



Interplay of genes in plant–pathogen interactions: *In planta* expression and docking studies of a beta 1,3 glucanase gene from *Piper colubrinum* and a glucanase inhibitor gene from *Phytophthora capsici*

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Abstract Oomycete pathogen, *Phytophthora capsici* is devastating for black pepper (*Piper nigrum* L.) and causes foot rot disease at all stages of plant growth. *Phytophthora* secretes a glucanase inhibitor protein (*GIP*), which is capable of inhibiting defence proteins like endoglucanases. In this particular study Quantitative PCR analysis, molecular docking studies and analysis of sequences of Glucanase inhibitor protein and beta-1,3 glucanase genes were done mainly depending on the data derived from *Phytophthora capsici* whole genome sequencing and *Piper colubrinum* RNA-sequencing (RNA-Seq). Amino acid sequence length of *GIP* gene from *P. capsici* was about 353 amino acids and that of glucanase *pcEGase* gene from *P. colubrinum* was about 312 amino acids. *GIP* gene from *P. capsici* showed high level of expression at early hours of the inoculation time period and *pcEGase* gene showed high level of expression at 16 hpi. High level of expression of *pcEGase* gene at 16 hpi is an indication that the *GIP* gene is successfully inhibited by the glucanase protein from the plant. Moreover insilico studies gave some hint on the importance of certain sites on the surfaces of both interacting proteins that might be having a role in binding of the two proteins and subsequent reactions thereof. Insilico analysis also conclusively proved that inhibition of glucanase inhibitor protein is mainly caused by recognition of an arginine as well as an isoleucine residue during the interaction of the two proteins.

Keywords *Piper colubrinum* · *Phytophthora capsici* · Black pepper · Molecular docking · qRT-PCR

Introduction

Foot rot is a serious disease affecting black pepper (*Piper nigrum* L.) and is caused by *Phytophthora capsici*. *Piper colubrinum*, an exotic and wild species related to black pepper, from South America is widely known to be resistant to *Phytophthora*.

Plant apoplast is a place where numerous proteases are synthesized, especially when the plant is attacked by a pathogen. Pathogens produce certain proteins called glucanase inhibitor proteins *GIPs* which eventually targets plant glucanases present in the apoplast. *GIPs* were initially cloned in *Phytophthora sojae* and the *GIPs* showed homology to the chymotrypsin class of serine proteases (Damasceno et al. 2008).

GIPs inhibits the beta-1,3-glucanases produced by a particular host, eventually protecting the oomycete cell wall from any degradation caused by the plant glucanases (York et al. 2004). To date, there is no information regarding *GIP* genes of *P. capsici* infecting black pepper counteracting on beta-1,3-glucanase from *P. colubrinum*, a highly resistant species. In this study, we report the characterization of these genes and their expression by quantitative reverse transcription polymerase chain reaction (qRT-PCR), *in planta* after inoculating with the concerned pathogen. An effort was also made to find out domains in *GIP* protein that binds to the beta-1,3-glucanase through molecular modeling and docking studies and, also the sequence characterization and expression analysis of both *GIP* and glucanase gene were indeed studied to get a comprehensive

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understanding of the interaction between the two proteins.

Methods

Phytophthora isolates and plant material

Phytophthora isolate, 05–06 from ICAR-IISR was used for conducting experiments and the mycelium obtained from the same isolate was used for RNA isolation from the pathogen. *P. colubrinum* plants grown under controlled condition were used for carrying out the experiment. The *Phytophthora* isolates were grown on carrot agar at 25 °C for 3 days. The leaves of *P. colubrinum* were cleaned with autoclaved DEPC water. The mycelia discs were prepared aseptically by cutting with 10 mm diameter cork borer at the periphery of the culture plates. The mycelia disc (10 mm diameter) from the carrot agar plates were inoculated on the lower side of the leaves, without detaching from the plant. It was then covered by sterile cotton wetted with DEPC water. For the qRT-PCR studies, inoculated parts of the leaves were collected at 2, 4, 8, 16, 24, 48 h post inoculation (hpi).

