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Full Length Article

Identification and Expression Analysis of a Homologue of PR-1 Gene in *Sorghum bicolor*

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Abstract

Trichothecenes, zearalenone (ZEN), fumonisins and aflatoxins are mycotoxins produced by different *Fusarium* and *Aspergillus* spp. during infection of susceptible crops such as maize. Antifungal peptides are crucial in restricting the growth of fungal hyphae into plant cells. In this study, a homologue of a PR-1 accession of *Sorghum bicolor* L. is identified and analysed for expression against a number of fungal strains. Based upon the amplification of a conserved region of nucleotide sequence, a complete cDNA clone for PR-1 homologue was obtained by rapid amplification of cDNA ends (RACE). The construction of a molecular phylogenetic analysis by Maximum Likelihood method coupled with an alignment of the nucleotide sequences of the RACE amplicons yielded a full-length coding sequence for a polypeptide chain, which shares more than 90% homology with the PR-1 like cDNA accessions. The deduced amino acid sequence corresponds to a protein of 145 amino acids with a predicted molecular weight of 15.572 KDa and an isoelectric point of 5.54; both of which are the characteristics of PR-1 proteins. RT-qPCR shows an up-regulated relative expression of PR-1 like expression in response to fungal infection as compared with the control plants. The results elucidate the existence of homologue of *SbPR-1* in sorghum which upon further characterization could be used for an enhanced in-built resistance in sorghum cultivars. It will lead to reduced fungal infections and an increase in production of feed and fodder sorghum.

Keywords: PR-1 proteins; Homologue; qPCR; Antifungal analysis

Introduction

Pathogenesis-related proteins (PRPs) restrict the growth of invading pathogens and play a vital role in the survival of plants against infectious agents and have been classified into 17 families (Sels *et al*., 2008; Fernandes *et al*., 2013). The physicochemical properties of PRPs permit their stable conformation against proteolysis in the sub-cellular compartments, cell wall or intercellular spaces (Pečenková *et al*., 2017). Thus each PR group represents member proteins sharing different antimicrobial and secondary metabolic enzyme activities e.g., PR-2 represents β-1, 3-glucanases while PR3, PR-4, PR-8 and PR-11 include chitinases (Sreeramanan *et al*., 2006; Kovács *et al*., 2012). The exact role and bioactivity pathway of PR-1 proteins still remains to be elucidated. However, the detection of a PR-1 protein in plant cell wall appositions suggests that PR-1 protein may have a role in strengthening of the host cell walls to prevent spread of the pathogen (Benhamou *et al*., 1993).

PR-1 and PR-10 gene families share homology at the level of nucleotide sequences. Thus, based upon sequence homology, parsley PR-1 protein was initially grouped as the type member of PR-10 family (Filipenko *et al*., 2013). However, it was later classified separately because PR-1 protein does not display RNase activity and is different from PR-10 proteins with respect to 3-dimensional protein structure.

The identification of novel gene sequences is based upon the identification and possible amplification of a conserved region already identified in a type member of a gene family. The prediction of function of a novel protein is mainly achieved through the alignment of its nucleotide sequences with those of the members of a particular pathogenesis related protein family. That's why the data sets comprising of nucleotide/amino acid accessions generally lack information about the characteristics of proteins verified with laboratory experiments. The current study aims to explore more members of the PR-1 family. Keeping in view the homology of sequences of nucleotides and amino acids between PR-1 and PR-10, the present study aims to identify a homologue of pathogenesis-related proteins of group PR-1/PR-10 in sorghum.

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Materials and Methods

Plant Material and Fungal Inoculations

Sorghum plants were grown in an open field according to the recommended agronomic practices (www.Pakissan.com). Thirty-day-old plants were used for inoculation with conidia of *Fusarium moniliforme*, *Fusarium graminearum*, *Aspergillus niger* and *Aspergillus flavus*, which were collected from 10-day-old colonies of fungal spores grown on an oatmeal agar medium. The top most leaves were sampled after 72 h of inoculation with fungal spores. Sterile disposable syringes (1 mL) were used to inoculate fungal spores into the leaf tissues by keeping the leaves horizontal during inoculation. For the purification of DNA and RNA for subsequent analysis, the leaf samples were stored at -20°C to prevent RNA degradation primarily. The control sample was collected from the un-inoculated plants by detaching some of the leaves at the same nodal level and stored at -80°C.

