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RESEARCH PAPER

Identification and *in-silico* characterization of *RPN10* gene as a candidate for genome editing to develop little leaf disease resistance in brinjal

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Abstract

Brinjal is an economically important nutrition-rich fruit vegetable. Among the various biotic stresses, the little leaf of brinjal caused by phytoplasma is a severe threat to its production. The disease occurrence has tremendously increased in the recent past causing almost 100% yield losses in severe conditions. Due to the non-availability of a strong resistance source in brinjal genotypes, its management is a challenging task. In the present study, 26S proteasome non-ATPase regulatory subunit 4 homolog or *RPN10* gene which is the target of SAP05 effector of phytoplasma is identified in phytoplasma resistant Kashi Uttam variety of brinjal. The *RPN10* gene in brinjal was amplified and sequenced. Evolutionary analysis showed that the *RPN10* gene is highly conserved in plant species which are natural hosts for phytoplasma. A comparison of motifs and domains in RPN10 protein of *Arabidopsis* and brinjal revealed 100% similarity. 3-dimensional protein structures were generated using I-TASSER server and structural comparison also showed perfect similarity between RPN10 protein in brinjal and *Arabidopsis*. The results in the study propose RPN10 protein in brinjal as the potential candidate gene target for genome editing to develop resistance against little leaf disease in brinjal.

Keywords: Brinjal, Little leaf disease, Phytoplasma, RPN10, Resistance.

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Introduction

Brinjal or eggplant (*Solanum melongena* L.) is one of the most important vegetables cultivated all over the world. Brinjal is a more popular fruit vegetable crop in South Asian countries like India, China, Bangladesh, Philippines and Pakistan (Rao and Kumar 2017). In 2021, worldwide brinjal production was 58.64 million tonnes from an area of 1.96 million hectares, of which almost 94% of production was contributed by Asian Countries (https://www.fao.org/faostat). Globally, India ranks second after China with a production of 12.87 MT of brinjal fruits. Brinjal is a nutritionally rich vegetable containing vitamins and minerals, amide proteins, water-soluble sugars, and fibers (Raigona *et al*., 2008; Talwar *et al*., 2023). In addition, it has medicinal value due to the presence of crucial antioxidant phytonutrients such as phenolic compounds and flavonoids (Koley *et al*., 2019). It has been used since the ancient period to treat various human disorders like asthma, bronchitis, diabetes, liver diseases, rheumatism, colilithiasis, leucorrhea, cholera and dysuria (Rotino *et al*., 2014). Brinjal is also an important cash crop for farmers helping their livelihood improvement. Thus, brinjal is a highly important vegetable crop for both farmers as well as consumers.

Production and quality of brinjal fruits is severely affected by various abiotic and biotic stresses. Among the various biotic stresses, the little leaf disease in brinjal is a

major threat for its production resulting in 40% economic losses, which may reach to 100% in severely infected plants (Rao and Kumar 2017). The little leaf disease is caused by a mycoplasma-like organism called phytoplasma which are bacterial pathogen devoid of cell walls and are restricted to the phloem of the plants. Phytoplasmas are transmitted by leaf hoppers, dodder and grafting, which results in stunted growth, flower virescence, phyllody, and proliferation of shoots and little leaves giving plants a typical witches' broom symptom (Rao *et al*., 2010).

There are no effective management strategies for little leaf of brinjal (LLB) disease and therefore, the best way of controlling the disease is to develop a host resistance in brinjal plants. However, almost all the elite cultivated varieties of brinjal are susceptible to LLB (Rao and Kumar 2017) necessitating the screening of large germplasm including wild species to identify the LLB resistance source that can be used in resistance breeding programs. Among the earliest studies, 75 genotypes of brinjal were surveyed for little leaf disease and none among them was found resistant to the disease (Mitra 1988). In addition, the study identified various biochemical parameters like respiration rate, carbohydrate content, activities of catalase, peroxidase, nitrate reductase and metabolic changes in sugar content, soluble protein content and total free amino acid concentration that occur in plants in order to detect the disease at early stage of infection. Recently, large-scale screening of brinjal genotypes including wild species was carried out for phytoplasma resistance where the Uttara variety and its derived line selection-10 and 17 different wild species showed resistance (Venkataravanappa *et al*., 2022). Nevertheless, mapping and characterization of phytoplasma resistance genes in wild species of brinjal is a difficult task. Further, transferring such genes from wild species to cultivated ones is challenging with the penalty of undesirable traits coming along with desirable traits. This necessitates the exploration of novel biotechnological tools to develop host plant resistance for LLB in brinjal.

