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Research Article

DNA Sequencing and Bioinformatics Analysis of Clone pOr78 from the Species Specific Suppression Subtractive Hybridization Library Constructed from Endemic Wild Rice Species *O. rhizomatis*

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Abstract

Oryza rhizomatis is an endemic wild rice species in Sri Lanka with some unique characteristics of biotic and abiotic stress resistance. Hence characterization of desirable novel genes found in *Oryza rhizomatis* will be useful for transferring traits to commercial varieties. Therefore, in this study, a species-specific cDNA library was constructed from the wild rice species *O. rhizomatis* to isolate and characterize novel genes which are specifically present in this species but absent in cultivated rice (*Oryza sativa*). Forty recombinant clones were randomly selected from the cDNA SSH library and the inserts were sequenced. Sequence analysis of all forty clones indicated that the suppression and hybridization procedures in the library construction were successful as most of the clones have significant alignment with other wild rice species than *O. sativa* used as a reference cDNA for construction of subtraction library in this study. Therefore the genes which were specifically expressed in the wild rice species *O. rhizomatis* enriched in the SSH library. From this study it was possible to characterize *O. rhizomatis* nsLTP1 proteins (non specific Lipid Transfer Protein 1) super family AAI_LTSS which are found to be involved in control of pathogen attack related responses in plants.

Keywords: cDNA library; *Oryza rhizomatis*; Sequence analysis; SSH library; clone pOr78

Introduction

Wild members of *Oryza* have been shown to be important sources of genes for improving new rice varieties with resistance to biotic and abiotic stress through plant biotechnology. *O. rhizomatis* was selected as a "tester" plant and a popular cultivated variety was selected as a "reference" plant for construction of species-specific cDNA library (Suppression Subtractive Hybridization (SSH) library) to isolate and characterize novel genes, which are specifically present in this wild rice species. *O. rhizomatis* is adapted to specific areas and highly resistant to drought, temperature, soil type and water quality and may have desirable genes to be used in varietal improvement programs. In addition *O. rhizomatis* is growing high salinity areas (Puttalam) and adapted to survive in adverse environmental conditions (drought) of Sri Lanka because of its thick root system and underground branched rhizome. *O. rhizomatis* occurs in the region where maximum diversity of the major rice pest (brown plant hopper, *Nilaparvata lugens)* is present (Vaughan, 1990) Therefore, it could possibly have new sources of genes resistance to this pest. *O. rhizomatis* is also resistant to Bacterial Leaf Blight (BLB) of rice caused by *Xanthomonas oryzae* (Shah *et al*, 2009). Bg352 was selected as a reference plant as it is a popular commercial (new improved) variety and cultivated in 23 districts in Sri Lanka. Therefore these two rice candidates were selected for this study.

Therefore, the objective of this research was construction of a species-specific cDNA Suppression Subtraction Hybridization (SSH) library from wild rice species *O. rhizomatis* and characterization of *O. rhizomatis* cDNA clones. Clones were randomly selected from the SSH library and sequence analysis was carried out. From this study it was possible to characterize *O. rhizomatis* nsLTP1 proteins (nonspecific Lipid Transfer Protein 1) which are found to be involved in control of plant defense responses.

Materials and Methods

Seeds of wild rice species (*Oryza rhizomatis*) and the cultivated variety (Bg 352) were obtained from PGRC, Peredeniya, Sri Lanka. Seeds were planted in pots and grown under greenhouse condition. After two weeks, younger healthy tender leaves were harvested for total RNA extraction. *Oryza rhizomatis* was used as tester and cultivated variety (Bg352) was used as reference plant.

Total RNA was extracted from the tester and the reference plant by TRIzol reagent (Invitrogen, USA) according to the manufacturers' instruction. Tester and the driver ds cDNA were prepared from two mRNA samples by reverse transcription. The double-stranded tester and the driver cDNAs were cut with a four- base- cutter restriction enzyme *Rsa*1. The tester cDNA was subdivided into two portions, and each was ligated with a different cDNA adapter. Adapters did not ligate to the driver cDNA.

The subtraction was performed by two subsequent hybridizations. For the first hybridization, tester cDNAs ligated to adapter 1 and adapter 2R, respectively, were each separately hybridized with driver cDNA. In the second hybridization both batches from the first hybridization were combined and hybridized with freshly denatured cDNA from the driver. The products of the hybridization were used as templates in the first PCR reaction using an oligonucleotide from the common region of both adapters1 and 2R as primers. The product mixture of the first PCR was then used as a template in nested PCR using primers derived from the unique regions of the adapters1 and 2R. PCR products were subjected to TA cloning by using TOPO cloning kit (Invitrogen). White colonies were randomly selected from the original plate and cultured them in LB medium containing 50µg/ml ampicillin. Plasmid DNA was extracted from randomly selected colonies and bidirectionally sequenced using DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, USA) and MegaBACE 100 DNA Analysis System. Sequence data were analysed using Bio-Edit software and searched over GenBank database at National Centre for Biotechnology Information (NCBI) USA (http://www.ncbi.nlm.nih.gov/) to find out similar sequences.

