluted for cloning. The eluted products were cloned in Topo® TA vector (Invitrogen, USA) according to manufacturer's protocol. Plasmids were isolated using Qiagen Plasmid Miniprep kit from three colonies and sequenced using M13F and M13R primers.

In silico analysis of cis-elements

The raw sequence data were processed to screen out vector, linking sequence and low quality sequences using MacVector version 10.6.0 (MacVector Inc., USA). The exons of the sequence for all the three cultivars were aligned to identify mutations, if any. In order to identify *cis*-acting elements in the promoter region (–1183 bp) that may be responsible for the differential expression, an analysis was performed using PLACE (http://www.dna.affrc.go.jp/PLACE). PLACE is a database of motifs found in plant *cis*-acting regulatory DNA elements, from previously published reports.

Results

Characterization of gall midge-induced cDNA clone

A cDNA clone (GenBank accession no. GQ487633), highly induced in the seedlings of rice cultivar Suraksha after infestation with avirulent gall midge biotype (GMB1), was isolated from the SSH cDNA library (unpublished results). On the basis of sequence data, the clone contained a 303 bp insert, the sequence of which revealed 80% and 99% homology with those of the previously reported PR-10 class of *PR* genes, *PBZ1* (GenBank accession no. D38170) and *RsOsPR10α* (GenBank accession no. AB127580) respectively. 3′RACE was performed

to clone the full-length gene. The partial cDNA clone obtained from the SSH library was 303 bp and the partial clone obtained through 3′RACE was 537 bp. Both these partial clones were aligned into a 760 bp consensus sequence after removing the vector and adapter sequences. This 760 bp consensus sequence was further characterized: from 1 to 34 bp is a 5′ untranslated region; from 35 to 517 bp an open reading frame (ORF) and from 518 to 760 bp is the 3′-untranslated region. The ORF from 35 to 517 bp coded for a polypeptide of 161 amino acids. The coding sequence of the gene showed 99% homology with *RsOsPR10α* at the nucleotide level and 100% similarity at the amino acid level. Comparison of full-length gene sequence with the published sequence of *RsOsPR10α* (GenBank accession no. AB127580) revealed four nucleotide substitutions (at 72 bp, 105 bp T to C, and at 275, 374 bp G to A). However, these changes did not alter the amino acid sequence.

Transcript levels of RsOsPR10α

Results (Figure 1) revealed that the levels of *RsOsPR10α* transcripts in Suraksha cultivar, in comparison with the uninfested control, increased significantly by 40.4 ($P < 0.05$) and 23.5-fold ($P < 0.05$) at 24 and 120 h after infestation respectively, with the avirulent biotype GMB1. The substantial increase in mRNA coincided with the appearance of necrosis or HR. However, the transcript levels in this cultivar did not show any significant change (1.3-fold and 1.0-fold at 24 and 120 h after infestation respectively, $P > 0.05$) following infestation with the virulent biotype GMB4M. The upregulation of the transcript was also not significant in the cultivar Kavya and the susceptible cultivar TN1, following infestation with either of the gall midge biotypes tested.

Transcript levels of OsPR1α and OsPR2

Levels of *OsPR1α* transcripts did not change significantly in TN1 and Kavya after 24 and 120 h of infestation with either of the biotypes (Figure 2). However, there was a significant ($P < 0.05$) reduction in the transcript level in Suraksha after 120 h of infestation with GMB4M. No such change was evident in this cultivar after infestation with GMB1. The transcript levels of *OsPR2* also did not register any significant change in all the three cultivars following infestation with either virulent or avirulent biotypes of the gall midge (Figure 3).

Analysis of cis-elements of RsOsPR10α

The *RsOsPR10α* promoter from all the three cultivars when aligned and analysed by PLACE (http://www.dna.affrc.go.jp/PLACE) identified several defence-related *cis*-binding elements such as W-box, RAV1AAT, ASF1 motif, etc. known to be binding sites of WRKY, RAV1 and bZIP proteins respectively. Among the three cultivars, four *cis*-binding elements were found mutated in Kavya (Figure 4). The first mutation at the PALBOXAPC site at –346 bp contained the motif CCGTCC in TN1 and Suraksha, while it was CCGTCG in Kavya. GATA and GT-1 putative binding site (CCATTATC) at –377 bp promoter region in TN1 and Suraksha had an altered motif CCATGATC in Kavya. Mutation was also observed for myb oncogene binding site at –1084 bp, from CTATCC in TN1 and Suraksha to CTGTCC in Kavya. Erd1/bZIP transcription factor binding site at the far –1122 bp position was mutated to ATGTC in Kavya, from ACGTC in TN1 and Suraksha.

