Original Research Article

In silico analysis of *SlNAC1* transcription factor, a positive modulator for Tomato leaf curl virus

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ABSTRACT

NAC proteins, part of the largest plant transcription factor family, play pivotal roles in plant development, and biotic and abiotic stresses. Among them, the *SlNAC1* protein in tomato serves as a Negative regulator, enhancing tolerance to cold, bacterial, and viral diseases. However, our knowledge of *SlNAC1* protein *in silico* analysis, evolutionary relationships, and protein interactions remains limited. We used *in silico* tools to study the *SlNAC1* protein in tomato. Phylogenetic analysis showed it is closely related to other *Solanaceae* family members. Motif analysis revealed seven conserved motifs shared among *Solanaceae* members. The presence of NLS confirms its abundance in the nucleus. Protein-protein interaction analysis predicted ten potential nuclear partners, including five uncharacterized proteins and others involved in plant development. *In silico* expression, analysis showed abundant SlNAC1 presence in both fruit and roots. This study provides the role of *SlNAC1* protein in tomato, shedding light on its evolutionary context and protein interactions.

Keywords: SlNAC1, Tomato leaf curl virus, phylogenetic analysis, in silico expression analysis.

1. INTRODUCTION

Transcription factors (TFs) are proteins that control gene expression by binding to specific regulatory elements in gene promoters, either by activating or repressing transcription [1]. In plants, this regulation is essential for processes like cellular development, signal transduction, and responses to environmental stress and pathogens [2, 3]. Identifying and understanding the relationship between TF and target genes is crucial for unraveling the intricate transcription network in plants [4]. Among the 58 TF families, six Transcription factor families play a prominent role in stress responses. Among these, *NAC* TFs actively participate in the response to biotic and abiotic stresses. A limited number of cases have provided a comprehensive account of the molecular mechanisms underlying these processes [5].

NAC proteins constitute the largest protein family and are distinguished by their distinctive structures, featuring a highly conserved N-terminal NAC domain and a notably diverse C-terminal region. The NAC domain was initially identified in four proteins: NAM, ATAF1, ATAF2, and CUC2 [6]. In contrast, the C-terminal domain of NAC proteins exhibits significant variations in both sequence and length, having a pivotal role in specifying the precise functions and activities of NAC proteins in the species [7]

NAC proteins are majorly involved in stress conditions because they influence the plant's response to stress. The alteration of *NAC* gene expression through overexpression or silencing leads to heightened or diminished resistance against pathogens [8, 9], and Overexpression of *ANAC019, ANAC055, ANAC72* in *Arabidopsis* [10], *SNAC2* [11]*, and OsNAC4* [12] in *O. Sativa* leads to drought and salt tolerance. Loss of function in ataf1-1 [13]

and rim1-1 [14] leads to attenuated penetration resistance toward Bgh attack *in A. thaliana* and resistance to rice dwarf virus (RDV) in *O. sativa*, respectively. overexpression of *HbNAC1*, *PtrNAC72* leads to the drought tolerance in a *h. brasiliensis* and *Arabidopsis* [15, 16] and overexpression of *HvSNAC1*, and *HvNAC6* leads to the resistance against *barley ramularia leaf spot* (RLS) disease and powdery mildew pathogen *Blumeria graminis f. sp. hordei* (Bgh) respectively in barley [17, 18] and the knockdown of the *TaNAC1* and enhances the resistance against Puccinia stripe rust in wheat [19]. This indicates that the NAC Transcription factor especially the NAC1 Transcription factor may exert both positive and negative regulatory effects on plant defence responses in biotic as well as abiotic stresses [20] The significant reduction in tomato production due to Tomato Leaf Curl Virus (TYLCV) necessitates the development of resistant tomato varieties, achievable through the application of genome editing tools by targeting recessive gene [21]

In tomato, *SlNAC1* gene expression increases exponentially by the replication enhancer (RE) of the *Tomato Leaf Curl Virus* (TYLCV), which helps the virus to accumulate more in the plant and is also enhanced by Pseudomonas infection, indicating the role of the *SlNAC1* protein as a positive modulator of *TYLCV* and Pseudomonas infection in plants [8, 22]. Knocking down this gene resulted in Cold and Drought tolerance and increased fruit shelf life in the tomato [23, 24]. This TF upon knocking down might harbour multi-stress tolerance in the plant, but unfortunately*, in silico* analysis was little explored.
In the present study, to understand the structural, functional, and evolutionary relationship of the *SlNAC1* protein in tomato, we conducted an *in silico* analysis of the *SlNAC1* protein in tomato in comparison with seven different model plants. This analysis will provide a reference point for the *SlNAC1* gene in tomato, as we have studied its interaction with other proteins and its molecular aspects.