DNA and RNA Isolation

Samples were ground in mortar and pestle using liquid nitrogen, and were used for DNA and RNA isolations according to the given protocol by GeneJET Plant Genomic DNA Purification Kit (Invitrogen) and PureLink™ RNA Mini Kit (Invitrogen), respectively. The quantity and quality were analyzed by NanoDrop ND-100 and agarose gel electrophoresis.

SuperScript® VILO cDNA Synthesis Kit and PowerUp™ SYBR® Green Master Mix (Thermo Scientific™) were used for quantitative RT-PCR (qRT-PCR). Primers were designed using the Primer 3 software according to the gene sequences in GenBank Database and were synthesized by Vivantis Technologies. Based upon the accession AY953127.1, the primers; F: TCTTGCAGTCATGCCGTTCAG, R: CCCTCGATGAGCGTGTTCTT represented an amplicon of 90 bp for *SbPR-1*. Before proceeding to qRT-PCR, primers were re-designed to amplify a major part of the coding region of the homologue of PR-1 gene in sorghum. The primer sequences for *SbPR-1*; F: TGGACCCTGGAGATTCCGT, R: GTCGACTCCACCTTCACCAC were designed according to the sequence deduced from rapid amplification of the cDNA ends (RACE) procedure in this study and had an amplicon of 344 bp. The primer pair for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) i.e. F. ATCAACGGCTTCGGAAGGAT and R. GTGGTGATGAAGGGGTCGTT was designed for a 98 bp long amplicon according to the sequence in accession X07156.1.

In conventional PCR, 25 μL PCR reaction volume contained 4.5 μL PCR grade water, 3 μL DNA sample, 12.5 μL master mix (Invitrogen) and 2.5 μL of each of forward and reverse primers containing 10 pmol/μL. The Verity^{TM} thermocycler was programmed for an initial denaturation at 95°C for 3 min. It was followed by 30–40 cycles of denaturation for 15 min at 94°C; 30 sec at 56°C (annealing) and for 30 sec at 72°C (extension). The final extension was carried at 72°C for 10 min. The PCR amplicons were analyzed on 2% agarose/ethidium bromide gel (Vivantis) along with 50 or 100 bp DNA ladder (Invitrogen) which was visualized by direct observation on a UV trans-illuminator. During gel electrophoresis run, each well contained 10 μL of the PCR product and the gel was electroplated for 1 hat 30 mA/100V.

RACE kits (Invitrogen, Cat # 18374058; 18373019) were used to determine the sequences located at the 5' and 3' ends of the amplicon under study. The protocol was followed as per provided manual. For 5'RACE, reverse primer coupled with abridged anchor primer was used while for 3'RACE, forward primer with the universal amplification primer was used. The amplicon was cleaned using GeneJET Gel Extraction and DNA Cleanup Micro Kit (Invitrogen, Cat # K2100-12) and all amplified products were sequenced at CEMB (Center for Excellence in Molecular Biology) facility, Pakistan.

Comparative Transcript Analysis by Quantitative RT-PCR

Transcript levels of mRNA were evaluated by qRT-PCR (Livak and Schmittgen, 2001) with *GAPDH* as an internal reference gene. The qPCR was performed ^{i}Cycler (Bio Rad) wherein each reaction vial of 25 μL contained 2.5 μL of each forward and reverse primer having concentration of 10 pmol/μL, 5 μL DNA sample, 12.5 μL SYBR Green master mix and 4.5 μL PCR grade water., Thermocycler (Bio Rad) was programmed for an initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation for 15 min at 94°C; 30 sec at 56°C (*GAPDH*), 58°C (*SbPR-1*) (annealing), for 30 sec at 72°C (extension) with the final extension at 72°C for 10 min. Primer dimerization was examined by using melt peak curve analysis with temperatures ranging from 50°C to 95°C. Three technical replicates were performed for each sample.