A recent report by Huang *et al*. (2021) shows the involvement of effector protein SAP05 of *Candidatus Phytoplasma asteris* in modulating the host plant morphology by interacting with SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) and GATA transcription factors via a receptor protein RPN10. This interaction leads to degradation of SPL and GATA transcription factors which are key to normal developmental processes of plants resulting in the Witches' Broom symptoms. Further, modification of the *RPN10* gene by genome editing resulted in disruption of this interaction making the plants resistant to phytoplasma. Considering the potential of the *RPN10* gene as a possible candidate for genome editing to develop LLB resistance in brinjal, we have identified a homolog of *RPN10* in brinjal and further carried out its *in-silico* characterization.

Materials and Methods

Plant material, RNA extraction and cDNA synthesis

Seeds of brinjal variety Kashi Uttam which is resistant to LLB were germinated on germination paper at 25°C in the germinator. The seedlings were then transplanted into portrays in autoclaved soilrite. Leaf samples of 30-day-old plants were collected in the liquid nitrogen and immediately stored in -80°C until further use for RNA extraction. Total RNA was extracted from the leaf samples using TRIzol reagent (Ambion, USA) following the standard protocol provided by the manufacturer. The quality and quantity of the extracted RNA was analyzed by gel electrophoresis and spectrophotometer (BioSpectrometer, Eppendorf, Germany). In 1-µg of total RNA was used for the synthesis of cDNA using iScript cDNA synthesis kit (Bio-Rad, USA) by following the manufacturer's instructions. The quality of cDNA was analyzed by gel electrophoresis.

Sequence retrieval, Primer designing, PCR Amplification and Sequencing

Arabidopsis thaliana regulatory particle non-ATPase 10 (*RPN10*) mRNA (NM_120024.3) sequence was used to identify the sequence of *RPN10* gene in brinjal using BLAST tool. However, due to no similarity result in brinjal, sequence of 26S proteasome non-ATPase regulatory subunit 4 homolog, which is the homolog of *AtRPN10* gene in tomato (XM_010318127.3) was used to retrieve the brinjal sequence from Sol Genomic Network database using BLAST tool. Based on the sequence of *RPN10* in brinjal, primers were designed for amplifying the full-length ORF of the gene from Kashi Uttam variety. PCR amplification of the RPN10 gene in brinjal was carried out using the cDNA as template and Vent DNA polymerase (New England Biolabs, USA) and PCR cycles as 95°C for 5 min followed by 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 1-minute and the final extension of 7 minutes at 72°C. The amplification product was analyzed by Gel electrophoresis and subsequently purified using a PCR purification kit (Qiagen, Germany). The sequence of the amplified gene was identified by Sanger sequencing at DNA Sequencing Facility, UDSC, New Delhi.

Phylogenetic analysis

For the evolutionary study of the RPN10 gene in different plant species, a total of 32 similar sequences were retrieved from NCBI. Multiple sequence alignment of a total of 34 sequences including brinjal and Arabidopsis was carried out using the MUSCLE tool in MEGA X software (Edgar 2004). A phylogenetic tree was generated in MGA X using the neighbor-joining method with 1000 bootstrap replications (Kumar *et al*., 2018).

Protein modeling, Structural comparison and Protein-protein docking

The amino acid sequence of RPN10 protein in *Arabidopsis* and brinjal was used as input for studying the motifs or domains present in the protein by using the SMART tool (http://smart.embl-heidelberg.de/). The 3-dimensional structure of the brinjal RPN10 protein was generated using the I-TASSER server with default settings (Yang *et al*., 2015). Only a partial 3-dimensional structure of *Arabidopsis* RPN10 protein bound to SAP05 effector was available in the RCSB-PDB database and therefore its complete structure was also generated by the I-TASSER server. Complete 3-dimensional structures of both proteins were then compared by using the UCSF-Chimera tool (Pettersen *et al*., 2004). The interaction of SAP05 effector of phytoplasma with RPN10 of *Arabidopsis* and Brinjal was studied by protein-protein docking using HDOCK server (Yan *et al*., 2017).