2. MATERIAL AND METHODS

2.1 Sequence retrieval, chromosomal location identification, MSA, and phylogenetic analysis of *NAC1* Transcription factors

The NAC1 transcription factor sequence from *Solanum lycopersicum* was obtained from the Plant Transcription Factor Database (PlantTFDB v5.0) (http://planttfdb.gao-lab.org) [25]. In addition, sequences from various model plants, such as *Arabidopsis thaliana and Oryza sativa subsp. Japonica, Solanum melongena, Zea mays, Capsicum annuum, Glycine max,* and *Sorghum bicolor*, were retrieved from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov) and UniProt databases (https://www.uniprot.org) [26]. Once these sequences were obtained, they were imported into InterProScan (https://www.ebi.ac.uk/interpro/search/sequence/) [27] to check for the presence of the NAC Domain.
Following the sequence analysis, a multiple sequence alignment was carried out using the Clustal Omega online software [https://www.ebi.ac.uk/Tools /msa/clust alo/] [28]. Subsequently, a phylogenetic tree was constructed using the Maximum Likelihood algorithm in the MEGA 11 software [29]. The resulting data were visualized and represented in a more informative graphical format using the Interactive Tree Of Life (iTOL) online tool (https://itol.embl.de/) [30].

2.2 Possible Motif Prediction and Physiochemical Analysis and protein-protein interaction study of NAC1 Transcription factor:

Several essential analyses were conducted to study the NAC1 Transcription factor. Initially, tools such as TBtool (https:// github. com/ CJ- Chen/ TBtoo ls) [31] and MG2C (http://mg2c.iask.in/mg2c_v2.1/) [32] were used to precisely locate the *NAC1* gene within the tomato genome. Subsequently, motif prediction was carried out using the MEME online tool

(https://meme-suite.org/meme/tools/meme) [33], with a focus on sequences from different model plants. The maximum and minimum motif widths were 50 and 6, respectively, while limiting the maximum number of motifs to 10. The physiochemical properties of the *SlNAC1* protein were analyzed. Parameters such as the theoretical isoelectric point (pI), molecular weight, aliphatic index, and Grand Average of hydropathicity (GRAVY) were calculated using the ExPASy Prot-Param tool (https://web.expasy.org/protparam/) [33], with the UniProt sequence as the reference. Finally, the protein-protein interaction network of the *SlNAC1* protein was explored using the STRING online software tool (https://string-db.org/), shedding light on potential functional associations and interactions with other proteins.

2.3 Secondary structure prediction, subcellular location identification, and *in silico* expression analysis of *SlNAC1* Transcription Factor Protein

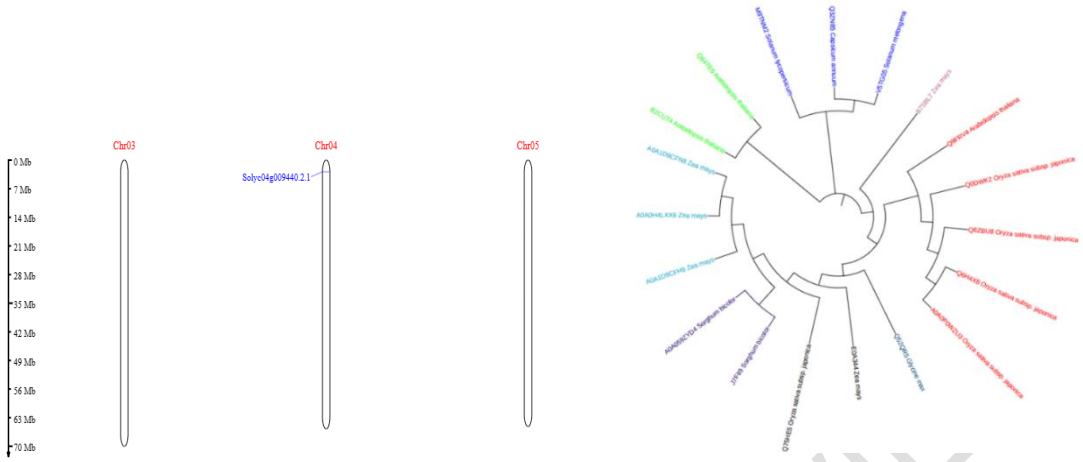
The SOPMA server [35] was used for the prediction of secondary structures of *SlNAC1* proteins with the output width set to 70, the number of conformational states to 4, similarity threshold to 8, and window width to 17. The subcellular location of the SlNAC1 Transcription factor protein was identified by using the TargetP2.0 online tool (https://services.healthtech.dtu.dk/services/TargetP-2.0/) [36], this tool particularly predicts the presence of N-terminal sequences: signal peptide (SP), mitochondrial transit peptide (mTP), chloroplast transit peptide (cTP) or thylakoid luminal transit peptide (lTP) this helps to determine the subcellular location. The tomato plant Electronic Fluorescent Pictograph Browsers (Tomato eFP browsers) (http://bar.utoronto.ca/eplant_tomato/) were used for the *in silico* expression analysis of the *SlNAC1* protein