Relative expression levels were determined using the average cycle threshold (Ct). Average Ct values were calculated from the triplicate experiment conducted for each gene, with the ΔCt value determined by subtracting the average Ct value of *SbPR-1*from the Ct value of *GAPDH*. Finally, the equation $2^{-\Delta\Delta CT}$ was used to estimate relative expression levels.

The information (or identification) and homology of nucleotide and amino acid sequences were investigated by using homology search with BLASTx and BLASTp in GenBank database. The phylogenetic tree was constructed by Maximum Likelihood Method**.** The Software used to generate multiple alignments is Molecular Evolutionary Genetic Analysis (MEGA), and Aligned by ClustalW). In the alignments the asterisk sign represents similar residues.

Results

The conventional PCR with sorghum gDNA and cDNA using the consensus primer pair yielded an amplicon of about 100 bp, which was subject to sequencing (Fig. 1 and 2). The alignment of the amplicon sequence in comparison with the predicted sorghum and maize accessions showed a high conservation of the nucleotide sequence across the selected accessions (Fig. 3). The deduced amino acid sequence was also aligned with the amino acid sequence of the selected accessions wherein most of the amino acids were found to be conserved across the accessions XM_021451108.1; XM_002467961.2; XM_002467963.2; XM_002467964.2 and AY751554.1. A significant outcome was that all the accessions with significant homology showed predicted pathogenesis related proteins primarily of the PR-1 group followed by PR-10 sorghum accessions (Fig. 4).

The closeness of accessions having high similarity index contributed by high query cover of 92% and homology with putative PR-1 gene was confirmed through construction of the evolutionary tree (Fig. 5).

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-1970.3339) is shown. The analysis involved 20 nucleotide sequences wherein all positions containing gaps and missing data were eliminated. There were a total of 399 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013). The dendrogram confirmed that (Putative *SbPR-1*) *SbPR-1* is considerably close to predicted *SbPR-1*-like gene, with whom it could be grouped.

The complete coding sequence along with 5' and 3' untranslated regions (UTRs) was determined by RACE method and the alignments of the nucleotide reads produced the complete coding sequence (Fig. 6). The sequencing of 90 bp amplicon obtained with primers based upon an accession of *Zea mays* PR-10 gene (AY953127.1), had yielded a 64 bp long nucleotide read due to the functional property of Sanger sequencer to skip the initial nucleotide sequence of amplicon on both of 3' and 5' ends of the target DNA. The sequence analysis could lead to a homologue of PR-1/PR-10 in sorghum because many members of the PR-1 and PR-10 in different plant species share polypeptide sequence as well as structural and functional properties. Thus the nucleotide sequence was subsequently translated into respective polypeptide sequence. In order to ascertain the identity of newly identified gene as a member of PR-1/PR-10 family, the coding nucleotide sequence was translated into peptide sequence with Expasy translate tool (http://web.expasy.org) and the polypeptide sequence from 64 bp amplicon, was explored within it. The highlighted sequence shows the presence of amino acid sequence deduced from 64 bp sequence within the polypeptide sequence of putative *SbPR-1* (P*SbPR-1*).

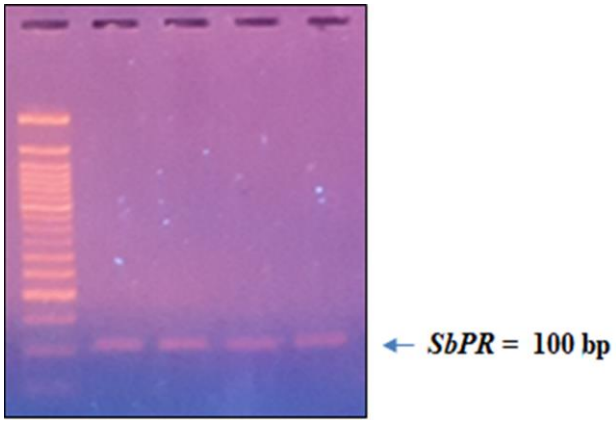

Fig. 1: Gel representation of putative *SbPR* gene fragment of 100 bp in the *Sorghum bicolor* with 50 bp ladder