Then blastn, blastx, and blastp programmes were performed for any homology with available sequences in NCBI and other databases. Further bioinformatics analysis of clones was carried out using different bioinformatics software.

Results and Discussion

Amino acid sequence of clone pOr78 from *O. rhizomatis* showed nsLTP1 domain (Fig. 1). nsLTPs is a basic protein, disulphide rich and can be divided into two sub families, nsLTP1 (~ 9 kDa) and nsLTP2 (~7kDa) (Kader, 1996). nsLTP1 transport cutin monomers while nsLTP2s are involved in the conveyance of the more rigid suberin monomers (Douliez *et al.*, 2000). But these two proteins belong to the AAI_LTSS (Alpha Amylase Inhibitors (AAI), Lipid Transfer (LT), and Seed Storage (SS)) super family (Seo *et al.*, 2010) (Fig. 2). Proteins in this family are known to play primary roles such as defending plants from pathogens and insects, lipid transport between intracellur membranes, and nutrient storage (Kader 1996). Sequencing of the clone pOr78 was performed and obtained sequence is shown in Table 1.

The longest ORF was in the frame +1 and *in-silico* translation of ORF resulted 98 amino acids.

The protein was predicted to have nsLTP1 domain (AAI_LTSS super family).

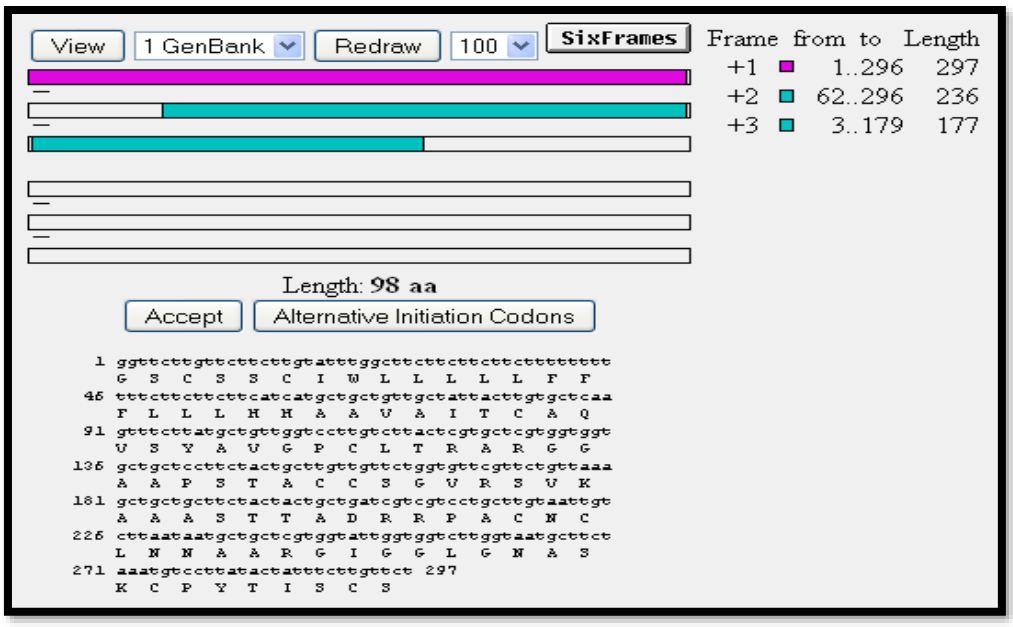


Fig. 1: ORF prediction for clone pOr78.

