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Molecular Cloning and *In silico* Analysis of Functional Homologues of Hypersensitive Response Gene(s) Induced During Pathogenesis of *Alternaria* Blight in Two Genotypes of *Brassica*

Arpita Mishra, Dinesh Pandey, Anshita Goel and Anil Kumar

Department of Molecular Biology & Genetic Engineering, G. B. Pant University of Agriculture & Technology, Pantnagar

Introduction

Plants possess a vast array of defense strategies to combat pathogen's growth and colonization. One of the most efficient and immediate defense reactions of plants against the pathogen is the hypersensitive response which is defined as "the rapid death of plant cells in association with restriction of pathogen growth" (Goodman and Novacky, 1994). It is characterized by the presence of brown, dead cells at the site of infection which limits the growth of biotrophic pathogens. HR is a highly concerted complex defense response and is followed by the production of reactive oxygen species (Lamb and Dixon, 1997), modification of ion fluxes (Levine et al., 1996), activation of defense through the synthesis of (Dangl et al., 1996; Dixon et al., 1994) signalling molecules such as jasmonic acid, salicylic acid, protein kinases. Salicylic acid accumulation leads to the onset of systemic acquired resistance in distal plant tissue (Ryals et al., 1996). These events are accompanied by the activation of several plant defense genes, local accumulation of pathogenesis related (PR) proteins, activation of transcription factors, degradation of proteins by the polyubiquitin system and programmed cell death.

Although the molecular mechanism leading to the establishment of HR remains to be obscure, a few components have recently been elucidated. The hsr *203J* gene of tobacco which is specially activated during the early stages of incompatible plant/pathogen interaction between tobacco and *R. solanacearum* has been shown to be a molecular marker of the hypersensitive response (Pontier et al., 1994). The expression of hsr *203J* gene has been found to be tightly correlated with HR mediated programmed cell death. For example, the expression of *Cladosporium fulvum* avirulence *avr9* gene product in the tomato line containing the *Cf-9* defense resistance gene led to cell death with rapid hsr *203J* gene activation (Pontier et al., 1998).

Rapeseed and mustard belonging to family Brassicaceae are one of the most important oilseed crops of India. However, the production and productivity of rapeseed and mustard has been greatly hampered by the disease "Alternaria blight" caused by a semibiotrophic pathogen *Alternaria brassicae* which is responsible for causing 30-70% yield loss in rapeseed and mustard at different locations of Northern India. Development of disease resistant lines so far, has been unsuccessful due to the absence of known source of resistant germplasm. It is being felt that biotechnological methods can successfully be utilized to develop resistant variety provided that the molecular mechanism of pathogenesis/defense is delineated. The molecular mechanism governing differential defense so far has not been studied at the molecular level implying hypersensitive response in *Brassica.* Present study is the first attempt to identify the hypersensitive response (hsr *203J*) gene homologue triggered during pathogenesis of Alternaria blight in susceptible and tolerant genotypes of *Brassica juncea* and to perform molecular cloning and in silico analysis for establishing their functional roles.

Materials and Methods

Seeds of tolerant genotype, *Brassica juncea* cv. PAB 9511 and susceptible genotype *Brassica juncea* cv. Varuna were sown in plastic pots under glass house conditions and artificially inoculated by spraying the spore suspension (10^4 spores ml^{-1}) derived from pure culture of Alternaria *brassicae*. Inoculated plants were incubated for 3 days in humid chamber at 90-100 percent RH followed by incubation, at the temperature ranging between minimum of 8°C and maximum 22°C. Healthy and infected leaves showing necrosis along with chlorosis symptoms from both varieties were harvested and used as source of plant material which was stored at -80°C until further use.