3. RESULTS AND DISCUSSION

3.1 Identification of evolutionary relationships

In this study, putative *NAC1* transcription factor (*NAC1*) genes were identified in the *Solanum lycopersicum* genome using *in silico* tools, revealing one gene, *SlNAC1* (Solyc04g009440.2.1), whose protein product contains the NAC conserved domain of 127 amino acids and its chromosomal location is represented in Fig 1a.

To explore the expansion of *NAC1* genes across different plant species, 18 full-length amino acid sequences from seven model plants *(Arabidopsis thaliana, Zea mays, Oryza sativa subsp. Japonica, Sorghum bicolor, Capsicum annuum, Solanum melongena, and Glycine max)* were used to construct a phylogenetic tree using the maximum likelihood method (Fig. 1b). A study conducted by Bhattacharjee *et al*.(2017) utilized in silico tools to identify a total of 99 *NAC* transcription factors (*NAC* TFs) in the *Solanum lycopersicum* genome. These NAC TFs were found to be distributed across various regions of the genome [37]. Across various species, different numbers of *NAC1* genes were found, including three in *Arabidopsis thaliana* and five in *Zea mays*. These results indicated that these transcription factors could be classified into three primary groups. One group consisted of *Arabidopsis* proteins with distinct motifs, whereas all *Solanaceae* plants fell into another main group. Additionally, all cereals and pulse crops were grouped together, sharing similar motifs with slight variations that led to the formation of smaller subgroups. The research conducted by Zhu *et al.* (2012) explored the evolutionary relationships among NAC transcription factors across nine major land plant lineages, revealing a high degree of conservation in their NAC domains across species, and proposed the existence of 21 subfamilies [38]. Furthermore, a study by Cenci *et al*., (2014) performed a phylogenetic analysis of NAC domain sequences from A. thaliana, V. vinifera, O. sativa, and M. acuminata. Their analysis identified 26 clusters that encompassed all NAC members of orthologous groups, as determined by expert comparison [39]. Bengoa Luoni *et al*., (2021) reported that the C-terminal region of NAC transcription factors is highly variable and species-specific, with this variability playing a critical role in stress responses[40]. Phylogenetic analysis demonstrated that *Solanaceae* plants share a common evolutionary history, making them somewhat distinct from other plant classes. However, they share some common motifs that connect them.



(a) (b)

Fig. 1. a) Chromosomal location of *NAC1* in *S. Lycopersicum* b) Phylogenetic map of *SlNAC1* Transcription factor in different model plants.

3.2 Physiochemical and motif analysis

The Physiochemical properties of *SlNAC1* were thoroughly examined using the ExPASy Prot-Param tool, and the resulting data are presented in Table 1. The SlNAC1 Transcription factor protein sequence contained 39 and 38 positive and negative amino acids, respectively. The isoelectric point was 7.6. It has been observed that the isoelectric point value of any protein has a significant impact on its subcellular localization and stability [41]. The research conducted by Nasrabadi *et al.* (2021) demonstrated that altering the physiochemical characteristics of plant proteins can influence both their technological functionality and biological properties [42]. Given that the *SlNAC1* protein functions as a suppressor, it is crucial to evaluate the consequences of modifying physiochemical properties in genome-edited crops when this factor is knocked down. There is the total of 10 different motifs were identified (Fig 2), and the first three motifs are in Table 2. and belongs to the highly conserved NAC domain. *Solanum Lycopersicum, capsicum annum,* and *solanum melongena*, three share a close association and collectively possess 7 motifs, these can be easily identified by the phylogenetic tree. A similar study conducted by Du et al. (2022) in *Kandelia obovate* revealed the total of 20 conserved motifs in the *KoNAC* protein. Additionally, through a comprehensive phylogenetic analysis, they were able to categorize the 79 KoNAC proteins into 16 distinct subgroups [43]