RNA extraction and cDNA synthesis

RNA isolation from both pathogen and plant materials were done based on Trizol method strictly following the manufacturers instruction. The extracted RNA was treated with DNase (Fermentas) to remove any traces of DNA as per the manufacturer instructions. The extracted RNA was treated with DNase (Fermentas) to remove any traces of DNA as per standard protocol. Biophotometer plus (Eppendorf) was used to quantify the extracted RNA and this was stored at –80 °C until further use. The cDNA was stored at –20 °C until further use.

qRT-PCR primer design

Actin genes were used as endogenous controls for conducting qRT-PCR for both plant and pathogen RNA's. Primers for the beta-1,3-glucanase and *GIP* genes were designed from the sequence databases maintained at IISR by using Primer 3 software (<http://primer3plus.com/>). Primer pairs (Table 1) were tested to check their specificity.

All the real time PCR reactions were performed using three independent biological replicates and three technical replicates for each of the individual biological replicates. qPCR analysis of genes were carried out at various time period intervals. Real time PCR analysis was done using SYBR Green PCR master mix (Qiagen) following manufacturer's instructions. (C_T) values or threshold cycle

values were computed using Rotor gene–Q software version 2.0.2 (Qiagen). Melt curve analysis was also performed to assess the specificity of the PCR products.

Bioinformatics analysis

Sequence data and phylogeny

β -1,3-glucanase gene from *P. colubrinum* has been obtained from gene ontology annotation done with transcriptome data and the pathogenic effector domain—glucanase inhibitor protein (GIP) has been identified from *P. capsici* 05–06 whole genome annotation data. ORFs were identified using CLC genomic workbench 7.0. The ORF start and stop position were trimmed using BioEdit version 7.0.5.3. Using CLC genomic workbench 7.0 Coding sequences (CDS) and six frame proteins were predicted. Functionally related sequences for each gene were then obtained for phylogenetic analysis using the blast hit. Phylogenetic analysis was done using MEGA5 with top hit data of delta Blast analysis. Secondary and tertiary structural study was conducted using Phyre2, I-tasser and Modeller 9.10.

Protein structure modeling

Beta-1, 3-glucanase protein of *P. colubrinum* model was built based on template 2B9L using Modeller 9.10. The template 2BL9 had the highest percent (70 %) similarities to the sequence of endo beta-1,3-glucanase of *H. brasiliensis* among other proteins.

A modeled structure of glucanase inhibitor protein from *P. capsici* was built based on template 2B9L. The template 2BL9 had the highest percent (40 %) similarities to the sequence of GIP among other proteins.

PSIPRED Protein Sequence Analysis Workbench (<http://bioinf.cs.ucl.ac.uk/psipred/>) was used to detect or predict the secondary structures of proteins. The active sites were predicted for both proteins using LIGSITE [cshttp://projects.biotec.tu-dresden.de/pocket/](http://projects.biotec.tu-dresden.de/pocket/). Structure models were validated using PROCHECK (Laskowski et al. 1993), *psi* and *phi* torsion angles were checked using the Ramchandran plot (Ramachandran et al. 1963) which provides a comprehensive check on the stereochemistry of a protein structures.