In order to design primers for amplification of hsr *203J* like genes, the nucleotide sequences of different hsr *203J* like genes were downloaded from NCBI and subjected to multiple sequence alignment using Mega align module of DNASTAR (Burland, 2000) to obtain a consensus sequences which was used to design primers using Primer3 software programme (Untergasser et al., 2007). The primer sequences used for amplification of hsr homologues from *Brassica* were 5'GTATTACCATGGCGGAGGATTC3'as forward primer and 5'CGGTGACTATGGGAGGACAAAC3' as reverse primer having T_m of 62°C and 64°C respectively. The RNA was isolated from infected leaves of susceptible (Varuna) and tolerant genotype (PAB-9511) of *Brassica juncea* by QIAGEN RNeasy Plant Mini Kit. The RNA obtained was quantified and analyzed on agarose gel electrophoresis (Maniatis et al., 1989). For the isolation of hsr203J like homologues, RT-PCR reactions were performed using QIAGEN One step RT-PCR kit. The amplified products were analyzed on agarose gel and gel eluted using Genei extraction kit. The amplified products were cloned in pGEM-Teasy vector (Promega,USA) as per the kit instructions. Eluted PCR amplicons and cloned fragments were directly sequenced.

The sequenced hsr *203J* like genes were subjected to homology search using BLASTX tool of NCBI (http://www.ncbi.nlm.nih.gov) (Pruitt et al., 2007). The gene sequence was translated into six possible reading frames using translate tool. (http://ca.expasy.org/tools/dna.html). The sequences were further analyzed by software called GENESCAN (Stormo, 2000) and FGENESH (Solovyev et al., 2006) for fishing out the hypothetical protein of that gene. Further,

***Corresponding author:** Dr Anil Kumar, Professor & Head,Department of Molecular Biology & Genetic Engineering, College of Basic Sciences & Humanities,G.B. Pant University of Agriculture & Technology,Pantnagar,U.S. Nagar-India, Fax - 05944-23328; E-mail: ak_gupta2k@rediffmail.com

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the ORF that might encode this hypothetical protein was revealed by its comparison with the six reading frames. The protein sequence was subjected to protein functional analysis using INTERPROSCAN and PFAM version 23.0 (Finn et al., 2006).The sequences of *Brassica* hsr *203J* like protein along with known hsr *203J* like genes from databases were aligned using clustalW (Thompson et al.,1994) and phylogenetic tree was constructed using UPGMA method. A tree was inferred by Bootstrap phylogenetic inference using MEGA3.1 (Tamura et al., 2007). The motifs present in these sequences were analyzed using MEME software version 3.5.7 (Bailey and Gribskov, 1998). For motif analysis, the selection of maximum number of motif was set to 30 with minimum width of 6 amino acids while other factors were of default selection. The predicted gene was compared with BAC clones of *Brassica rapa* genome for finding out full length gene encoding hsr *203J* like protein. Identified four BAC clones were screened for in silico characterization of *cis* acting elements in upstream region using PLANTCARE tool. For structural comparison, all four hsr *203J* like protein sequences were modelled using homology modelling tool, SWISSMODEL. The hsr *203J* like protein structures from tolerant and susceptible varieties were visualized and superimposed using SWISSPDB viewer.

Result and Discussion

In silico analysis of hsr gene homologues

Efforts were made to isolate and clone hsr *203J* like homologues from *Brassica* leaves at late stage of infection of Alternaria blight disease. The expressed hsr *203J* like gene sequences from infected leaves of tolerant and susceptible variety of *Brassica juncea* were amplified by RT-PCR by using gene specific primer. A single amplicon of ~400 bp was observed in *Brassica juncea* cv Varuna while three amplicons of ~400, 600 and 800 bp were observed in *Brassica juncea* cv PAB 9511 from infected leaves (Figure 1). All four amplicons obtained were gel eluted, purified and sequenced. All four nucleotide sequences were subjected to BLASTx to reveal the similarity at protein level with other existing hsr *203J* like proteins respectively. The predicted hsr *203J* like protein sequences from *Brassica juncea* cv Varuna showed 80% whereas the three sequences designated as hsr *203J* R_1, hsr *203J* R_2 and hsr *203J* R_3 from *Brassica juncea* cv PAB9511 respectively showed 79%, 70% and 72% homology with *Arabidopsis*