By examining protein motifs and tracing their evolutionary relationships, valuable insights can be obtained. It is evident that *SlNAC1* shares its evolutionary history with members of the *Solanaceae* family and shares common conserved regions with other crop families. There is a total of four motifs (Table 2) which were conserved in all the sequences of the model plant. Of these four motifs, the first three motifs are part of the NAC domain. With the exception of the *Solanaceae* family, motifs 8 and 10 were lost in the other studied species. This information can serve as a starting point to delve into the mysterious role of the *NAC1* transcription factor in various crops. In some crops, it may confer resistance, whereas in others, it may act as a susceptibility factor [44]. This divergence could be influenced by specific motifs with distinct plant-specific roles that require closer examination

Table 1 Physiochemical properties of *SlNAC1* protein.

Properties	*SlNAC1* Protein
Amino acids	301
MW(DA)	34775.2
Positively charged amino acids	39
Negatively charged amino acids	38
Theoretical Pi	7.6
Extinct coefficient	54235
Instability index	38.46
Aliphatic index	58.7
GRAVY	-0.824

Table 2: Conserved motifs discovered in *SlNAC1*.

Sr No.	MOTIFS	Width
1	VDLYKFDPWDLPERALFGEKEWYFFSPRDRKYPNGSRPNRAAGNGYWKAT	50
2	RGRTLGIKKALVFYAGKAPRGVKTDWIMHEYR	32
3	ELNLPPGFRFHPTDDELVEHYLCRKAAGQ	29
4	RLDDWVLCRLYNKKNEWEKMQ	21
5	DMATSHSHSHSQSHSHSWGETRTPESEIVDNDPFPELDSFPAFQDPAMMM	50
6	DGCSAKSSBLFVDLSYDDIQGMYSGLDMLPP	31
7	GEDFYSSLFASPRVKGNQPA	20
8	DFQHNYLDGLLNPPFETQMQQQNCNIDQFNSFQDMFJPMQK	41
9	JPVPIIAE	8
10	TRMHTTNSSSGSEHVLSPCDKEVQSAPKW	29

