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RESEARCH ARTICLE

Isolation and characterization of partial *sec*A gene from coconut root wilt and arecanut yellow leaf disease phytoplasma and assessment of its possible use for sero diagnosis

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ABSTRACT: A 425 bp fragment of phytoplasma *sec*A gene encoding SecA, a component of the Sec protein translocation system, was isolated and characterized from coconut root wilt disease and arecanut yellow leaf disease phytoplasma, both belonging to the 16SrXI-B sub group phytoplasma. The gene showed 99% nucleotide identity with sugarcane grassy shoot phytoplasma *sec*A gene. In the phylogenetic analysis of the translated SecA protein sequence, all the 16SrXI group phytoplasmas clustered together. The three dimensional structure of the protein was predicted using Moddler 9.13. The validation of the predicted structure indicated that 83.6% of the amino acids lie in the favored region indicating that the predicted structure was ideal. The possible antigenic domains in the protein were also predicted using the tool SVM TriP. The antigenicity prediction of the SecA protein fragment showed the presence of 4 possible antigenic domains. The *sec*A gene was expressed in pET vector and the protein was used for the development of polyclonal antiserum in rabbit. Because the titre of the phytoplasma was very less, the color development in ELISA was less intense. A technique for isolation of higher number of phytoplasma cells has to be standardized.

Key words: Antiserum development, ELISA, Phytoplasma SecA, structure prediction

Coconut palm (*Cocos nucifera* L.) is one the most beneficial plantation crops in the tropical countries of the world and is rightly known as 'Kalpavriksha' in Sanskrit meaning 'wish fulfilling tree'. Around the world the coconut production and productivity has been greatly affected by poor management practices and pest and diseases. The root wilt disease is a major threat to coconut cultivation in south India, especially Kerala and is also attributed as the major reason for low coconut productivity in the state. The diagnostic symptoms include flaccidity of leaflets, yellowing of older leaves, necrosis of leaflets and deterioration and decay of root system. The leaflets curve inwardly to produce the typical rib symptom so that the whole frond develops a cup like appearance. Abnormal shedding of buttons and immature nuts are also noticed. The root wilt affected palms become susceptible to diseases like leaf rot and pests like rhinoceros beetle and red palm weevil. The symptoms are only obvious in palms that are more than 30 months old (Butler, 1908).

Manimekalai *et al.* (2010a,b) reported the association of 16SrXI group phytoplasma with the coconut root wilt disease and arecanut yellow leaf disease in India. Phytoplasma is obligate parasitic bacteria resembling mycoplasma in pleomorphic constitution due to the lack of cell wall and is grouped under the class 'Mollicutes'. Detection and identification of phytoplasma is necessary for accurate disease diagnosis (Nejat and Vadamalai, 2010).

The cytoplasmic membrane of phytoplasma contains different transporter proteins which are thought to interfere directly with the cytoplasm of the host plant and insect cells (Kakizawa *et al.*, 2001). Several proteins in the membrane preparations are strongly antigenic (Seddas *et al.*, 1996), the amp genes encoding immunodominant proteins are candidates of the phytoplasma components involved in host-pathogen interactions. The cell surface proteins help in interaction with host cell and are involved in disease transmission (Kakizawa *et al.*, 2009). Another factor determining pathogenicity is a transportation system, Sec translocation system (SecA, SecY, dnaK, yidC, ftsY, ffh, grpE, groES and groEL) which are involved in secretion of phytoplasmal proteins directly into the host cell (Kakizawa *et al.*, 2001) could be helpful in understanding the mechanism of phytoplasmal pathogenicity.

The present work was undertaken to isolate and characterize the *sec*A gene of coconut root wilt disease and arecanut yellow leaf disease phytoplasmas. The aim was also to predict the protein structure, possible antigenic domains and to determine level of conservation with closely related phytoplasmas. The possible application of the SecA in serodiagnostics of coconut root wilt and arecanut yellow leaf phytoplasmas was also analyzed.