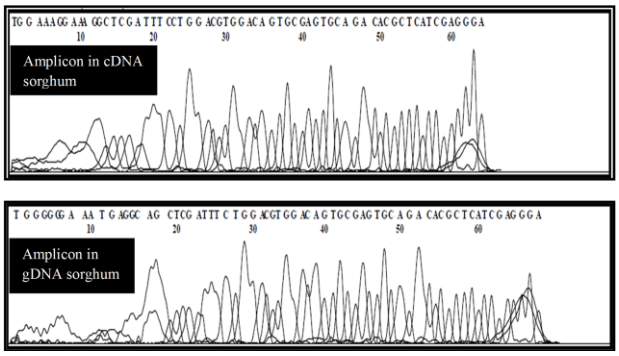

Fig. 2: Sequences of amplicon obtained in gDNA and cDNA of *Sorghum bicolor*

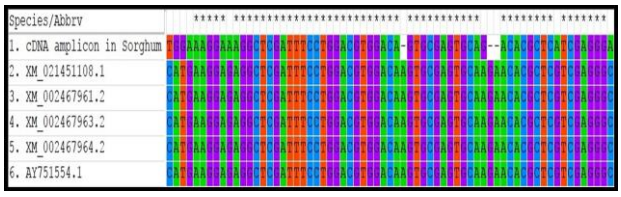

Fig. 3: An alignment of cDNA sequence with highly related accessions of *Sorghum bicolor*

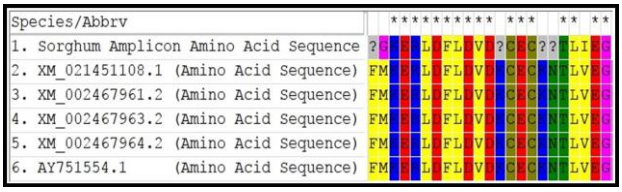

Fig. 4: An alignment of the deduced amino acid sequences with highly related accessions of sorghum

Furthermore, the comparative homology of P*SbPR-1* with pathogenesis-related protein accessions confirmed it to be highly close to members of the PR-1 family (Fig. 7).