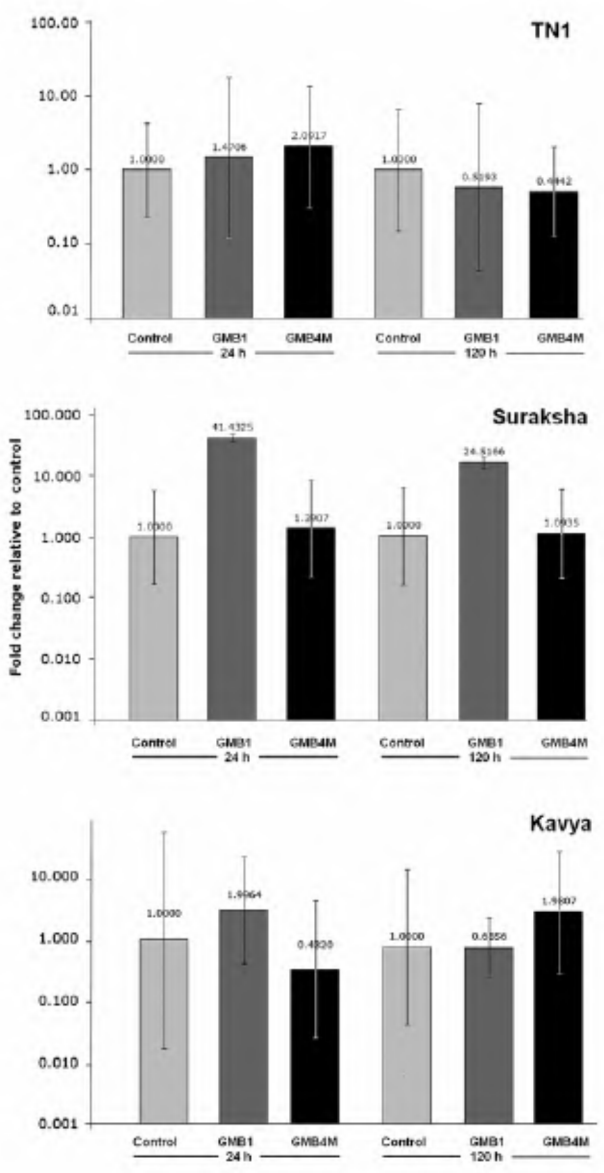

Figure 1. Expression profile of *RsOsPR10α* in three rice cultivars at 24 and 120 h of infestation with gall midge biotypes GMB1 or GMB4M.

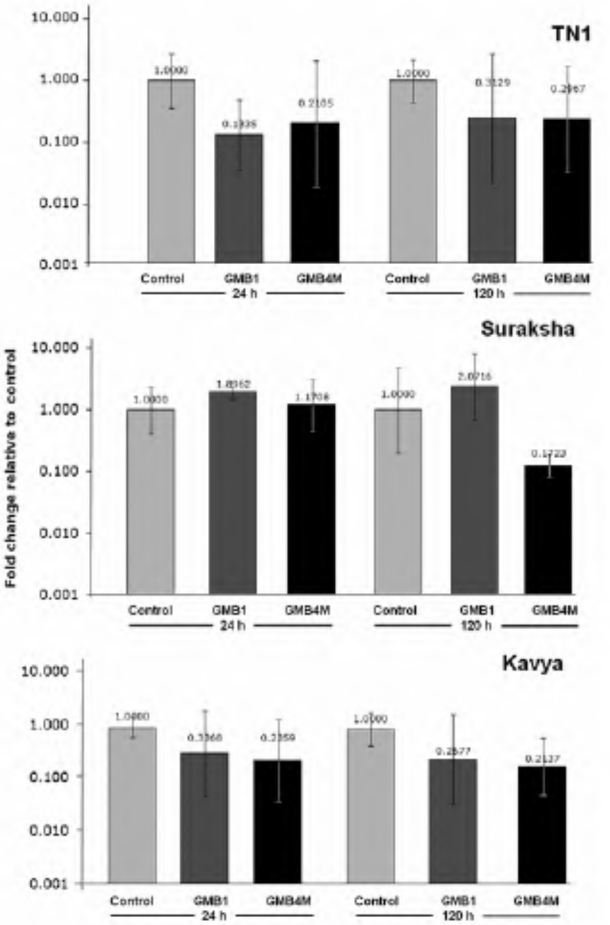

Figure 2. Expression profile of *OsPR1α* in three rice cultivars at 24 and 120 h of infestation with gall midge biotypes, GMB1 or GMB4M.