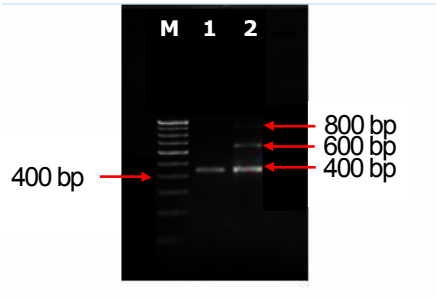

Figure 1: RT-PCR amplification of *hsr* like gene from infected leaf of *Brassica juncea* cv Varuna and *Brassica juncea* cv PAB 9511. M; 100 bp ladder, 1; *Brassica juncea cv Varuna* , 2; *Brassica juncea cv* PAB 9511.

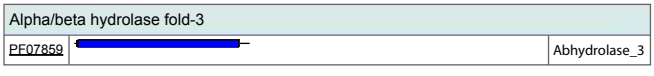

Figure 2: Presence of αβ hydrolase in *Brassica hsr* as revealed by InterProScan.

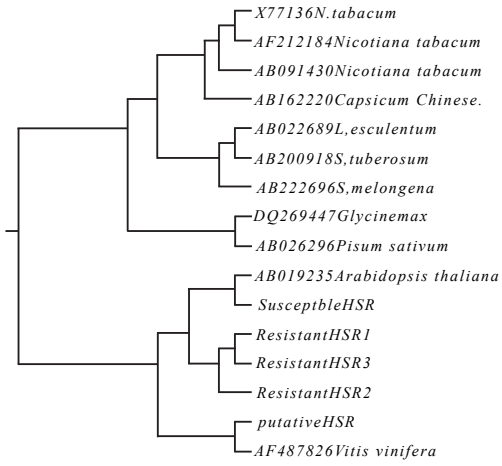

Figure 3: Phylogenetic tree construction using UPGMA method.

hsr *203J* like protein. Hence these sequences were considered to encode the homologues of hsr *203J* like proteins in *Brassica*. These sequences were submitted to NCBI database and were assigned the accession numbers FJ357150, FJ812381, FJ812382 and FJ812383 respectively. The differential induction of hsr *203J* gene homologues during HR mediated cell death in response to pathogen infection in susceptible and tolerant cultivars of *Brassica juncea* suggests its role in defense against Alternaria blight pathogen, however their actual functions are yet to be validated through knock out and knock in based functional approaches.

GENESCAN (Stormo, 2000) and FGENESH (Solovyev et al., 2006) were utilized for fishing out the hypothetical protein of that gene. This hypothetical protein was used as input parameter for INTERPROSCAN and PFAM version 23.0 (Finn et al., 2006) for finding their functional domains. It was observed that the protein sequence possess Alpha/beta hydrolase (abhydrolase) domain (Figure 2) which was also present in all hsr like proteins available in the database.

The αβhydrolase domain was common to a number of hydrolytic enzymes of widely different phylogenetic origin and catalytic function. All the four protein sequences along with known hsr *203J* like protein sequences subjected to multiple sequence alignment and phylogenetic tree construction using UPGMA method revealed two major clusters. All four sequences were found to be clustered close to *Arabidopsis thaliana* hsr *203J* like proteins while other lying in other cluster (Figure 3). It is worth to recall here that *Arabidopsis thaliana* also belongs to Brassicaceae to which *Brassica* spp. belong. Hence, this analysis reveals the role of species specific hsr *203J* like homologues in triggering the defense response against various pathogens.