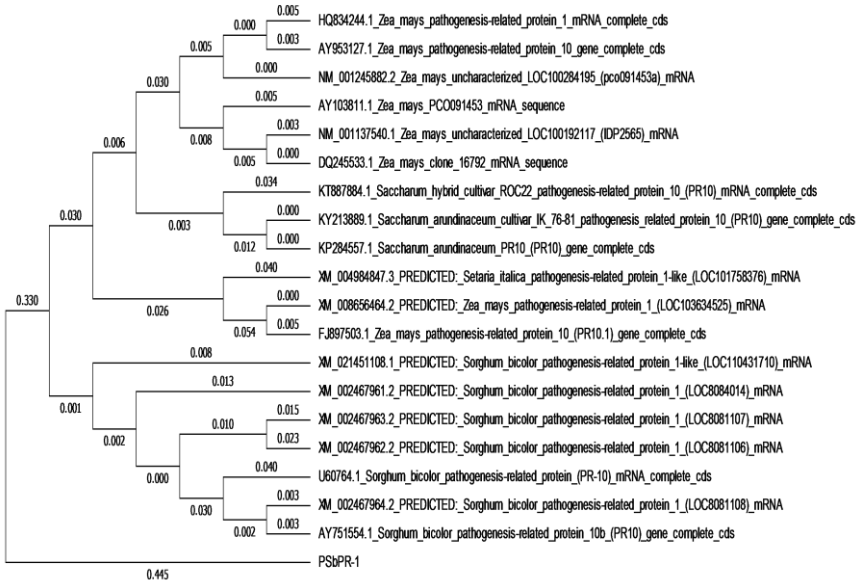

Fig. 5: Molecular phylogenetic analysis by Maximum Likelihood method

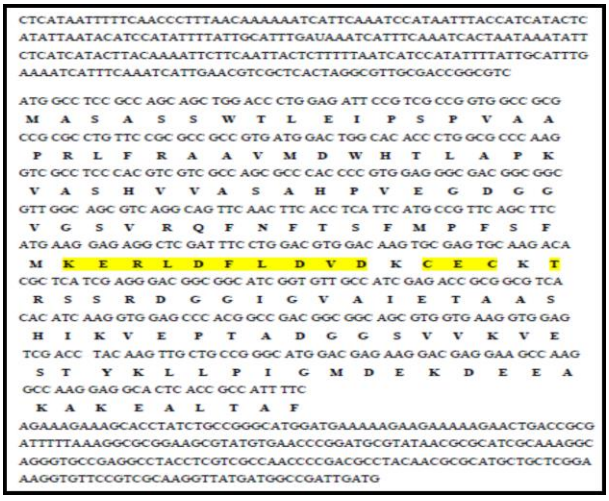

Fig. 6: The complete gene sequence of putative predicted *SbPR-1* gene in *Sorghum bicolor* with sequences derived by 5' and 3'RACE procedure. The alignments were done with Sequence Manipulation Suite (Ver. 2)

Keeping in view the predicted role of closely related accessions, role of *SbPR-1*-like gene in antifungal biochemical pathways was assessed by qRT-PCR of the sorghum tissues infected with different fungal strains. Preceding to qPCR, the annealing specificity of the primers was confirmed by conventional PCR. A single amplicon of predicted length was obtained in gel electrophoresis result. The validated set of primer was used in qPCR (Fig. 8, 9a and b). The amplified product exhibited an identical melt peak curve for all the samples, which showed the robustness of primers annealing at the specific template on target DNA. For qPCR, the Ct value of *SbPR-1* was normalized to the Ct

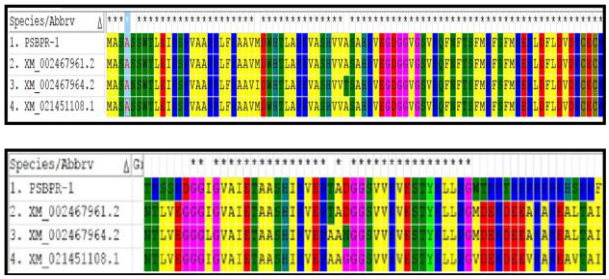

Fig. 7: A comparative alignment of the deduced polypeptide sequence of putative SbPR-1 with accessions of PR-1 family showing close homology in the molecular phylogenetic analysis

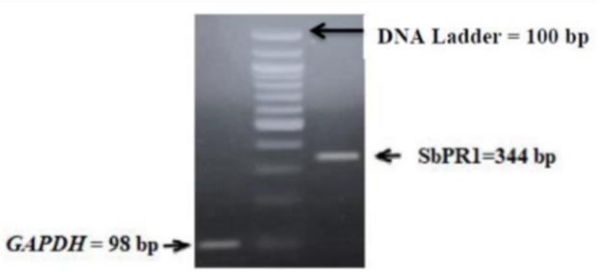

Fig. 8: Gel photograph of amplicons for *GAPDH* and *SbPR-1* with 100 bp ladder

value of *GAPDH* and the relative expression of *SbPR-1* was compared with the control plants (Fig. 10). The experiments were performed with 56 samples with each sub-divided into three technical repeats and statistically analyzed using Student's t-test ($P<0.05$). Bars indicate standard error of the mean (SE).