Table 1: Sequencing result of the clone pOr78

1	GGTTCTTGTT	CTTCTTGTAT	TTGGCTTCTT	CTTCTTCTTT	TTTTTTTTCT	TCTTCTTCAT
61	CATGCTGCTG	TTGCTATTAC	TTGTGCTCAA	GTTTCTTATG	CTGTTGGTCC	TTGTCTTACT
121	CGTGCTCGTG	GTGGTGCTGC	TCCTTCTACT	GCTTGTTGTT	CTGGTGTTCG	TTCTGTTAAA
181	GCTGCTGCTT	CTACTACTGC	TGATCGTCGT	CCTGCTTGTA	ATTGTCTTAA	TAATGCTGCT
241	CGTGGTATTG	GTGGTCTTGG	TAATGCTTCT	AAATGTCCTT	ATACTATTTC	TTGTTCT

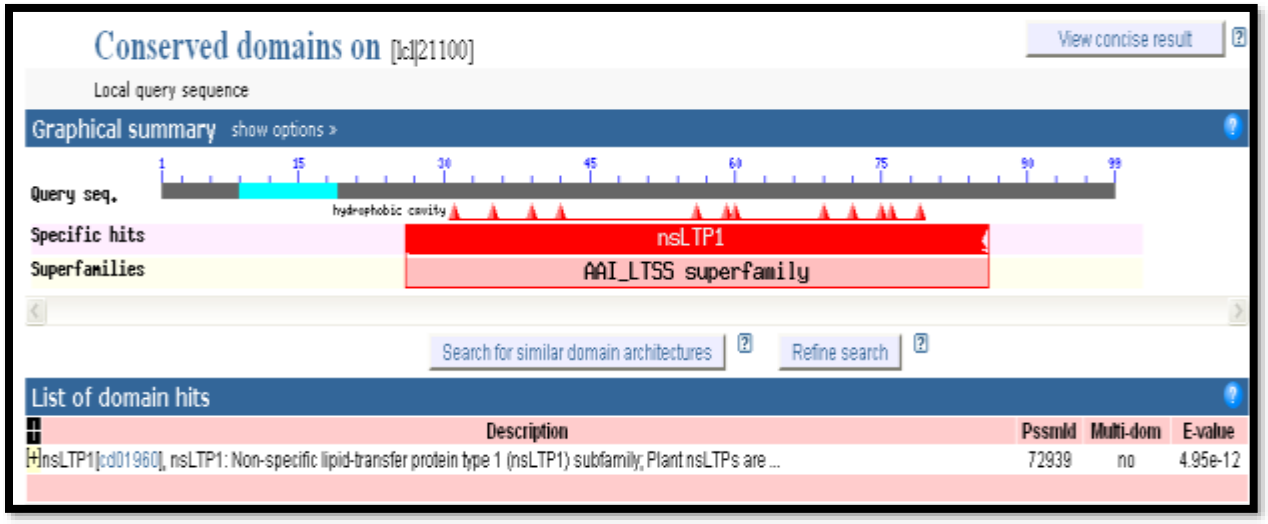


Fig. 2: Conserved domain present in clone pOr78

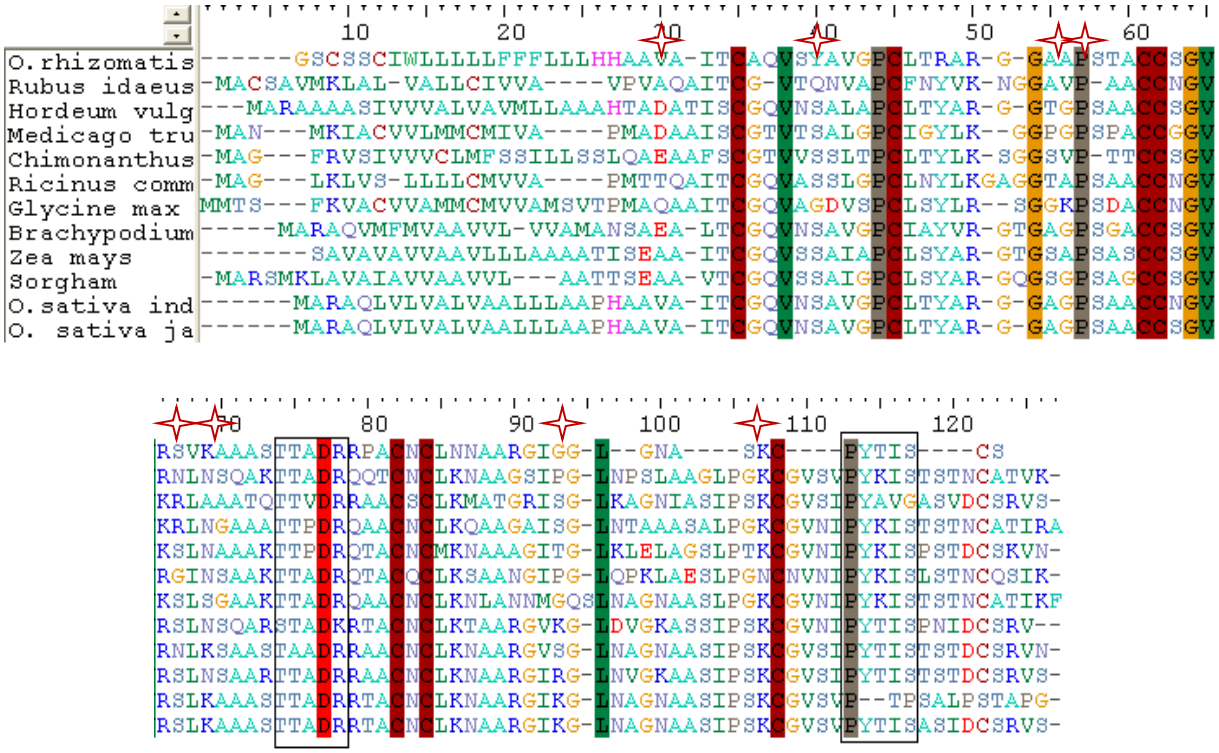


Fig. 3: Comparision of aminoacid sequences of lipid transfer protein of some plants.

Other plant species also reported to have nsLTP1. Aminoacid sequences of nsLTP1s of other plant species and rice genus contain two highly conserved regions with eight-cysteine motif were (same region in all plant nsLPT1) obtained from the data bank and compared with the nsLTP1 of *O. rhizomatis*. Plants nsLTP1s have high sequence identity (Fig. 3). Two highly conserved regions ^{74}T/S-X-X-D-R/K^{79} and ^{113}P-Y-X-I-S^{117} (in box) of rice nsLTP1 were observed (Douliez *et al.*,, 2000). This same concerved regions observerd in *O. rhizomatis* protein (pOr78). That contribute significantly to lipid binding (Cheng *et al.*, 2004).

Among all known sequences of nsLTP1s, all are characterized by an eight-cysteine motif (8CM) occured in a strictly conserved pattern and form disulphide bridges. *O. rhizomatis* nsLTP also showed similar disulphide bonding pattern (Fig. 4) of nsLTP1.

The four helices (H1-H4) bundle is stabilized by four disulfide bonds and forms a tunnel-like hydrophobic cavity/pocket surrounded by the C terminal loop (Fig. 5) that can bind different types of ligands: hydrophobic molecules, fatty acids or lipids (Shin *et al.