Results and Discussion

Brinjal is an important crop for farmers and consumers especially in India and Asian countries. However, its yield and quality are drastically affected by major diseases including little leaf disease. LLB is highly difficult to manage by agronomic practices or by use of chemicals. Many weeds act as reservoirs of phytoplasmas and therefore, proper weed management has to be adopted to control LLB disease. One of the effective management strategies is to control the insect vector leafhopper by repeated spray of insecticides (Sohi *et al*., 1974; Mall *et al*., 2011). However, more use of insecticides increases the production cost as well as it is harmful to the consumers and the environment. Host plant resistance is thus far the most effective approach for LLB management. However, there is a lack of strong resistance sources for LLB in brinjal germplasm. Recently, the target of phytoplasma effector SAP05 has been identified in *Arabidopsis* and the mechanism of susceptibility is very well documented (Huang *et al*., 2021). In addition, the results demonstrated that genome editing mediated modification of the target protein RPN10 could lead to resistance against little leaf disease in *Arabidopsis*. Wang *et al*. (2022) suggested that the RPN10 protein is an ideal target for exploitation to develop immunity in plants against phytoplasma and other bacterial pathogens. CRISPR/Cas9-based genome editing has been widely used for developing disease resistance in several crops including vegetables (Karkute *et al*., 2017; Zaidi *et al*., 2020; Barka and Lee, 2022). Taking leads from these reports, the present study has been carried out to identify and characterize RPN10 gene in brinjal for further utilization in developing LLB resistance.

For this purpose, a good-quality RNA was obtained from leaf tissues of Kashi Uttam variety of brinjal and cDNA was synthesized. BLAST search in NCBI database using *Arabidopsis* RPN10 gene sequence (NM_120024) did not provide any results for brinjal, however, a similar sequence was obtained in tomato (XM_010318126). Therefore, the sequence of tomato 26S proteasome non-ATPase regulatory subunit 4 homolog was used as a query for BLAST search in the Solanaceae-specific database Sol Genomics Network. In this database, a gene "Similar to RPN10 26S proteasome non-ATPase regulatory subunit 4 homologs" (SMEL_000g031640.1) was retrieved from the brinjal cDNA database. This cDNA sequence was 1163 bp. With the help of this sequence, primer pair for full-length cDNA amplification including start and stop codons were designed and used for PCR amplification using Kashi Uttam brinjal cDNA which resulted in the amplicon of more than 1Kb in size. Sequencing data of this gene obtained by Sanger sequencing revealed the sequence size of exactly 1163 bp which was then translated using ExPASy translate tool and a complete RPN10 protein sequence of 387 amino acids was obtained.

For evolutionary analysis of RPN10 gene and its association with phytoplasma disease, RPN10 protein sequences of a total of 34 plant species including *Arabidopsis* were used and an unrooted phylogenetic tree was generated (Figure 1). The tree formed several clades where related species were grouped in a single clade. For instance, all the Solanaceae family species such as tomato, potato, brinjal, capsicum, datura, Nicotiana, etc. formed a large single clade with small subgroups according to the evolutionary relatedness of individual species. *Arabidopsis* could be seen as an outlier in the tree due to the significant difference of its RPN10 protein with that of brinjal and all other species used in the tree. Interestingly, the literature survey revealed that almost all these species are host for phytoplasma, which further confirms the association of RPN10 gene with phytoplasma and it is conserved in plant species during evolution.

Arabidopsis RPN10 is a well-characterized host susceptibility protein interacting with SAP05 effector of phytoplasma. To qualify for analogous function as a host susceptible factor to phytoplasma, any homolog of RPN10 must have the same motifs and domains as well as a similar 3-dimensional protein structure (Petrey *et al*., 2009). SMART analysis of RPN10 protein showed the presence of one vWA and 3 UIM motifs at exactly similar positions in Arabidopsis and brinjal (Figure 2), thereby supporting that the two proteins may have same functions. The vWA domain is crucial for interaction with SAP05 effector protein. The RCSB-PDB database contains the X-ray crystallographic structure of *Arabidopsis* RPN10 protein bound to SAP05 effector protein (PDB ID 8JTL). However, this is only the partial structure of the protein. Complete 3-dimensional structures of RPN10 protein in both *Arabidopsis* and brinjal were developed in I-TASSER server (Figure 3) The partial PDB structure of *Arabidopsis* RPN10 helped to check the accuracy