These sequences were further subjected to MEME program for motif analysis. Motif 2, 3, 12, 14, 7 were found to be conserved in all four hsr *203J* like sequence as well as *Arabidopsis thaliana* bringing them together in one cluster (Figure 4). Motif 2 and 3 encode αβ hydrolase domain and esterase domain which are universally present in all hsr *203J* like proteins. Motif 12 which is present in αβ hydrolase domain and in N-myristoylation site of proteins is present only in susceptible variety (Varuna) as well as in *Arabidopsis* hsr *203J* like protein. Myristoylation plays a vital role in membrane targeting and signal transduction in plant responses to environmental stress (Podell and Gribskov, 2004). This motif might be responsible for putting

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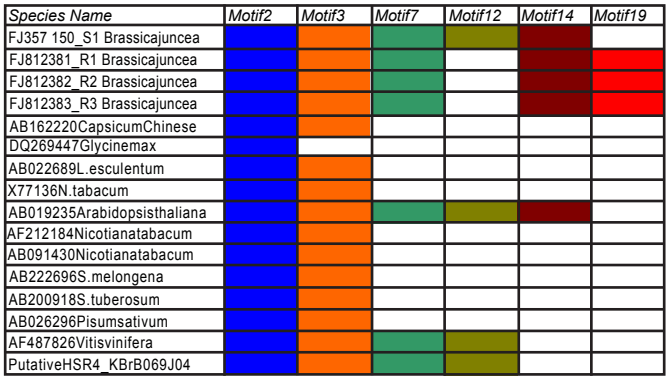

Figure 4: Schematic distribution of respective conserved motifs in *hsr203J* like proteins identified by means of MEME software.

Motif	Function	Signature sequence
2	(Alpha/beta hydrolase)	YYTFYTRMARDANAIIVSVFYRLAPEHRLAPAACDDGFA
3	Esterase	SYADFNRVFLIGTSSGGNIVHQVAIRAGEEDLSP
7	*N-glycosylation site*	IRGMILHHPFFGGEERNGSEMRLANDQVC
12	*Abhydrolase(alpha/ beta hydrolase) N-myristoylation site.*	YYHGGGFCLCSVDWQ
14	BIG1(bacterial immunoglobulin-like (Ig) domain 1) involved in bacterial pathogenecity	NSDDGWIG
19	*Protein prenyltransferases alpha subunit,*	YLYCVAEKDVIKDREMEFC

Table 1: Multilevel consensus sequences for the MEME defined motifs observed among different *hsr203J* like proteins.

them together in one cluster. Motif 19 is present only in hsr *203J* like protein from tolerant genotype (PAB9511) of *Brassica juncea* and absent in hsr *203J* like protein of Varuna as well as of other crop plants (Table 1). Motif 19 encodes protein prenyltransferases alpha subunit, which catalyzes the transfer of an isoprenyl moiety to a cysteine at or near the C-terminus of several eukaryotic proteins. Recent research has led to an explosion of information concerning prenylation signals, prenyl transferase enzymes and the role of prenylation in protein-membrane interactions. Experiments have examined the role of prenylation in protein function and the results suggest that protein prenylation may be involved in facilitating proper sub-cellular localization, promoting protein-protein and protein-membrane interactions and regulating protein function (Cox and Der, 1992). Motif 14 is present in BIG1 domain which is immunoglobulin-like (Ig) domain present in bacterial adhesion molecules involved in pathogenecity. Hence, the presence of motif 14 in hsr *203J* like protein is suggestive of their defensive role during pathogenesis. The predicted gene was compared with BAC clones of *Brassica rapa* genome for finding out full length gene encoding hsr *203J* like protein. Four full length hsr *203J* like genes were identified in four BAC clones (no KBrB032A21, KBrB034A02, KBrB026C23, KBrB069J04) of *Brassica rapa* and screened for analysis of *cis* acting elements in upstream region (-1000/+200) using PLANTCARE tool and trans acting factors in downstream region by plant transcription factor database. Several *cis* acting elements like W box, TC rich repeats and TATA box which represent sites for interaction of defense and pathogenesis related transcription factors were identified in upstream region of putative hsr *203J* like gene present within the BAC clone of *Brassica rapa* (Figure 5). This finding is supported by observation of similar kinds of *cis* acting elements in hsr *203J* like proteins by other workers. For example, in silico analysis of hsr *203J* like sequence in tobacco also revealed the presence of W boxes in promoter region that act as important element required for elicitor responsive expression of defense genes (Rushton and Somssich, 1998). It has been reported that the transcriptional factor called WRKY proteins, bind to W boxes and activate plant defense. In plants, a common TAATNN core motif is known to bind many homeodomain proteins and have been identified in parsley pathogenesis -related gene pr-2 (Korfhage et al., 1995). This motif has been found five times in hsr203J like promoter in tobacco (Pontier et al., 2001).