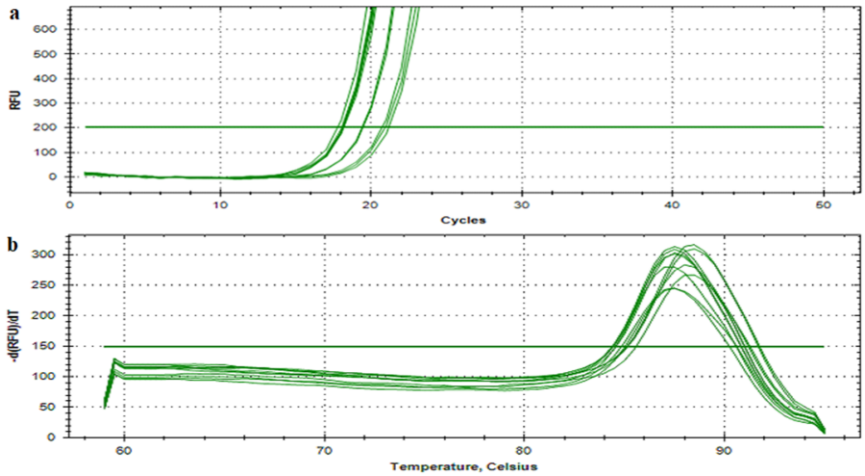

Fig. 9: Amplification and melt curve analysis for *GAPDH* and *SbPR-1*

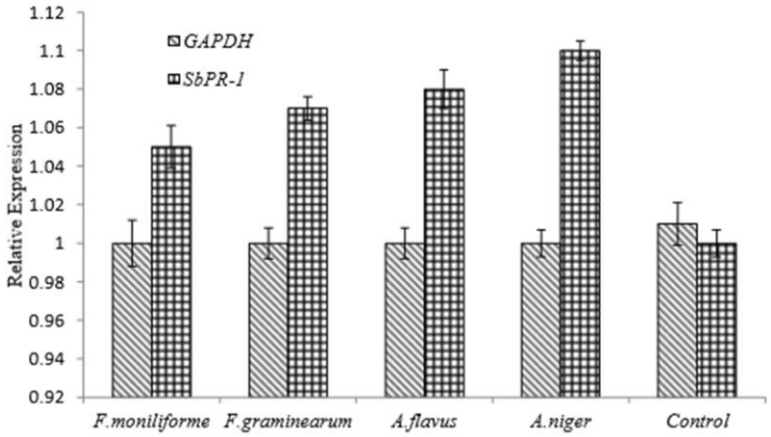

Fig. 10: The expression analysis of *SbPR-1* in response to the selected fungal infections in leaf tissues of *Sorghum bicolor* after 72 h of inoculation of fungal spores

Discussion

The role of pathogenesis-related proteins (PRPs) against fungal infections in plants is critical for the survival, growth and multiplication of plant cells. PR-1 proteins are the most abundant of PR proteins which are commonly used as molecular markers in sorghum breeding for resistance against fungal pathogenicity. The identification of novel pathogenesis related proteins and their functions can be carried out by comparison of the sequence homology of nucleotides and/or amino acids (Heike *et al.*, 2002). Moreover, there is a high likeliness of conservation of the functionally active residues over evolution and homology of proteins' functions can also be predicted by using a phylogenetic tree to rank the residues by evolutionary cadre (Del Sol Mesa *et al*., 2003). Thus, protein sequences are homologous if they have descended from a common ancestral sequence as is the case of sorghum and maize in this study.

In order to identify a novel pathogenesis-related gene in sorghum, consensus primers were designed based primarily on a maize PR-10 sequence to increase our chances of finding an amplicon either in PR-1 or a PR-10 accession. The sequence of the conventional PCR amplicon was used to identify the homology of the sequence with different PR accessions. According to the Sanger sequencing protocol, the peaks at the beginning of the read are usually unresolved and small and thus are skipped from reading at the 5' end of the subject DNA strand. Thus 64 bp and 68 bp nucleotides read were obtained for cDNA and gDNA respectively. By using $BLAST_X$ interrogation, the cDNA sequence showed significant homology and conservation of the nucleotide residues across a number of accessions. A similarity of 90% and E value of $8E^{-15}$ was found for Predicted *S. bicolor* PR-1 like cDNA accessions followed by four *S. bicolor* PR-10 accessions with decreased homology. The sequences were used to establish a phylogenetic tree. It was necessary to observe if the sequenced region corresponded to a PR accession.