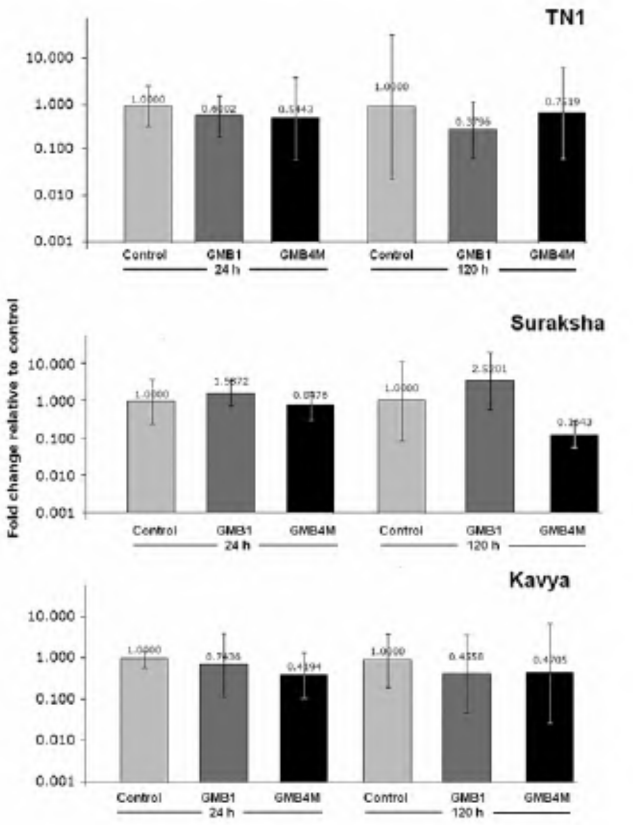

Figure 3. Expression profile of *OsPR2* in three rice cultivars at 24 and 120 h of infestation with gall midge biotypes, GMB1 or GMB4M.

Discussion

PR genes are involved in plant resistance against pathogens involving HR[10]. Plant defence against insect herbivores, however, has been generally distinct from that noted against plant pathogens. The response of plants against leaf-feeding insects has been found, akin to wounding, to be associated with the jasmonic acid pathway, while defence against sucking insects like aphids and gall formers is associated with the salicylic acid pathway[6,22]. Studies on rice defence against the sap-sucking brown planthopper suggested that defence mechanisms against this insect differed from those against chewing insects[23,24].

Rice–gall midge interaction observed in the present study involved upregulation of *RsOsPR10α* by 40.4 and 23.5-fold in incompatible interaction of Suraksha with GMB1. Since such upregulation was not seen in incompatible interaction of Kavya with GMB1, it strongly suggested that the mechanism of resistance was genotype-specific.

In contrast to the several reports on resistance interactions in plants accompanied with hypersensitivity, reports on HR-independent resistance are few. *Rx*-mediated resistance against PVX virus in potato[7] and *Mlg*-mediated resistance against powdery mildew in barley[25] are two such reports. Similarly, our experiments also suggested HR-independent mechanism to be involved in the Kavya–GMB1 interaction. No change in the expression pattern of *RsOsPR10α* in Kavya demonstrated here suggested an alternative pathway of HR-independent resistance response in rice against gall midge.

In spite of clear indication of involvement of *RsOsPR10α* in HR-mediated gall midge resistance in the selected cultivar, our results did not show similar distinct trends with other genes coding other pathogenesis-related proteins, e.g. *OsPR1α* and *OsPR2*. A significant reduction in the transcript level of *OsPR1α* in Suraksha after 120 h of infestation with GMB4M involving compatible interaction is suggestive of its role in susceptibility. However, suppression of *OsPR1α* in our study was of lower magnitude and cultivar-specific.

Four putative mutations in the *cis*-acting elements of *RsOsPR10α* allele in Kavya promoter could be responsible for its lack of induction. PALBOXAPC site, found to be mutated at position –346 has been reported to be