*, 1995). The hydrocarbon tails of the lipids are inserted into the hydrophobic cavity of nsLTP1 while the head groups of the lipids protrude out of the binding pocket and points towards the solvent. The binding pocket of the lipid-free protein shows high plasticity. Its volume may expand from about 200 to 750 angstrom upon lipid binding (Lee *et al.*, 1998). According to the findings of Cheng *et al.*, (2004) it was found that the volume of the binding cavity of rice nsLTP1 complex depends on the lipid binding situation. A lipid with a longer aliphatic chain binds more tightly with the protein and has a smaller binding cavity volume. Cavity volume of *O. rhizomatis* (257 Cubic Angstroms) is greater than *O. sativa japonica* (178 Cubic Angstroms) and smaller than *O. sativa indica* (518 Cubic Angstroms). Therefore *O. rhizomatis* has longer aliphatic chain binding affinity than *O. sativa japonica* group but lesser than *O. sativa indica* group.

Three crystal structures of rice nsLTP1 from *O. sativa*, complexed with myristic (MYR), palmite (PAL), or stearic acid (STE) were determined. The nsLTP1-MYR and nsLTP-STE complexes bind a single fatty acid while the nsLTP1-PAL complex binds two molecules of fatty acids (Cheng *et al.*, 2004). According to the output of ScanProsite server *O. rhizomatis* nsLTP1 can be categorized as myristic complex. The server did not predict ay palmite and stearic acid sites in *O. rhizomatis*. Four N-myristoylation sites were identified in *O. rhizomatis* at the sequence 1-6 (GScsSC), 45-50 (GAapST), 84-89 (GGlgNA), and 85-90 (GLgnAS). Four N- myristoylation sites were predicted in other rice subspecies also.

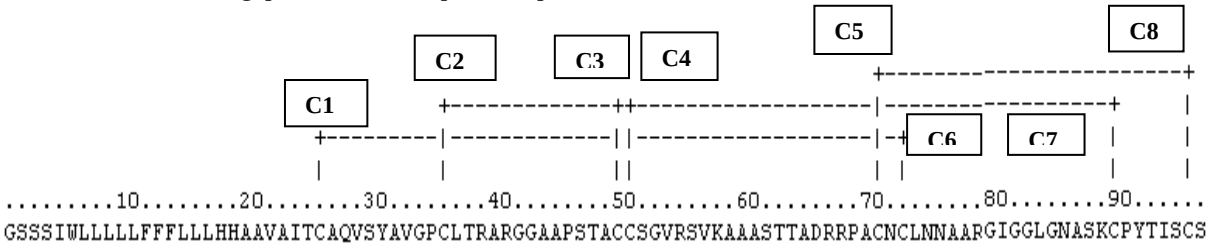


Fig. 4: Disulphide bonding pattern of nsLTP1 of *O. rhizomatis*.