An amino acid sequence deduced from nucleotide sequence showed its conservation across PR-1 accessions. The complete coding sequence of putative *SbPR-1* gene was determined by RACE methodology. The sequence was translated into amino acids sequence followed by its comparison across selected PR-accessions to identify the presence of amino acids deduced previously from the 64 bp nucleotides sequence. The translation of the coding sequence yields a 145 amino acids residues long polypeptide of 15.572 KDa and an isoelectric point of 5.54. It is in accordance with the characteristics of PR-1 proteins which have molecular weight ranging from 14-17 and a high variability in isoelectric points (Yashoda and Rajalaxmi, 2013). It is noted that all of the PR-1-like proteins are from sorghum and are remarkably similar (over 90% identity). The NCBI Blast of the sequence coding for peptide chains (highlighted in Fig. 6) showed its 99% homology with the accession XM_002467961.2 where in the query cover was 100%. However the length of amino acid sequence is small in *SbPR-1* as compared with a polypeptide length of 189 residues in accession XM_002467961.2. The estimated molecular mass, pI and homology of nucleotide and amino acid sequence of *SbPR-1* with the existing accessions identify the sequence as a novel homologue of PR-1 genes in sorghum.

Beside the use of sequence homology for describing the function of new genes, experimental evidence is required to validate the function of the genes. Gene functions at experimental level can be obtained from expression patterns by quantifying the level of mRNA and/or protein in specific tissues under given pathogenic stress. Since mRNAs represent the expressed regions of the gene, it is possible to establish a link between the growth of fungal hyphae into the plant cells and an activation of the molecular chaperones involved in plant immunity against infection, thus resulting in the enhanced expression of the responsible mRNAs. PR-1 proteins have been reported to enhance antifungal response in many crop plants such as cowpea, soybean, barley, wheat, pepper, rice, maize, tobacco etc. (Casacuberta *et al*., 1991; Uknes *et al*., 1992; Sarowar *et al*., 2005; Vega-Sanchez *et al*., 2005; Makandar *et al*., 2006) Similarly, Immuno-cytochemical studies by Santen *et al*. (2005) revealed that the PR-1 protein accumulated on the mesophyll cell walls in barley leaves infected by *Bipolaris sorokiniana*. In this study, RT-PCR confirmed up-regulation of PR-1 like protein in response to fungal infection and in comparison to control plants, the levels of relative expression were 1.05x, 1.10x, 1.08x and 1.07x in plants infected with *F. moniliforme*, *A. niger*, *A. flavus* and *F. graminearum* respectively. Since there was a slight up-regulation in all the samples studied and the fact that the resistance to growth and spread of fungal hyphae comprises of a multitude of activated genes wherein a contributory role is played by different polypeptides, it can be concluded that *SbPR-1* plays a participatory role in defence against fungal attack in sorghum. Similar results about antifungal response were reported in a study by Van Loon *et al*. (2006) wherein an in-significant enhancement in PR-1 gene expression was observed in transgenic tomato and tobacco for resistance to *Phytophthora infestans*. It must be mentioned here that antifungal response is a polygenic trait and no direct relationship between the quantity of pathogenesis related proteins and antifungal response has yet been established to date.

In conclusion, the results of this study elucidate the existence of a homologue of *SbPR-1* in sorghum which shows an inducible expression against fungal pathogens. Based on the conservation of nucleotide and amino acid sequences in closely related accessions coupled with expression analysis in response to fungal pathogenicity, the homologue can be classified as a member of the PR-1 group. Using recombinant DNA technology, the enhanced expression of this inducible defence related genes and their accumulation in sorghum hybrids will contribute to better feed and fodder.

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