Results

Expression analysis of the genes

All qPCR primer pairs designed for gene expression produced specific products of expected size as revealed by the

Table 1 Primer sequences used for this study by quantitative reverse transcription PCR followed by inoculation with *Phytophthora capsici* on *Piper colubrinum*

Sl. no	Primer name	Length	Sequence	Amplicon size
1.	<i>Phytophthora</i> GIP	20 bp	F AAGGCGCTCAAGTCTACCAC R ACGAGCGTAAATAGCCGGAG	198
2.	<i>Phytophthora</i> actin	20 bp	F CCCATCTACGAGGGTTACGC R CCGTGGTGGTGAACGAGTAA	121
3.	<i>Piper colubrinum</i> actin	20 bp	F CACATCTGCTGGAAGGTGCT R CACTACTGCAGAGCGGGAAA	143
4.	Beta-1,3-Glucanase	24 bp 20 bp	F CGATGGGTACGTACGTGAACAGTG R CCGCCTACAAAATGACATC	190

conventional PCR assay. *P. capsici* GIP gene was found to be expressed in both mycelia used for challenge inoculation and in planta. Significant expression of GIP following *P. capsici* inoculation was observed at 2 hpi, and the expression was enhanced further to maximum level at 4 hpi and thereafter it declined from 8 hpi onwards. Thus the highest level of expression of GIP gene was observed during the initial hours of infection (Fig. 1a). The beta-1,3-glucanase gene from *P. colubrinum* exhibited lower expression up to 4 hpi and significant increase in expression was observed by 8 hpi (Fig. 1b) and then a sharp increase in expression was shown at 16 hpi. Gradual down regulation of the gene was exhibited in the later phases particularly at 24 and 48 hpi.

Sequence data and phylogeny

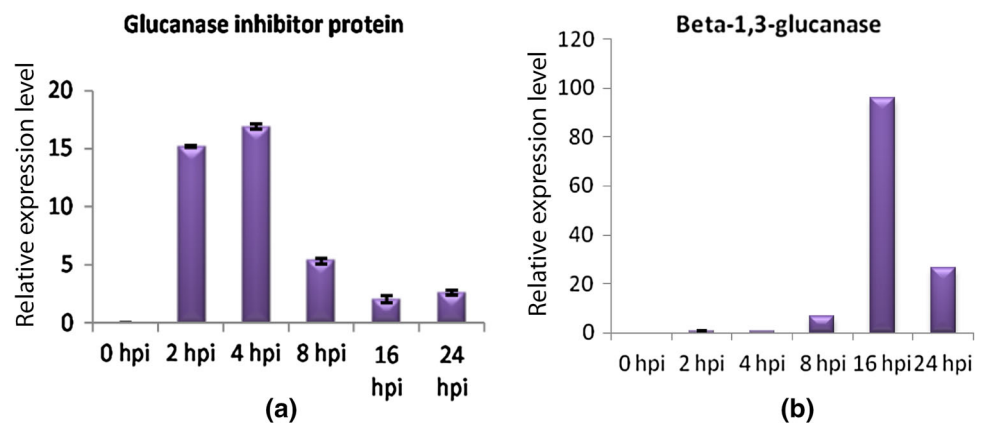
The ORF prediction gives out beta-1,3-glucanase from *P. colubrinum* with length, 864 and Glucanase inhibitor protein of *P. capsici* with length 1899. The CDS prediction gives out length of 818 for beta-1,3-glucanase from *P. colubrinum* and 1059 for *P. capsici* GIP. The translation of these CDS gives out beta-1,3-glucanase with 273 amino acid and GIP with 353 amino acid. Phylogenetic analysis

was done using PSI-blast for each gene. Phylogenetic tree was drawn for each functional genes separately; beta-1,3-glucanase with other *Piper* species (Fig. 2) and GIP with other *Phytophthora* species (Fig. 3) and Phylogenetic trees shows similarity of these genes with evolutionarily related ones.