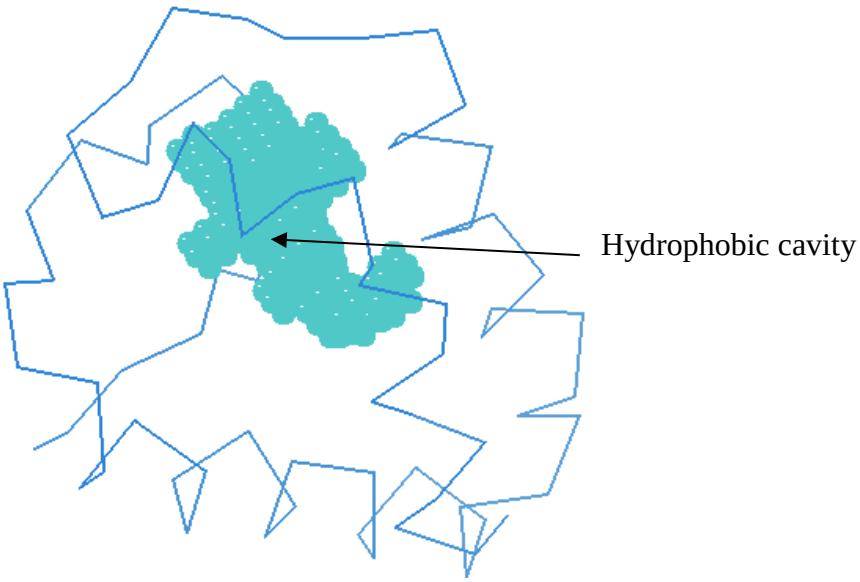


Fig. 5: Hydrophobic cavity of nsLTP1 of *O. rhizomatis*.

Cheng *et al.* (2004) found that the nsLTP1-PAL dual lipid bound complex has the biggest hydrophobic cavity among the three rice nsLTP1 complexes but it is only bigger than that of the nsLTP1-MYR complex, a single lipid bound complex and they concluded from their findings that nsLTP1-STE complex has the smallest hydrophobic cavity and have strongest interaction between the ligand and the protein

All four protein structures showed (Fig. 6) four helix bundle folding and long C-terminal loop. The overall structures of nsLTP1 have a long C-terminal loop and this C-terminal loop region is elastic in order to accommodate a diverse range of lipid molecules. nsLTP1 of *O. rhizomatis* has somewhat shorter C-terminal (Fig. 6) when compared to other rice subgenus nsLTP1 (*japonica and indica*).

According to targetp v1.1 server prediction result, nsLPT1 of all plant species had highest score for SP that is sequences contain signal peptide for secretory pathway (Table 2).