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MATERIALS AND METHODS

Sample collection and DNA extraction

Spindle leaf samples of root wilt disease symptomatic coconut palms were collected from Kayankulam (Kerala state). Spindle leaf samples from yellow leaf disease symptomatic arecanut palms were collected from Sullia district, Karnataka. Sample collected from a disease free area, Kidu (Karnataka state), was used as the negative control. Sugarcane showing the grassy shoot disease symptom, obtained from Sugarcane Breeding Institute, Coimbatore, was used as positive control. The DNA was extracted from 3g fresh tissue sample using modified phytoplasma enrichment protocol (Ahrens and Seemuller, 1992). PVPP (2010) was added in the extraction buffer. The homogenized samples were incubated at 4°C for 5 min before proceeding for extraction. The DNA was finally dissolved in TE buffer (pH 8) and checked on 0.8% agarose gel. The DNA concentration was measured in spectrophotometer and diluted to 25 ng/µl for further analysis.

Primers for *secA* gene and PCR amplification

The coconut RWD and arecanut YLD phytoplasma belong to 16SrXI group. Their 16Sr rRNA gene sequences show similarity to the16SrXI and 16SrXIV group members. Taking this in to consideration, we designed *secA* gene specific semi-nested primers, cocsf (GACGAAGGAAGAACGCCTTTAAT)/ cocsr (TAGCAGT TCCTGTCATGCCTG) - cocsnf (TATATGGATGCTAA TCGTTTTGC) / cocsr based on available sequences of napier grass stunt phytoplasma (EU168750) and Malaysian bermuda grass white leaf phytoplasma (FJ755004) *sec A* gene sequences using FASTPCR 6.1 software (Kalendar *et al.,* 2009). Primers were validated using Primer-BLAST and BlastN (Altschul *et al*., 1990).

The PCR assays were performed in 15 µl volume containing 50 ng of DNA template, 0.2 µM of each primer, 150 µM of each dNTPs, 0.5U of Taq DNA polymerase (Bangalore Genei) and 1X PCR buffer with 1.5 mM $MgCl_2$. First round amplification with primers cocsf / cocsr was performed to 35 cycles in a Veritiflex (Applied Bio-systems) thermocycler under following conditions: initial denaturation at 95°C for 2 min followed by 35 cycles of 1min denaturation at 94°C, 1min annealing at 52°C and 1 min and 30 s primer extension at 72°C followed by a final extension at 72°C for 10 min. The products of the first PCR were diluted 1:4 with sterile water and 2 µl of each dilution was used as template during 35 cycles of PCR with nested primer pair cocsnf/cocsr respectively. DNA extracted from grassy shoot diseased sugarcane was used as the positive control while negative control contained sterile water substituted for test DNA.

Cloning and sequencing

The PCR fragments of expected sizes were excised from the gel, purified using QIAGEN gel extraction kit, cloned in to pTZ57R/T vector (M/s MBI Fermentas Inc.) and sequenced with M13 forward and reverse primers. Vector sequence was removed from the target nucleotide sequences using VecScreen (Altschul *et al*., 1997) and trimmed sequences were subjected to similarity search using the local alignment search algorithm, blastn (Altschul *et al*., 1997).

Multiple Sequence Alignment and Phylogeny

The phytoplasma *secA* gene sequences were retrieved from the GenBank database. A total of 42 sequences of phytoplasma *sec*A gene from phytoplasma belonging to different 16Sr groups were taken and subjected to multiple sequence alignment using MEGA software package.

Since the RWD and YLD phytoplasmas showed more similarity with 16SrXI and 16Sr XIV groups, five sequences belonging to these groups, i.e. sugarcane grassy shoot (DQ459440), Malaysian Bermuda grass white leaf (FJ755004), Napier grass stunt (EU168750), coconut root wilt disease (JX394030) and arecanut yellow leaf disease (JX394029) phytoplasma *secA* sequences were retrieved and translated using Transeq (EMBL-EBI). Then the sequences were subjected to multiple sequence alignment using ClustalW.