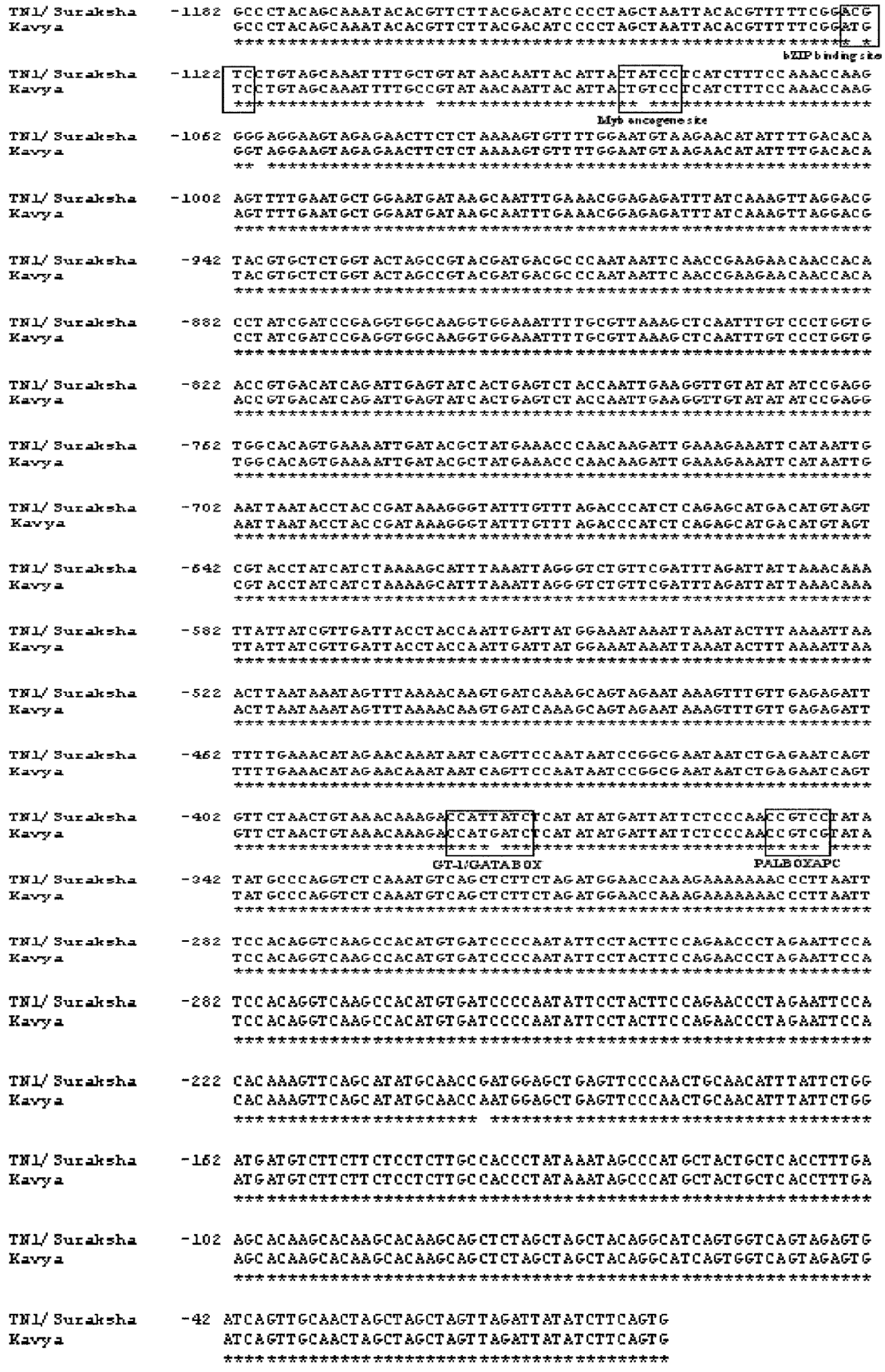

Figure 4. Sequence alignment of *RsOsPR10α* promoter region from the three rice cultivars TN1 (GenBank accession no. GQ487631), Suraksha (GenBank accession no. GQ487631) and Kavya (GenBank accession no. GQ487632).

necessary, but not sufficient, for elicitor or light-mediated phenylalanine ammonia-lyase gene activation[26]. GT-elements have both a positive and negative function in modulating cell type-specific transcription[27]. Mutation of GT-1 binding sites in the *PR1a* promoter influenced the level of inducible gene expression *in vivo* in tobacco against tobacco mosaic virus[28]. Third mutation was found at the –1084 position, which is the putative site for GATA and myb oncoprotein binding domain (GGATA) that has been reported to work as a transcriptional activator. The myb oncoprotein has been reported to be involved in hypersensitive response reaction against several pathogens[29]. The *erd1* binding site is reported to be required for the gene function in *Arabidopsis*[30]. Earlier studies have suggested bZIP class of transcriptional factors to be involved in activation of *PR* genes. Several *bZIP* transcription factor genes are reported to be involved in pathogen defence, such as members of *Arabidopsis thaliana TGA* family[31]. Based on site-directed mutagenesis, Hwang *et al.*[32] demonstrated that mutations in the *cis*-acting sites in the promoter region of *OsPR10a* are responsible for lack of induction of the gene by salicylic acid. Therefore, it is suggested that one or more of the mutated *cis*-acting sites may be responsible for lack of induction of *RsOsPR10α* in Kavya following GMB1 infestation. Lack of *RsOsPR10α* gene induction in the rice cultivar TN1, despite no mutations in the *cis*-binding elements suggested that one or several of the altered upstream genes between resistant and susceptible cultivars may be responsible.

In conclusion, the gene *RsOsPR10α* is induced in the meristematic tissue upon gall midge infestation leading to HR-mediated resistance. Secondly, mechanism of rice resistance against the gall midge shares similarity with that against pathogens. A molecular basis for the reported genetically divergent mechanisms of resistance against gall midge, with or without HR mediation, is evident in these studies.

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