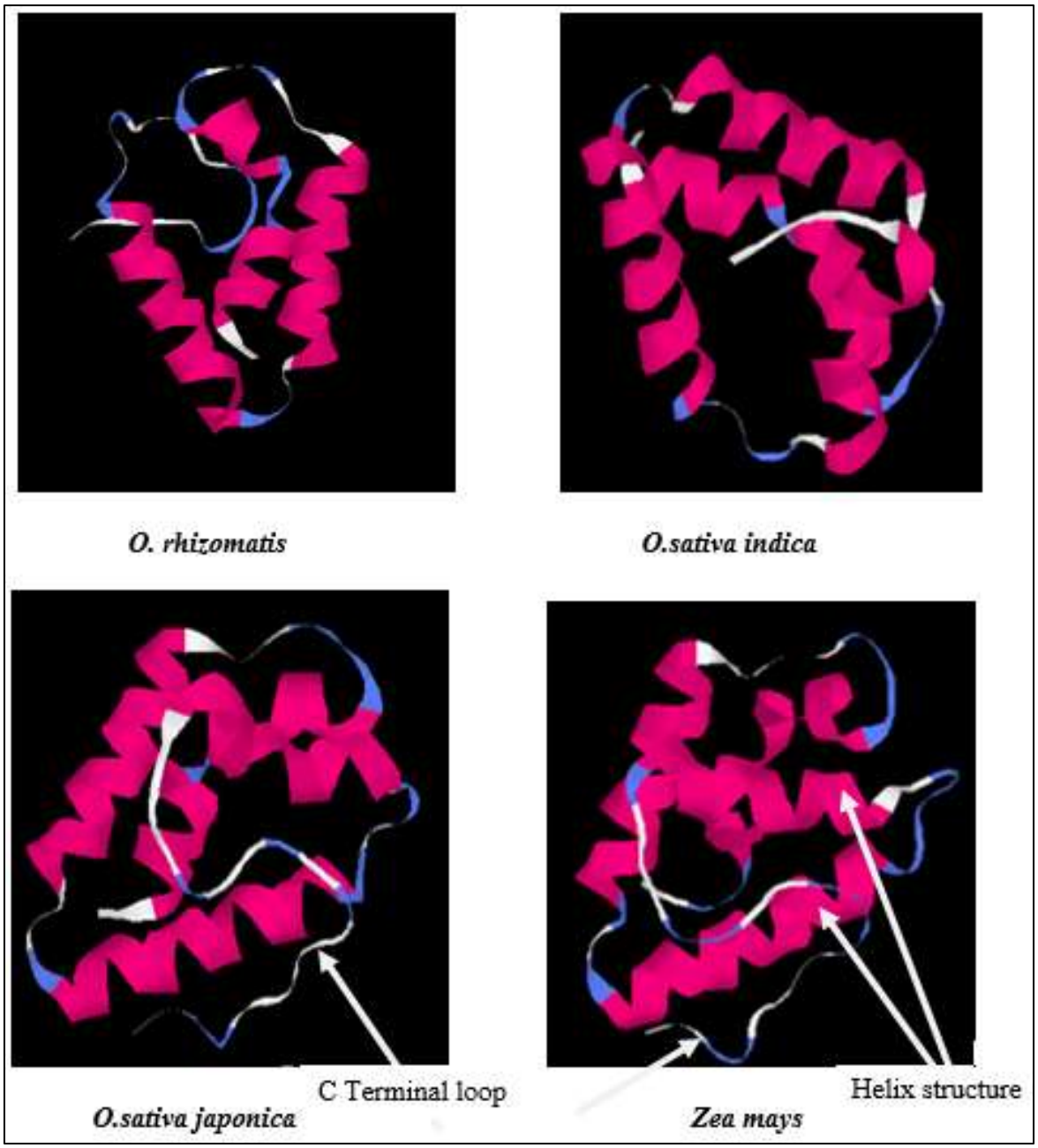
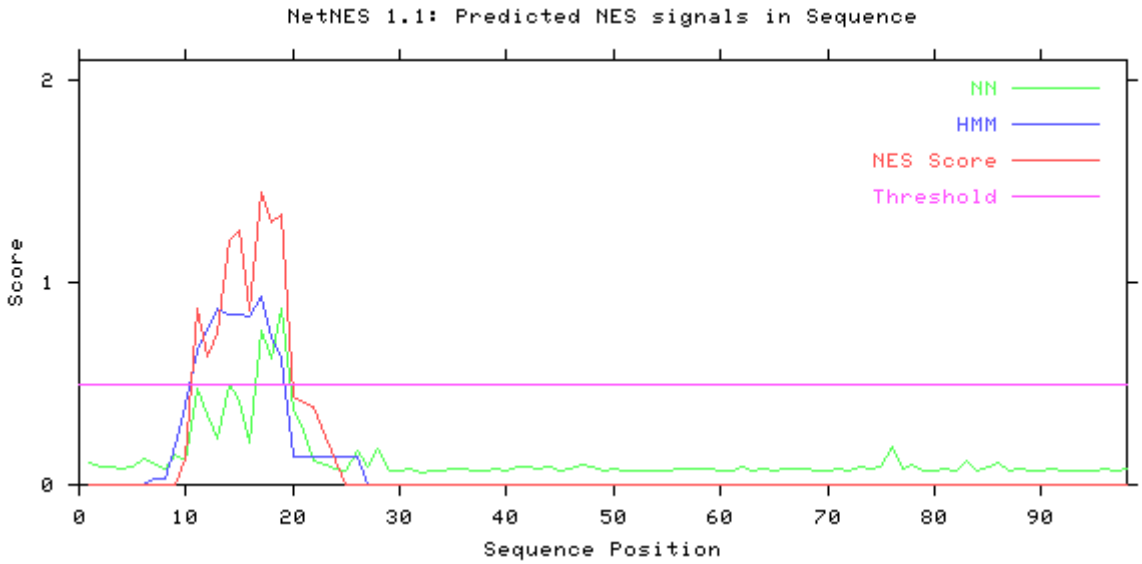


Fig. 6: 3D Ribbon diagram (CPHmodels 3.0 server prediction) of nsLTP1 of *O. rhizomatis* , *O. sativa* and *Zea mays*.