Protein structure prediction and evaluation

Modelled structure of beta-1,3-glucanase protein revealed that, out of 273 residues, almost 88 % were modelled with by single highest scoring template and with 100.0 % confidence. Verify_3D predicts 99.59 % residues to have an average score of >0.2 and Errat factor was 78.319. Modelled structure of glucanase inhibitor protein from *P. capsici* revealed that out of 353 residues, 96 % was modeled utilizing the highest scoring template. PROCHECK analysis was done for testing the modeled structures internal consistency and reliability. Ramachandran plot analysis of *pcEGase* indicated that 83.7 % residues belong to most favored region and 16.3 % residues in additional allowed regions. Altogether 0.0 % residues were found in both generously allowed regions and disallowed regions. Analysis by Ramachandran plot in GIP showed that, 84.3 %

Fig. 1 Real time PCR results show relative expression levels (Y axis) of glucanase inhibitor protein (a) and beta-1,3-glucanase (b)



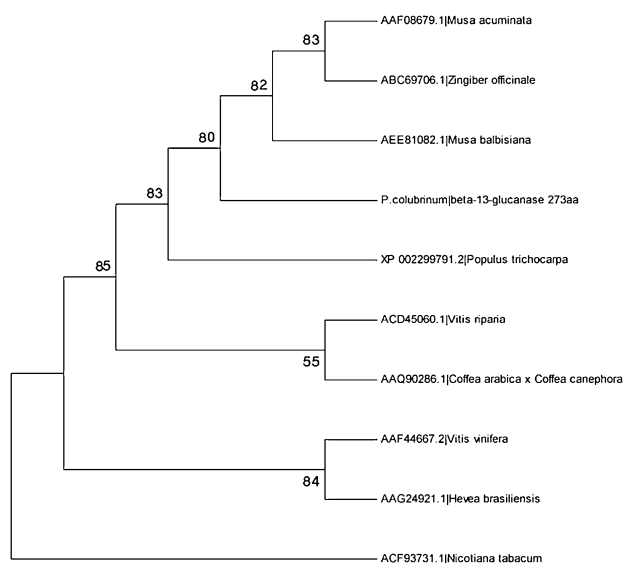


Fig. 2 Phylogenetic tree for beta-1,3-glucanase

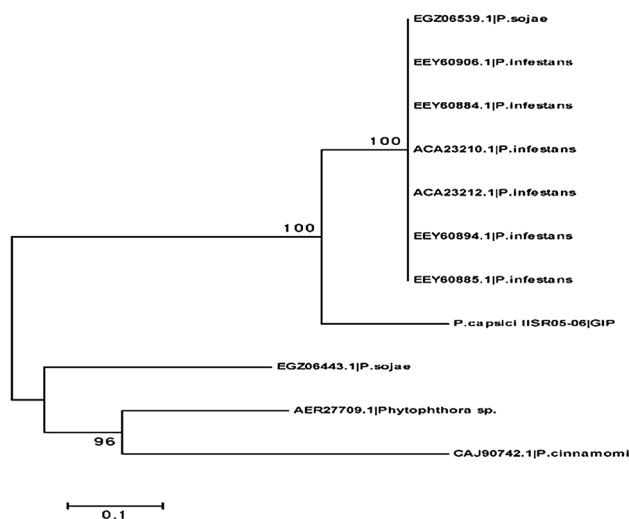


Fig. 3 Phylogenetic tree for *GIP* gene

residues were in the most favored region, 14.7 % residues belong to additional allowed regions, 0.5 % of residues were found to be in generously allowed regions and disallowed regions had about 0.5 % residues (Fig. 4a, b).

Docking studies of beta-1,3-glucanase with glucanase inhibitor protein

Docking of Glucanase Inhibitor Protein to sites on Endo- β -1,3-Glucanases was done using Cluspro 2.0. This particular interaction between two proteins was analyzed using Swiss PDB viewer and PyMOL v1.7.2, for having better information about what are all the key residues that are involved in the binding process. The docked complex comprising *pcEGase* and *GIP*, revealed 21 polar hydrogen bond

interactions. The interaction and binding of the inhibitor to target enzyme was between following residues, as given in (Table 2). The modeling studies indicate that, *GIP* inhibition is mediated by an obstruction in glucanase active site (Figs. 5, 6).