Protein structure prediction

Protein secondary structure prediction: Secondary structure prediction was done using GOR server. GOR is (Garnier-Osguthorpe-Robson) an online secondary structure prediction server.

Protein secondary structure analysis: Secondary structure in proteins consists of local inter-residue interactions mediated by hydrogen bonds. Predicted secondary structure was analyzed for its features. Composition of different secondary structures such as alpha helix, beta sheets, random coils and extended strands were analyzed.

Protein tertiary structure prediction: Since the selected proteins had average sequence homology with the proteins in the publicly available database such as PDB, tertiary structure prediction was done by homology method. The structure prediction tool is Modeller 9.13 (http://salilab.org/modeller/)

Protein structure validation: Structure validation of the predicted protein was done with the help of Ramachandran plot obtained from protein preparation wizard in the Schrodinger package. Amino acids in the disallowed regions were identified and their corresponding region in the 3D predicted model was refined to rectify the error. As for comparison of the quality of the refined structure, SAVES (http://nihserver.mbi.ucla.edu/SAVES/) structure validation server was used. PROCHECK checks the stereo chemical quality of the protein by analyzing the residue by residue geometry and overall geometry. Ramachandran plot for amino acid distribution in the PROCHECK module was analyzed.

Antigenicity prediction

A fragment of SecA protein containing 100 amino acid residues was selected for prediction of possible antigenic domains. The online software SVM Trip was used for antigenicity prediction (Yao *et al*., 2012).

Production of antisera and ELISA

The amplified *sec*A segment was expressed through pET-32a (Novagen, Darmstadt, Germany) vector. The expressed protein were purified and checked on SDS PAGE. The expressed immunogen were injected to rabbit. After 45 days, antiserum was collected and purified.

The direct antigen coating indirect ELISA was performed in Tarsons 96 well microplate. 200 mg spindle leaf sample was ground in 2ml carbonate/bicarbonate coating buffer (CB), pH 9.6. Different volumes of leaf extract were coated on to microtitre plate (50ul, 100ul and 150ul). Diluted antigen, 10µl/ml in CB buffer was added as positive control in separate well (add 100 µl). 100 µl of CB buffer was used as negative control. Each sample had three replications. Incubated the plate overnight at 4°C. Plates were then washed and blocked by the addition of Phosphate Buffered Saline Tween-20 (PBST) containing 1% Bovine serum albumin (PBST-BSA), incubated at room temperature for 1 hr and washed. The primary antibody (diluted in CB buffer 1:10000) was applied and incubated at room temperature for 1 hr and washed. For each sample, the assay was also carried out using the pre immune serum instead of the primary antibody. The secondary antibody, goat anti-rabbit-HRP conjugated, was diluted 1:15000 in 1% PBST-BSA. 100µl diluted secondary antibody was added and incubated at room temperature for 1 h and washed. Then, 0.25% substrate, Tetra Methyl Benzidine (TMB), was added and incubated for 5 minutes at room temperature. The reaction was stopped by adding stop solution (5.93% sulphuric acid). The colour change was read at 450 nm filter in Tecan ELISA plate reader using Tecan i-control software.

RESULTS

PCR amplification and sequence analysis

Semi nested primers cocsf/ cocsr- cocsnf/ cocsr amplified a 425 bp fragment of *secA* gene from RWD coconut and YLD arecanut DNA samples. The partial *sec*A sequence showed 99% nucleotide identity with SCGS phytoplasma (DQ459440) and 88% nucleotide identity with NGS phytoplasma (EU168750) and 87% nucleotide identity with Malaysian BGWL phytoplasma (FJ755004) *secA* genes. Representative sequences were deposited in the GenBank database (JX394030, JX394029).