Table 2: Sub cellular localization of nsLTP1 of some plant. (targetp v1.1 prediction)

Name	Len	cTP	mTP	SP	other	Loc	RC
O. rhizomatis	99	0.013	0.019	0.908	0.007	S	1
Rubus	116	0.008	0.008	0.986	0.025	S	1
Hordeum	120	0.015	0.007	0.935	0.014	S	1
Medigaco	116	0.022	0.005	0.967	0.051	S	1
Chimonanthus	119	0.035	0.013	0.954	0.004	S	1
Ricinus	116	0.012	0.017	0.915	0.018	S	1
Glycine	122	0.003	0.011	0.975	0.035	S	1
Brachypodium	116	0.016	0.013	0.961	0.010	S	1
Zea	116	0.125	0.006	0.560	0.015	S	3
Sorgham	119	0.014	0.007	0.898	0.014	S	1
O. sativa indica	114	0.008	0.030	0.862	0.006	S	1
O. sativa japonica	116	0.009	0.024	0.860	0.006	S	1

C Chloroplast, i.e. the sequence contains cTP, a chloroplast transit peptide; M Mitochondrion, i.e. the sequence contains mTP, a mitochondrial targeting peptide; S Secretory pathway, i.e. the sequence contains SP, a signal peptide.



Sequence-11-L	0.477	0.661	0.874	Yes
Sequence-12-L	0.344	0.774	0.630	Yes
Sequence-13-L	0.229	0.870	0.750	Yes
Sequence-14-F	0.496	0.845	1.208	Yes
Sequence-15-F	0.414	0.847	1.263	Yes
Sequence-16-F	0.209	0.829	0.863	Yes
Sequence-17-L	0.766	0.936	1.443	Yes
Sequence-18-L	0.623	0.722	1.297	Yes
Sequence-19-L	0.874	0.620	1.342	Yes

Fig. 7: Protein localization of nsLTP1.

According to the NetNES server prediction result protein localization of nsLTP1 was predicted. If the calculated score 'NES score' exceeds the threshold, then that particular residue is expected to participate in a nuclear export signal. This is denoted with a 'Yes' in the column 'Predicted'. Above output shows amino acid residues 11- 19 are participating in nuclear export signal of nsLTP1 of *O. rhizomats* whereas amino acid resides 8-17 are participating in *O. sativa indica* as well as *O. sativa japonica* subspecies.

According to the STRING 9.0 server results, this protein had functional interactions with many other proteins however most interacting protein/s cannot be predicted as score values for the interactions were almost the same (Fig. 9).

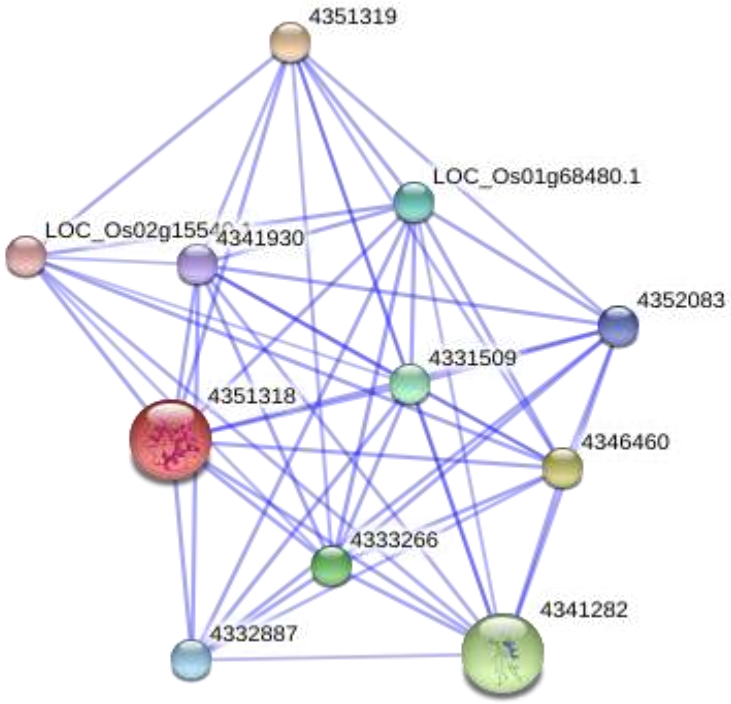


Fig. 8: Confidence view of protein-protein interactions of nsLTP1 of *O. rhizomatis*.