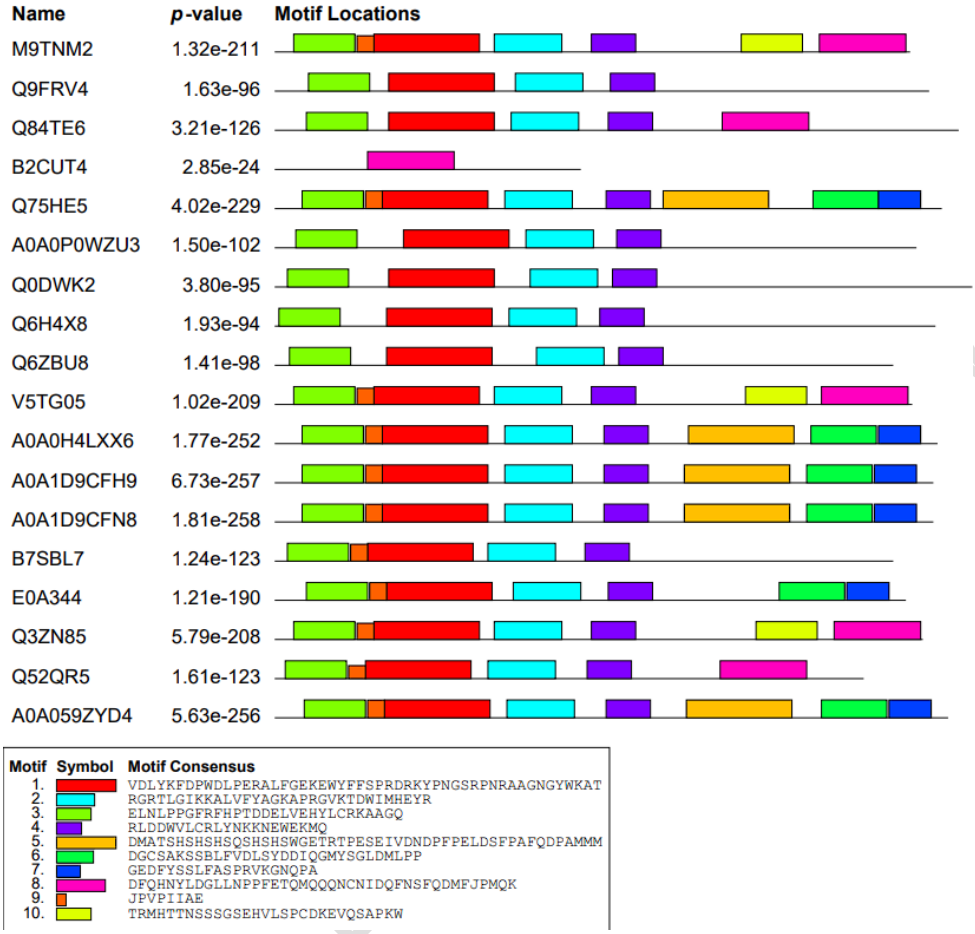


Fig 2. Motif identification in the seven-model plant NAC1 Transcription factor and *NAC1* Transcription factor of *S. Lycopersicum*.

3.3 Secondary structure prediction, subcellular location identification, and *in silico* expression analysis.

The process involved utilizing the SOPMA tool with a retrieved sequence as input to predict the secondary structure, which is presented in Table 3. Subsequently, subcellular localization of the Transcription Factor was determined, predominantly in the nucleus, without the presence of mitochondrial, thylakoid lumen, or chloroplast transfer peptides in the protein sequence. Transcription factors play a crucial role in the nucleus by facilitating transcription processes and other essential biological functions. A study reported that the NAC domain typically contains the Nuclear Localization Signal (NLS) and rarely other extra-nuclear signal peptides [45]; however, in the case of the *SlNAC1* protein, it is missing, which indicates its limited role up to the nucleus. To confirm the in silico subcellular localization predictions, GFP tags were fused with the KoNAC proteins, and subsequent examination was conducted using confocal microscopy. The fluorescent signals obtained delineated the subcellular localization of the NAC proteins within the nucleus [43]. Additionally, the results obtained from the Tomato eFP browsers indicated significant *SlNAC1* gene activity across various tissues, with the highest expression observed in fruits, followed by roots, vegetative meristem, developing fruit, leaves, stems, and unopened flowers, as illustrated in Fig. 3. An in vivo expression study of NAC1 TF found that it was highly expressed in the root and

ripened fruits of crops such as tobacco and wheat [20]. This analysis provides valuable insights into optimal plant tissues for studying the expression of this specific protein, which can be particularly useful for investigating protein product mutants.

Table 3: Secondary structure prediction of *SlNAC1* transcription factor.

Secondary structure element	Percentage
Alpha helix (%)	19.60%
Beta turn (%)	2.99%
Extended strand (%)	9.63%
Random coil (%)	67.77%