Discussion

Plants have developed an immune system which is switched on by certain microbe-associated molecules or pathogen associated molecules (PAMP/MAMP) and is therefore called PAMP-triggered immunity (Thomma et al. 2011). In PAMP-triggered immunity (PTI), hydrolases like endo glucanase and hydrolase inhibitors like glucanase inhibitor proteins are important players involved in resistance response of the plant. After pathogen recognition, plants secrete hydrolases, such as β -1,3-glucanase to degrade components of the pathogen cell wall, which is one of the PAMP-triggered defense responses. The degraded cell wall fragments are used as PAMPs to further boost PTI (Shetty et al. 2009). The recognition of a pathogen boosts levels of hydrolases in plants and the abundance in levels of these proteins can be associated with disease resistance. The quantitative or RNA transcript expression levels of glucanase inhibitor from *P. capsici* was studied in planta through real time PCR and the expression levels of beta-1,3-glucanase induced in *P. colubrinum* was studied in parallel. This study was done to understand the interaction mechanism of *GIP* with *pcEGases* in the resistant host plant and also to know about their role in pathogenesis.

In the current study, expression of *P. capsici GIP* was found at an increased level during early hours of infection, which can be attributed to initial success in counteracting the β -1,3-glucanase gene from *P. colubrinum*. The glucanase inhibitor *GIP1* from *P. sojae* was found to inhibit endoglucanase from soybean (Misa-Villamil and van der Hoorn 2008). The temporal expression patterns of *PiGIP* genes in *P. infestans* after the pathogen inoculation were observed throughout, starting from the inoculation to different stages of the pathogen, using semi quantitative reverse transcription-polymerase chain reaction (RT-PCR) and *PiGIP* transcripts were found to be up-regulated in planta (Torto et al. 2002). Ham et al. (1997) found out that *P. sojae GIPs* inhibits soybean endoglucanase activity. Temporal expression studies on *GIP* family from *P. infestans* revealed that the genes exhibited distinct patterns of expression during an infection process (Damasceno et al. 2008). Immunoblot analysis showed the presence of *PiGIPs* and *EGases* in the tomato apoplast during infection (Damasceno et al. 2008). Studies on different black pepper cultivars revealed higher expression of β -1,3-glucanase in tolerant varieties of black pepper (Stephen et al. 2001;