Multiple Sequence Alignment and Phylogeny

In the multiple sequence alignment of s*ec*A gene sequences of different group phytoplasmas, the 16SrXI and 16SrXIV group members remained together. So we selected these five sequences and translated them and aligned. Sequence conservation was observed in the alignment (Fig. 1). In the *SecA* protein based phylogenetic tree analysis, the 16SrXI group members clustered together while the 16SrXIV group members remained separate (Fig. 2).

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CLUSTAL 2.1 multiple sequence alignment

Malaysian_BGWL_FJ755004      -------------DEARTPLIISSYAKKEKRFYIDANRFAKVLKPNHYII 37
NGS_EU168750                 -------------DEGRTPLIISDYAKKGQKFYMDANRFAKILKTHHYII 37
Arecanut_YLD_JX394029        --------------------------------YMDTNRFAKILKPHHYII 18
Coconut_RWD_JX394030         -------------DEGRTPLIISSYAKKEKKFYMDANRFAKILKPHHYII 37
SCGS_DQ459440                GYVIIDEVDSVLIDEARTPLIISSYAKKEKKFYMDANRFAKILKPHHYII 50
                                                             *:*:*****:**.:****

Malaysian_BGWL_FJ755004      DLESDTIELTEEGIKKGEDFFRIPNLYDSNNIILLHCIKNALKANFIMEK 87
NGS_EU168750                 DLETNTIELTEEGIKKGESFFRISNFYNSNNIVLLHCIKNALKAHYIMSK 87
Arecanut_YLD_JX394029        DLEANSIELTEEGIKKGENFFKIPNLYDSNNIVLLHCIKNALKAHFIMNK 68
Coconut_RWD_JX394030         DLEANSIELTEEGIKKGENFFKIPNLYDSNNIVLLHCKKNALKAHFIMNK 87
SCGS_DQ459440                DLEANSIELTEEGIKKGENFFKIPNLYDSNNIVLLHCIKNALKAHFIMNK 100
                             ***:::************.**:*.*:*:****:**** ******::**.*

Malaysian_BGWL_FJ755004      NKDYLVSNNQILIIDQFTGRILEGRQFSDGLHQALEAKERCVIKEETEIA 137
NGS_EU168750                 NKDYLVSKNNILIIDEFTGRILEGRQFSDGLHQALEAKEGCIIKEETEIA 137
Arecanut_YLD_JX394029        NKDYLVYKNNVLIIDQFTGRTLEGRQFSDGLHQALEAKEGCIIKEETEIA 118
Coconut_RWD_JX394030         NKDYLVYKNNVLIIDQFTGRTLEGRQFSDGLHQALEAKEGCIIKEETEIA 137
SCGS_DQ459440                NKDYLVYKNNVLIIDQFTGRTLEGRQFSDGLHQALEAKEGV--------- 141
                             ****** :*::****:**** *****************

Malaysian_BGWL_FJ755004      ATITYQNFFRIYKKISGMTGTAKT 161
NGS_EU168750                 ATITYQNFFRIYKKISGMTGTAKT 161
Arecanut_YLD_JX394029        ATITYQNFFRIYKKISGMTGTAK- 141
Coconut_RWD_JX394030         ATITYQNFFRIYKKISGMTGTAX- 160
SCGS_DQ459440                ------------------------
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Fig. 1. Multiple sequence alignment of partial SecA of 16SrXI and 16SrXIV group phytoplasmas