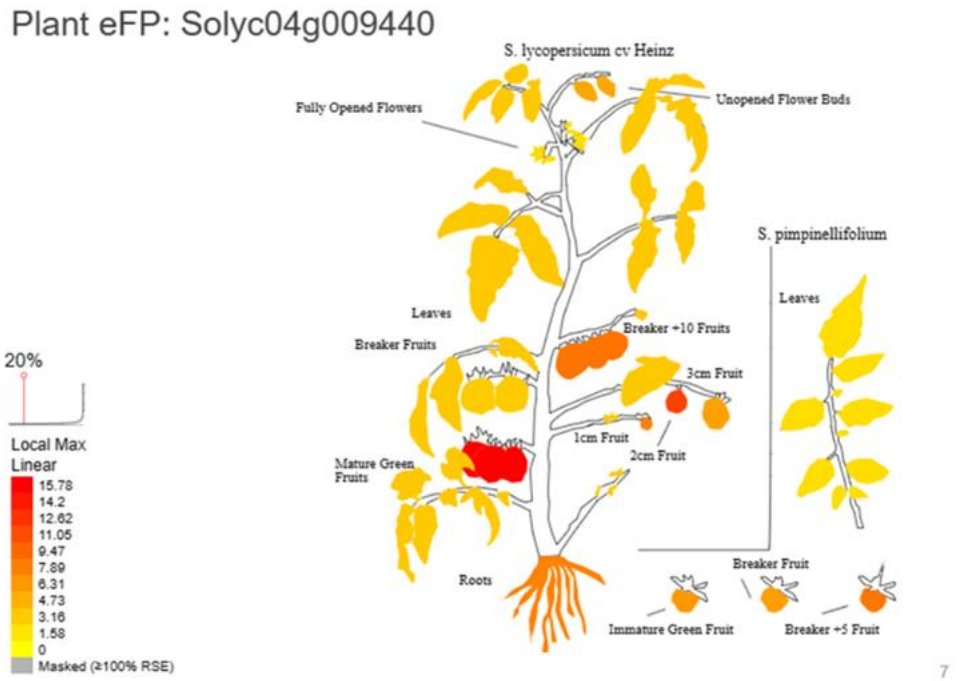


Fig. 3. *In silico* expression characterization of tomato *SlNAC1* transcription factor.

3.4 In silico study of protein-protein interactions of *the SlNAC1* Transcription Factor

The protein sequence was input into the String software, which then identified the sequence with the highest similarity, *A0A3Q7FYL7*, exhibiting a remarkable 94.7% identity, a bit score of 474.6, and an e-value of 5.2e-134. This analysis generated a network graph (Fig. 4) with 11 nodes and 48 edges. Predictions also identified 10 functional partners, of which five were proteins with uncharacterized functions, while the rest are detailed in Table 4. ZmABI19, a protein containing the transcription factor-B3 domain found in Zea mays, plays a direct role in governing essential factors that coordinate endosperm and embryo development and

grain filling in maize [46]. Auxin response factor protein is a transcription factor that plays an active role in plant growth and development by regulating the expression of auxin response genes. An interaction study conducted for the *CaNAC* genes in pepper, interaction network revealed its interaction with the 48 genes which have a significant role in biotic, abiotic stress tolerance and the growth and development in plants [47], Similarly, in the case of the SlNAC47 transcription factor, a similar interaction study unveiled its interactions with proteins that are involved in hormonal responses [48]. This significantly simplifies our ability to anticipate the outcomes of genome editing experiments aimed at knocking down target protein molecules. As the CRISPR-Cas9 system has OFF-Target as a limitation, it is very important to predict which proteins might be affected.

Table 4. List of Functional Partners of *SlNAC1* Transcription Factor.

Primary accession	Name
A0A3Q7J310	TF-B3 domain-containing protein.
ARF6	Auxin_resp domain-containing protein.
A0A3Q7H9Z3	Auxin response factor
A0A3Q7I5Z7	PHD_Oberon domain-containing protein.
A0A3Q7EAX8	Phytocyanin domain-containing protein.

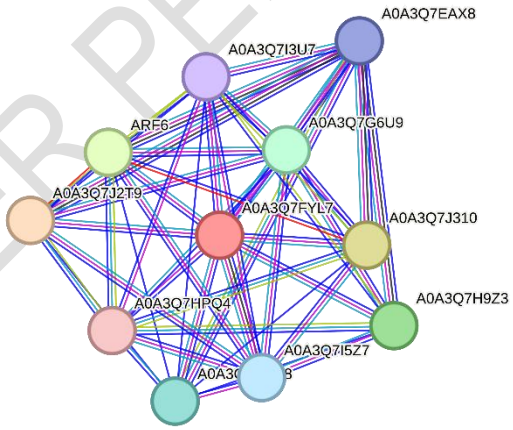


Fig. 4. Network Graph.

4. CONCLUSION

The present study unveiled the evolutionary relationship between our target gene and members of the *Solanaceae* group, as evidenced by the sharing of all seven predicted motifs. Additionally, through physiochemical analysis and *in silico* expression profiling, we explained the chemical properties of this gene and identified potential target sites for further

qualitative and quantitative protein studies. Furthermore, our investigation into protein-protein interactions revealed crucial associations with proteins that play pivotal roles in plant developmental processes. These interactions hold the promise of providing insights into the potential consequences of knocking down this gene in plants using the CRISPR-Cas9 technology.

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