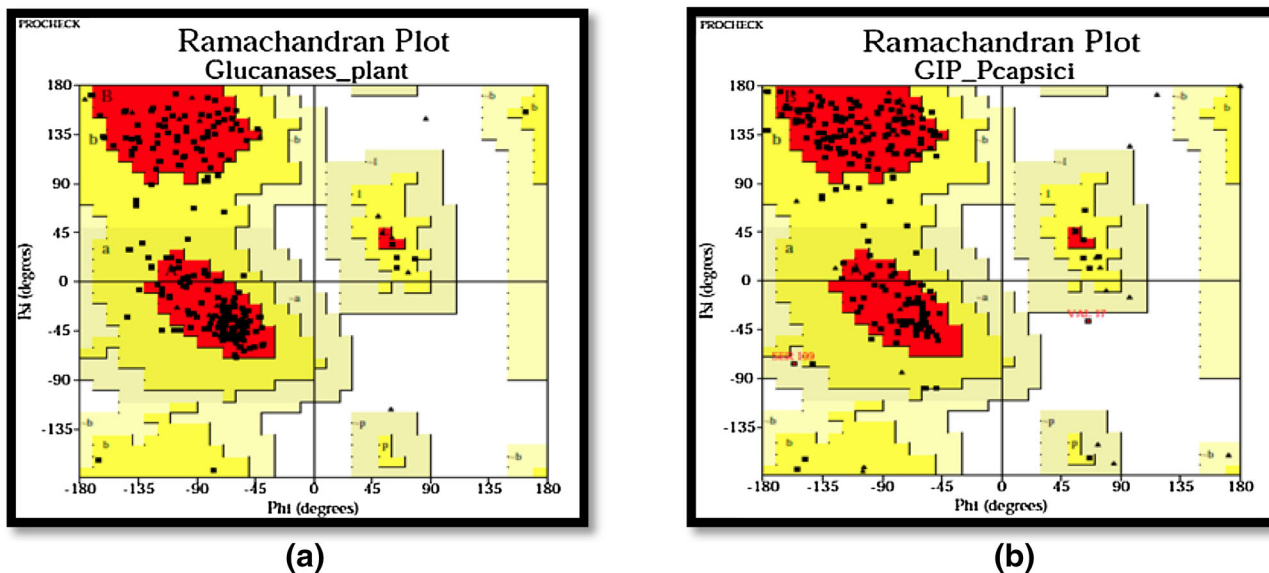


Fig. 4 **a** Ramachandran plot of beta-1,3-glucanase, **b** Ramachandran plot of GIP gene

Table 2 List of interacting residues of *GIP* and *pcEGase* and the number of hydrogen bonds associated with each residue

Serial no.	Interacting residue of GIP	Interacting residue of EGase	No. of H-bond
1	ASP47	ARG238	2
2	LYS44	GLN236	1
3	LYS44	ALA234	1
4	SER67	SER219	1
5	THR205	LEU222	1
6	GLU252	ALA272	1
7	GLU252	SER271	1
8	THR242	THR269	1
9	SER243	THR269	1
10	SER199	GLY33	2
11	LYS198	CYS35	2
12	GLY194	ARG36	2
13	GLU195	ARG36	2
14	ILU196	ARG36	1
15	GLN24	ASN57	1
16	GLN24	SER55	1

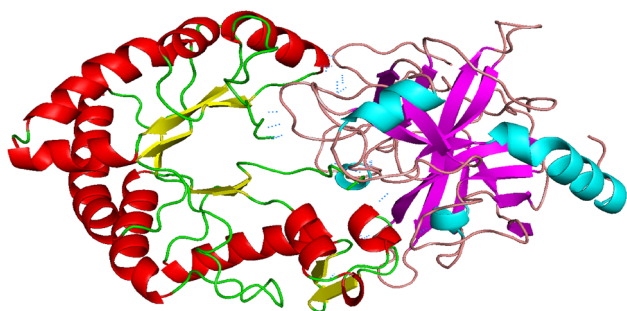
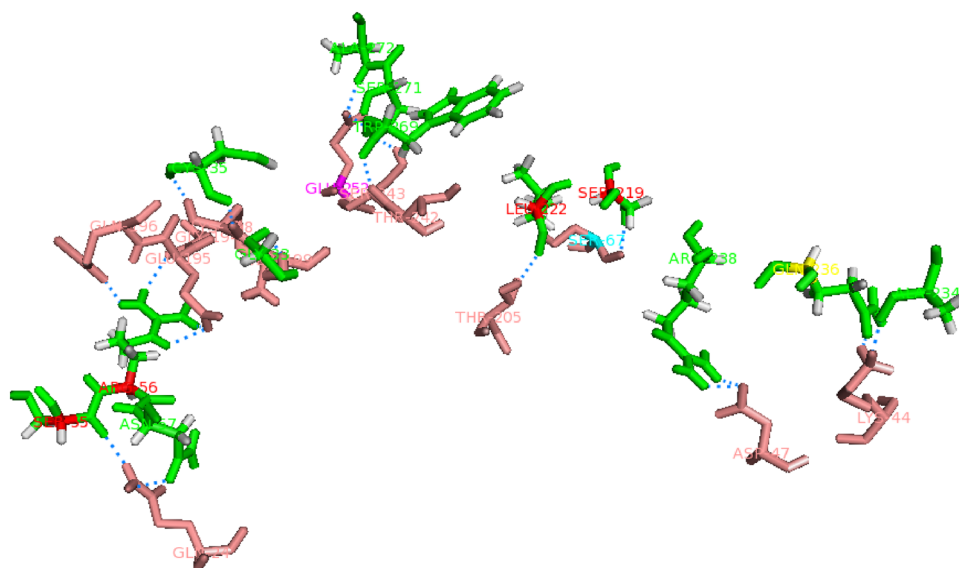