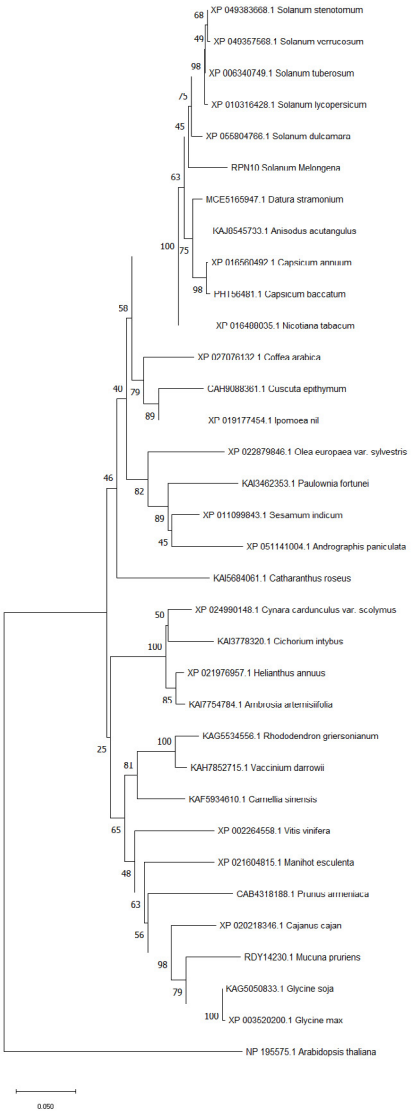
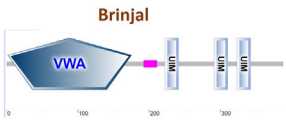


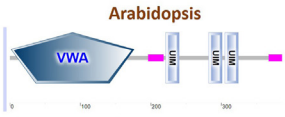
Figure 1: Phylogenetic tree showing evolutionary relationship of RPN10 protein in different plant species

and quality of the structures obtained from I-TASSER as they were perfectly matched after overlapping (Figure 4A), indicating that the obtained 3-D structures are of high quality. Brinjal and *Arabidopsis* RPN10 protein structures when superimposed in UCSF-Chimera, showed perfect overlapping (Figure 4B). Thus, presence of vWA and UIM domains and also the similar 3-D structure supports that the RPN10 protein in brinjal is the homolog of *Arabidopsis* RPN10, which could act as host susceptibility factor by interacting with the SAP05 effector of phytoplasma.

Further, protein-protein docking of RPN10 protein with SAP05 effector by HDOCK server revealed that binding of SAP05 to RPN10 of Kashi Uttam variety of brinjal is weak and not exactly at the active site compared to that in *Arabidopsis* explaining the possible mechanism of resistance in Kashi Uttam (Figure 5). Docking score of interaction was -257.23 with 0.8952 confidence score in case of *Arabidopsis*



Name	Start	End	E-value
VWA	2	175	1.09e-14
low complexity	194	214	N/A
UIM	222	241	14.2
UIM	291	310	1.66
UIM	323	342	0.128



Name	Start	End	1.08e-9
VWA	2	176	1.08e-9
low complexity	196	218	N/A
UIM	221	240	12.4
UIM	282	301	28.5
UIM	305	324	0.0171
low complexity	367	386	N/A

Figure 2: Domain structure analysis of RPN10 protein in brinjal and Arabidopsis by SMART tool

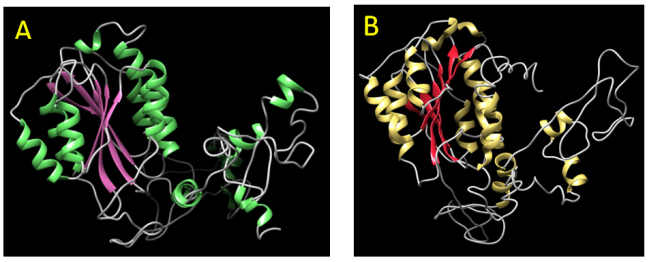


Figure 3: Structure of RPN10 protein generated by I-TASSER server. A-Arabidopsis, B-Brinjal

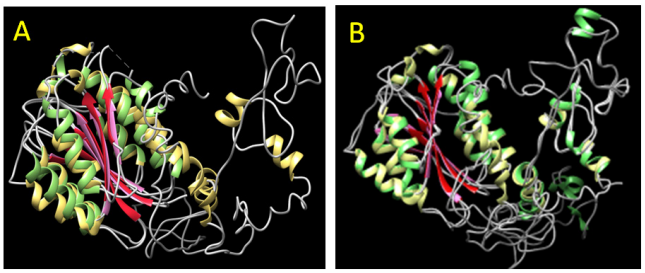


Figure 4: Superimposition of 3-D structures of RPN10 protein in brinjal and Arabidopsis.
A-vWA domain of Arabidopsis RPN10 (X-ray crystallography) and RPN10 of brinjal generated by I-TASSER. B-RPN10 structures of Arabidopsis and brinjal generated by I-TASSER