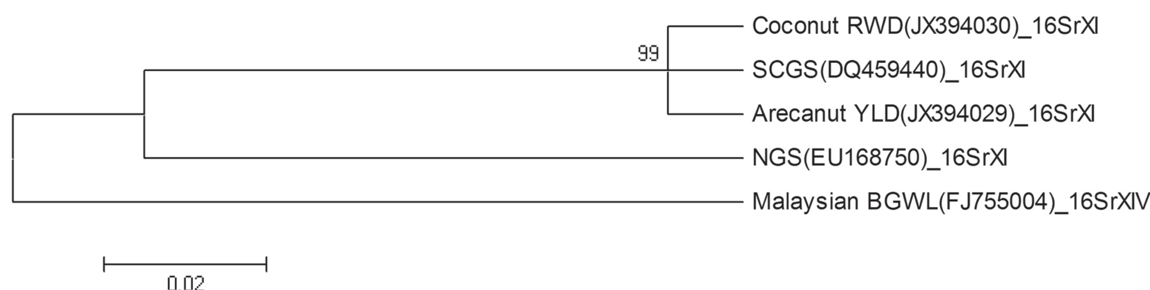

Fig 2. Phylogenetic tree constructed using neighbor joining method with MEGA software showing phylogenetic relationship of 16SrXI and 16SrXIV group phytoplasmas based on partial SecA protein. Bootstrap values are expressed as percentage of 1000 replications.
Coconut RWD, SCGS, Arecanut YLD, and NGS: '*Ca* P oryzae'; Malaysian BGWL: '*Ca* P cynodontis'

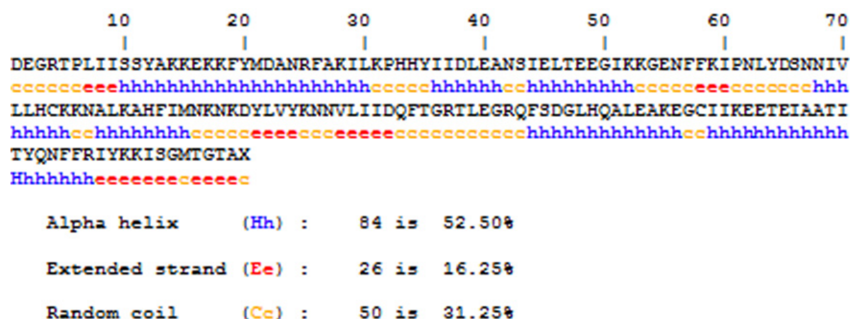

Fig. 3. Secondary structure of SecA protein

Protein structure prediction

The protein secondary structure was predicted by using GOR-4. 52.50% of the SecA protein structure contains alpha helix, which is composed of 84 amino acids, 31.25% of the structure composed of random coils (50 residues) and 16.25% (26 residues) is composed of extended strands (Fig. 3). The tertiary structure prediction was done by Homology modeling method. The template used was SecA protein of *Bacillus subtilis* (PDB id: 3IQM_A). Number of helices in the structure is 16, number of strands is 6 and number of turn is 6 (Fig. 4).

Fig. 4. The tertiary structure of SecA protein predicted by using Modeller 9.13

The predicted model structure has been validated by Ramachandran plot and it reveals the quality of the model. The ideal structure has most of residues present in favored region (82.9%) and some residues in allowed region (17.1%). There is no residue found in disallowed region (Fig. 5).

Antigenicity prediction, polyclonal antiserum development and ELISA

The results for the prediction of possible antigenic domains in SecA protein fragment showed the presence of 4 possible antigenic domains. The highest prediction score of 1 was obtained for a region between 11 and 22 amino acids (Table 1). In the ELISA, the antigen and antibody showed intense color formation. But in case of the samples, the color formed is less intense, due to lower titre of the phytoplasma and hence, the sample preparation for extracting most of the phytoplasma cells has to be standardized.