Analysis of downstream sequence by Plant TFDB tool (Figure 6) indicated the presence of NAC domain which has recently been demonstrated to be present in the proteins involved in mediating various biotic and abiotic stress responses in plants. For example, a few NAC genes, such as At NAC072(RD26), At NAC019 At NAC055 from *Arabidopsis* (Fujita et al., 2004), Os NAC 6 from rice and Bn NAC from *Brassica* (Hegedus et al., 2003) were found to be involved in plant's response to various environmental stresses. Presence of NAC domain in hsr *203J* like protein suggests its possible involvement in HR resulting from plant pathogen interaction. This is supported by the recent finding in which Os NAC 4 is found to be induced during HR cell death against *Acidovorax avenae* in cultured rice cells (Kaneda et al., 2007).

These studies implicated that hsr *203J* like proteins of *Brassica*

Figure 5: Presence of *cis* acting elements in upstream region of putative *hsr203J* like BAC clone of *Brassica rapa*.

```
>OsIBCD033988|os indica 581 NAC
          Length = 581

 Score = 88.2 bits (217), Expect = 7e-19
 Identities = 58/143 (40%), Positives = 79/143 (55%), Gaps = 15/143 (10%)

Query: 2    IRLYYHGGFVLCSVDLQVFHDFCSDMARDLN-AIVASPSYRLAPEHRLPAAYDDGAEAL 60
            + +++HGGGF L        ++H F + +A +L+ A + S     LAPEHRLPAA D G  AL
Sbjct : 95   VLVHFHGGGFCLSHAAWSLYHRFYARLAVELDVAGIVSVVLPLAPEHRLPAAIDAGHAAL 154

Query: 61   EWIRNSGDG---WIGSH--------ADLSNAFLMGTSAGGNLAYNVGIR---SAASDLSP 106
              W+R+   G    I  H          AD  S  FL+G SAGG L  +NV   R    + A  L P
Sbjct : 155  LWLRDVACGTSDTIAHHAVERLRDAADFSRVFLIGDSAGGVLVHNVAARAGEAGAEALDP 214

Query: 107  LRIRGMILHHPFFGGEERSGSEM 129
             +R+ G  +L HP F    E+S SE+
Sbjct : 215  IRLAGGVLLHPGFILPEKSPSEL 237
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Figure 6: Analysis of *trans* acting elements in down stream region of putative *hsr* BAC clone by Plant TFDB tool.

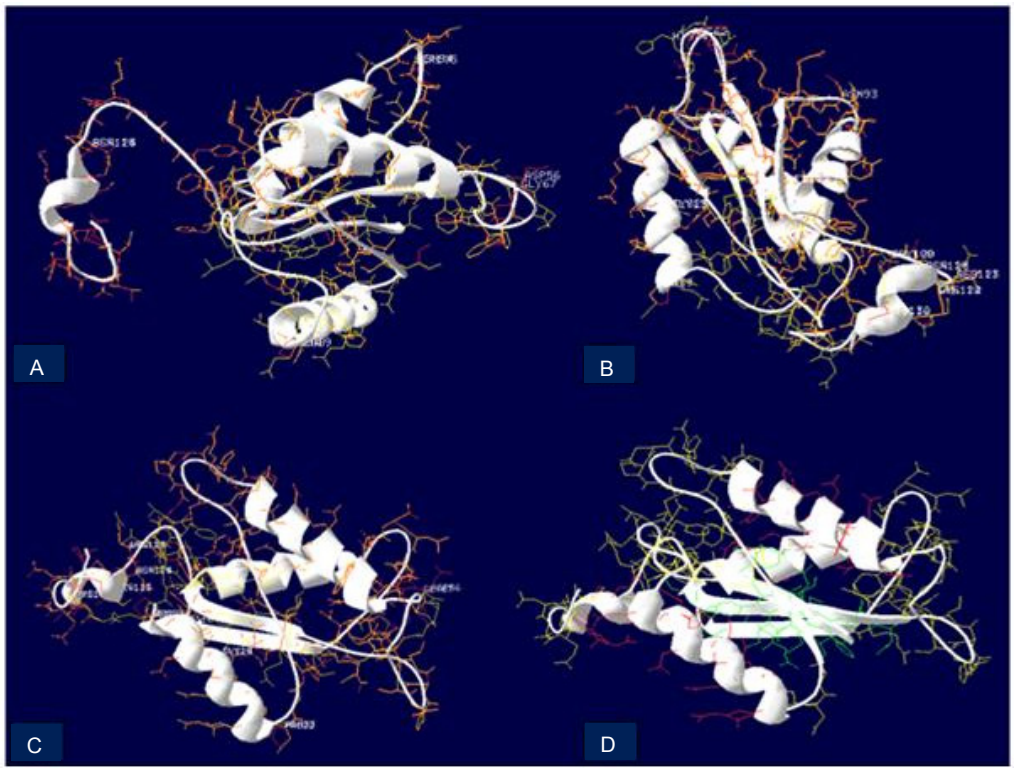

Figure 7: Super imposition of protein structures encoded by *hsr*R_1 (A) *hsr*R_2 (B) *hsr*R_3 (C) from *Brassica juncea* cv PAB 9511 with (D) *hsr* from *Brassica juncea* cv Varuna.