Fig. 5 Model of GIP-EGase complex interaction. Polar covalent hydrogen bond interactions are shown in blue dashed lines. GIP shown as Helix Sheet Loop and EGase shown in Helix Sheet Loop (color figure online)

Nazeem et al. 2008; Vandana et al. 2014). A variety of proteins that is indeed associated with attack, defense, and counter defense are produced during *Phytophthora*–plant interaction (Kamoun 2006).

The maximum expression of beta-1,3-glucanase gene observed during 16 hpi indicate the preparation undertaken by the plant to counteract on the challenge caused by the pathogen through the increased expression of glucanase inhibitor gene (*GIPs*) during initial stages of infection. Nazeem et al. (2008) detected presence of defense related proteins including beta-1,3-glucanase in black pepper as well as in *P. colubrinum* in response to *P. capsici* infection. Being an incompatible interaction,

Fig. 6 Polar covalent Interaction of GIP with EGase active site residues. The interactions are shown in blue dashed lines (color figure online)



the suppression of defense-related endo-glucanase by GIP of *P. capsici* was presumably not successful at later stages (after 16hpi) and hypersensitive reaction was observed in the inoculation sites by 24 hpi. Two isozymes, *EGaseA* and *EGaseB* were found in soybean which differed in their response to (GIPs) produced by *P. sojae* (Bishop et al. 2005). Based on above studies we may expect the possibility of the presence of more isoforms of beta-1,3-glucanase in *P. colubrinum* also, which have to be studied in detail in the future. The expression of both *EGases* and *GIPs* in tomato apoplast points towards a scenario of these proteins being involved in a molecular arms race in plants in general (Dawkins and Krebs 1979).

Thus the binding of GIP to *pcEGase* shows resemblance to a trypsin-like manner binding, which occurs by recognition of an arginine residue on the substrate (Damasceno et al. 2008), as revealed by docking studies which was based on template 2BL9. The template 2BL9 had the highest percent (70 %) similarities to the sequence of endo beta-1,3-glucanase of *H. brasiliensis* and it also has the highest percent (40 %) similarities to the sequence of GIP among other proteins. That was the important reason, the particular template was chosen for docking studies.

The positively selected sites in both *GIPs* and *pcEGases* are evolving rapidly and are found to be in close proximity to each other. This indeed points out to an arms race which is supposed to be taking place between the pathogen and the host (Damasceno et al. 2008). Positively selected residue on *EGase* protein modifies the interaction with GIP and thereby influences the glycan hydrolytic activity (Bishop et al. 2005). This might be the reason for the successful counteraction shown by *pcEGase* upon inhibition by GIP and thereby its expression increases

subsequently at an increased level resulting in a resistance response to the pathogen.

Conclusion

The results showed the important role played by β -1,3-glucanase in *P. colubrinum* with respect to the response towards *P. capsici* and the gene could be directly linked to the high level of resistance besides other major genes involved in the interaction. In silico studies of *pcEGase* and GIP, especially docking experiments carried out revealed that GIP caused inhibition at the active site of *pcEGase*. But studies have proved that the positively selected *EGases* residues are capable of overcoming the inhibition caused by GIP and these proteins are able to express successfully in order to challenge the pathogen. The same situation arises here also where *EGase* expression is found to be very high during the interaction with the pathogen which might be one of the reasons for the resistance response shown by *P. colubrinum*. Studies on more genes in *P. capsici* and counteracting genes in *P. colubrinum* will help to understand the incompatible *Piper-Phytophthora* interactions more thoroughly.

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