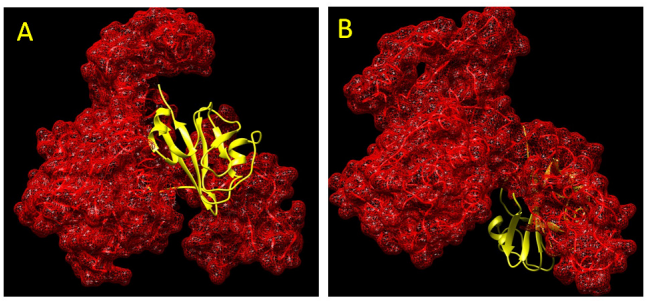


Figure 5: Interaction of SAP05 effector protein of phytoplasma with RPN10 protein.
A-Interaction with Arabidopsis RPN10 protein. B-Interaction with brinjal RPN10 protein

RPN10 whereas it was comparatively less (-234.78) with 0.845 confidence score in case of brinjal RPN10. Weak binding of SAP05 effector to RPN10 protein may not lead

to subsequent degradation of SPL and GATA transcription factors making plants resistant to phytoplasma (Huang *et al*., 2021). The results need to be validated for confirmation of the resistance nature of this allele to utilize it in the breeding program to transfer phytoplasma resistance trait to cultivated lines through marker-assisted breeding. Nevertheless, with the availability of high-efficiency transformation and regeneration protocols in brinjal, CRISPR/Cas9 mediated genome editing can be effectively employed to create mutant alleles at vWA domain to disrupt the SAP05 interaction and transgene-free edited line for RPN10 gene can be developed in brinjal with durable resistance against little leaf disease.

Conclusion

Little leaf disease in brinjal is an economic threat to farmers and therefore, it is essential to develop LLB-resistant cultivars in brinjal for sustainable production. Phytoplasma modulates the plant morphology through SAP05-mediated degradation of plant transcription factor via RPN10 protein. Developing alleles of RPN10 protein that can be no longer recognized by SAP05 effector of phytoplasma is an effective strategy to develop resistant cultivars. The RPN10 gene of brinjal is thus the potential candidate for targeting by CRISPR/Cas9 tool to develop LLB resistance brinjal varieties.

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सारांश

बैंगन एक आर्थिक रूप से महत्वपूर्ण एवं पोषण से भरपूर सब्जी है। विभिन्न जैविक तनावों में से एक, फाइटोप्लाज्मा के कारण होने वाली बैंगन की छोटी पत्ती रोग बैंगन के उत्पादन के लिए एक गंभीर खतरा है। हाल के दिनों में इस बीमारी का प्रकोप काफी बढ़ गया है, जिससे गंभीर परिस्थितियों में 100% तक उपज का नुकसान हुआ है। बैंगन के जीनोटाइप में प्रभावी रोग प्रतिरोधी स्रोत उपलब्ध न होने के कारण इसका प्रबंधन एक चुनौतीपूर्ण कार्य है। प्रस्तुत अध्ययन में, बैंगन की फाइटोप्लाज्मा प्रतिरोधी किस्म काशी उत्तम में 26S प्रोटीयासोम नॉन-एटीपीएज रेगुलेटरी सबयूनिट 4 होमोलॉग या आरपीएन10 जीन की पहचान की गई है, जो फाइटोप्लाज्मा के SAP05 प्रभावक का लक्ष्य है। बैंगन में आरपीएन10 जीन को प्रवर्धित और अनुक्रमित किया गया और विकासवादी विश्लेषण से ज्ञात हुआ है कि आरपीएन10 जीन पौधों की प्रजातियों में अत्यधिक संरक्षित है जो फाइटोप्लाज्मा के लिए प्राकृतिक पोषिता हैं। एराबिडोप्सिस और बैंगन के आरपीएन10 प्रोटीन में रूपांकनों और डोमेन की तुलना से 100% समानता का पता चला। I-TASSER सर्वर का उपयोग करके 3-आयामी प्रोटीन संरचनाएं बनाई गईं और संरचनात्मक तुलना में बैंगन और अरेबिडोप्सिस के आरपीएन10 प्रोटीन में पूर्ण समानता भी प्राप्त हुई । अध्ययन के नतीजे बैंगन में आरपीएन10 प्रोटीन को बैंगन में छोटी पत्ती की बीमारी के खिलाफ प्रतिरोध विकसित करने के लिए जीनोम संपादन के लिए संभावित उम्मीदवार जीन लक्ष्य के रूप में प्रस्तावित करते हैं।