might be regulated by various intracellular signalling proteins during defense response against *Alternaria brassicae* pathogen during pathogenesis of Alternaria blight. The modelled protein structures of resistant and susceptible hsr like proteins were visualized and superimposed using SWISSPDB viewer. The results indicate that these proteins differ with each other at several amino acid residues (Figure 7 and Table 2). These structural differences might lead to differential functions of different homologues of hsr *203J* like proteins in *Brassica* during susceptibility and resistance response.

Conclusions

Hypersensitive response, a defense reaction of plants against the pathogen can be studied through analyzing the expression of its marker gene(s) like hsr *203J*. Present study reveals the identification and cloning of hsr *203J* homologues from tolerant and susceptible genotypes of *Brassica juncea* through RT-PCR analysis. Interestingly, it is the first report to identify structurally different hsr *203J* homologues in response to Alternaria blight infection in *Brassica*. In silico analysis of the sequences isolated from susceptible and tolerant

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Susceptible	Resistant(R1)	Resistant(R2)	Resistant(R3)
Gln (19)	Pro	Pro	Pro
Gly (67)	Asp	Asp	Asp
Ser (105)	Asn	Asn	-
Ser (125)	Asn	-	Asn
Cys (25)	-	Gly	Gly
Asn (33)	-	-	Pro
Leu (114)	-	Met	Met
His (115)	-	Gln	Leu
His (116)	-	-	Leu
Arg (124)	-	-	Lys
Gly (126)	-	-	Arg
Arg (130)	-	-	Lys
Gly (120)	-	Val	-
Glu (123)	-	Asp	-
Arg(124)	-	Lys	-
Ser (125)	-	Asn	-
Arg (130)	-	Lys	-
His (74)	-	Arg	-

Table 2: Amino acid differences in structures of *hsr* proteins.

genotypes of *Brassica juncea* showed the presence of conserved abhydrolase domain having role in cell death. Motif analysis indicated that motif 19 that functions in prenylation is found exclusively in tolerant genotype and motif 12 having myristoylation site was found in susceptible genotypes. Various defense related important *cis* and *trans* acting factors were also found in these homologues. This suggests that these hsr *203J* like homologues of *Brassica* play important role in differential defense response against Alternaria blight- a recalcitrant disease caused by *Alternaria brassicae*.

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