DISCUSSION

In south India, the RWD is a major debilitating disease of coconut causing great economic loss. The YLD of arecanut is another major production constraint faced by farmers in south India, especially Kerala and Karnataka. In our earlier works we have detected and characterized the phytoplasma associated with these two major diseases (Manimekalai *et al.*, 2010a,b). The SecA gene of phytoplasma has now been in focus due to its ability to make phytoplasma detection more convenient. Many workers have characterized the phytoplasma *sec*A gene (Hodgetts *et al.*, 2008; Mehdi *et al.*, 2012; Kakizawa

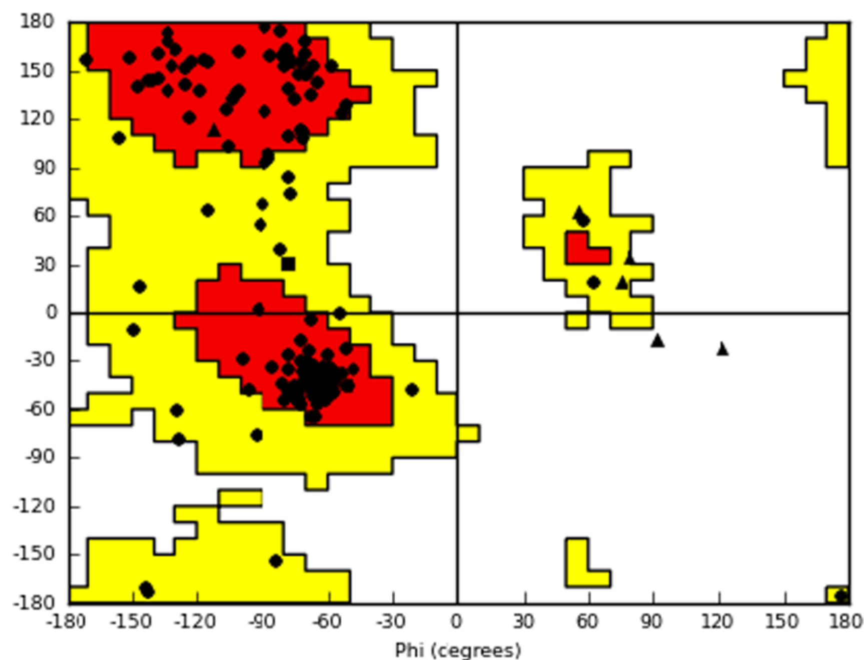

Fig. 5. SecA protein structure validation based on the Ramachandran plot

Table 1. Antigenicity regions in SecA peptide

Rank	Location	Epitope	Score[1]
1	11 - 22	YIIDLEANSIEL	1.000
2	45 - 56	IVLLHCIKNALK	0.455
3	73 - 84	NVLIIDQFTGRT	0.313
4	89 - 100	QFSDGLHQALEA	0.256

1 Score calculated by the prediction method. Predictions done SVM TriP.

et al., 2001) and universal primers are also available of amplification of this gene. Since we could not amplify the RWD and YLD *sec*A genes using the earlier reported primers, we designed our own set of semi nested primers.

Multiple sequence alignment and phylogenic analysis of SecA protein sequences also identified phytoplasmal similarity with the 16SrXI and XIV group members with more similarity with the 16SrXI group. This is in consistency with the 16S rDNA based characterization. Sec protein system present across the bacterial kingdom constitutes the membrane transporters. Kakizawa *et al*. (2001) reported that *sec*A gene is expressed only in phloem tissues of infected plants that phytoplasmas are known to inhabit. In our work we could identify antigenic domains associated with the fragment of protein characterized in the study. The presence of antigenic domains hint that the protein is a candidate for developing polyclonal antiserum for diagnostics for phytoplasma. Hence efforts were also undertaken to raise antiserum against the expressed SecA protein. As the titre of the phytoplasma being less in perennial plantation crop like, coconut and arecanut, there will be low concentration of expressed SecA protein in the samples. And this could be the possible reason for less color development in ELISA. In a similar report, Hodgetts *et al.* (2014) developed monoclonal antibody against SecA of Cape St. Paul wilt phytoplasma but could not detect the pathogen from diseased samples. Hence, the technique for extraction of more phytoplasma cells / proteins has to be standardized for better results in serological diagnostics.

CONFLICT OF INTEREST

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