Predicted Functional Partners:		Score
4351319	LTPL13 - Protease inhibitor/seed storage/LTP family protein precursor, expressed; Plant non-spe [...] (123 aa)	0.532
4346460	PAP fibrillin family domain containing protein, expressed (319 aa)	0.532
4341282	40S ribosomal protein S24, putative, expressed (138 aa)	0.532
4333266	cbbY, putative, expressed (320 aa)	0.532
4331509	catalase domain containing protein, expressed; Occurs in almost all aerobically respiring organ [...] (492 aa)	0.532
LOC_Os01g68480.1	thioredoxin, putative, expressed (187 aa)	0.532
4332887	mTERF domain containing protein, expressed (301 aa)	0.530
4352083	3-beta hydroxysteroid dehydrogenase/isomerase family protein, putative, expressed (376 aa)	0.527
4341930	expressed protein (189 aa)	0.527
LOC_Os02g15540.1	expressed protein (137 aa)	0.527

Fig. 9: Functional interactions of nsLTP1 of *O. rhizomatis* with other proteins.

The transmembrane region of LTP1 of *O. rhizomatis* was estimated by TMHMM server (Fig. 10). TMHMM server predicted only one transmembrane helix that has probability over the default threshold value. Prediction analysis of the hydrophobicity of the deduced amino acid sequences indicated that *O. rhizomatis* LTP1 contained one specific transmembrane spanning domain like other rice subspecies between the amino acids 7-29.

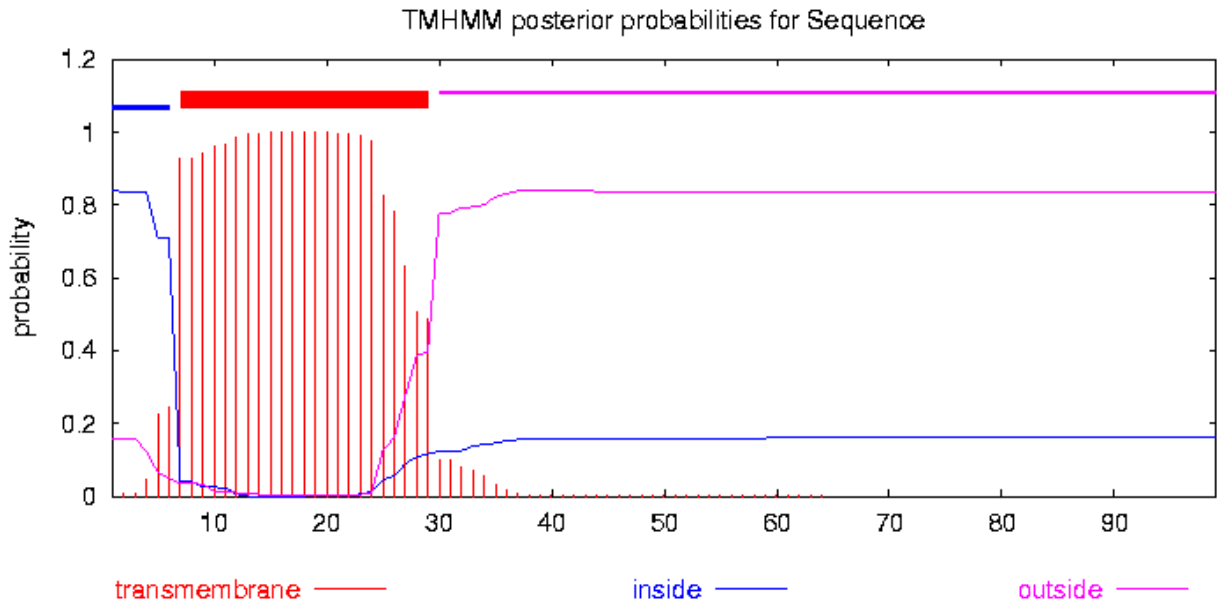


Fig. 10: The transmembrane region of nsLTP1 of *O. rhizomatis*.

Conclusions

According to the above structural analysis, *O. rhizomatis* has the nsLTP1 super family AAI_LTSS which involved in pathogen attack related responses in plants. Therefore this protein may involve in control of plant defense responses of the wild rice species *O.rhizomatis*. *Oryza rhizomatis* has been shown to be important sources of genes for disease resistance and the genes can be used to develop new rice varieties through plant biotechnology. Hence characterization of desirable novel genes responsible for above attributes will be useful for transferring traits to commercial varieties by modern biotechnological approaches.

Acknowledgement

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