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In-silico identification and structural validation of *RCD1*-like *SRO* gene family member and analysis of its role in modulation of abiotic stress responses in *Withania coagulans* (L.) Dunal

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***Abstract:WcRCD1* is a member of plant-specific gene family SRO (SIMILAR-TO-RCD-ONE), which is known to participate in the regulation of different developmental processes and stress adaptations. However, knowledge of the systematic characterization of SROs in *Withania coagulans* (Rishyagandha) is still unknown. Therefore, an in-silico approach was conducted for the identification of *WcRCD1* genes in *W. coagulans*. The *WcRCD1* was identified as having PARP and RST domain via transcriptomic data set from the SRA database. Further, the phylogenetic tree and multiple sequence alignment suggest the proximity & evolutionary divergence between *WcRCD1* and other *SRO* genes from related SRO members of other plant species. Motif analysis in *WcRCD1* with other *SRO* genes revealed the presence of 6 conserved motifs out of 16. Further, protein-protein interaction of *WcRCD1* showed putative interaction with *DREB2A, DREB2B, DREB2C, NAC* 13, *NAC046*, poly (ADP-ribose) polymerase (PARP), TAF 2 and SOS1. The GO annotation studies were carried out to depict *WcRCD1* role in oxidative stress and drought stress tolerance. Further, *WcRCD1* putative protein model was submitted after validation by the SAVES server on the protein model database. The in-silico analysis was also performed using microarray data of model plant *Arabidopsis SRO* genes (*AtSRO2*, *AtSRO5*, and *AtRCD1*) against abiotic stress and in various developmental stages to predict its probable involvement in various processes. The RT-PCR analysis in *W. coagulans* validated its putative role in abiotic stress tolerance which is more pronounced in drought stress and SEM microscopy analysis affirmed its possible role in stomatal closure. Overall, the current work lays the foundation for future studies on the function of *SRO* genes and their involvement in abiotic stress tolerance in *W. coagulans*.**

Keywords: Abiotic stress, Transcriptome, SRO gene, Bioinformatics, Expression analysis, Transcription factor, Phylogenetic assessment.

I. INTRODUCTION

The Plant can survive a variety of environmental alterations by inducing stress-related gene responses to activate a series of transcription factors and other genes (Fujita *et al*., 2006; Shinozaki & Shinozaki, 1997). In plant-specific development processes, varied transcription factors (TFs) family members have their role in the regulation of stress responses to the external environment. At the genomic level some of the transcription factors have been already isolated, such as NAC (NAM, ATAF, and CUC) (Rai *et al*., 2020), AP2/ERF (APETALA2/ethylene-responsive element binding factor), WRKY (Pandey *et al*., 2019), DOF (DNA-binding with one finger) and ARF (Auxin Response Factor) (Yamasaki et al., 2013). Among these TFs NAC TF has been reported to be regulated by SRO (SIMILAR-TO-RCD-ONE). Using the SRA dataset, one of the important *SRO* gene families, which regulates stress responses has been also identified in several plant species. The *SRO* gene has been characterized for its role in various developmental and stress tolerance processes. The SRO proteins contain a poly (ADP- ribose) polymerase catalytic PARP (PF00644) and RST domain (PF12174) at C-terminus. Sometimes, a few members of the SRO family also contain WWE (PF02825) domain at N-terminus (Liu et al., 2021).Most of the research on the SRO protein family has been focused on the model plant Arabidopsis thaliana. Studies show that six *SRO* homologous genes were identified in *Arabidopsis*, namely *AtSRO1-AtSRO6* with *RCD1* domain.

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The *RCD1* domain is responsible for oxidative stress and hormone-influenced gene expression coordination (Jaspers & Kangasjärvi, 2010). The *RCD1* is the first gene that was observed in oxidative stress-sensitive yeast strains lacking transcription factor YAP1 which is important in coping with the adverse effects of oxidative stress. As a consequence of mutation in the *RCD1* gene, there is an increase in characters governed by a single gene showing its pleiotropic nature, such as reduction of ROS under UV radiation, early flowering, and also enhanced production of ethylene, jasmonic acid resulting in abnormalities in development and growth by also an alteration in morphology under salt stress (Shapiguzov *et al*., 2019). It is also observed that *RCD1* along with SOS1 interacts with several transcription factors with a putative role in development and stress responses which were demonstrated in various *RCD1* mutant studies (Shapiguzov *et al*., 2019). In DREB2A mutant *RCD1* interaction participates in protein stability under various stress conditions and loss of function in the *RCD1* gene suggested the improper function of the DREB2A in variable environmental stress-changing conditions (Vainonen *et al*., 2012).

The mechanistic function of the *SRO* gene under abiotic stress and its role in tolerance is already explored. In various reports of other plant species like *OsSRO1c*, the role in water stress which induces the NAC TF enhanced the H_2O_2 formation and was also involved in the closure of the stomatal aperture (You et al., 2013). In maize, it was found that under drought stress conditions there was induction in the expression of the *ZmSRO1e&ZmSRO1f* (Jiang *et al*., 2020). Studies on Arabidopsis thaliana and *Solanum lycopersicum* have revealed that the expression of *SRO1&SRO5* increases in osmotic and salt stress (Babajani *et al*., 2009). Additionally, in the apple (*Malus domestica*) plant, it was out of 6 *SRO* genes one gene *MdRCD1* have a significant function in stomatal and ABA signaling, and has a prominent role in abiotic stress tolerance (Li *et al*., 2017). In bananas (*Musa* spp.), it was found that has a significant role against abiotic stress tolerance via mechanistic phytohormonal signaling. The *SRO4* was found in the study interacting with *MaMYB4&MaNAC6* through DNA binding PARP thereby altering the downstream signaling processes (Blue *et al*., 2021). These above findings make a concrete base for the *SRO* gene family to be a transcriptional controller against environmental constraints which needs thorough investigation to decipher their regulatory role.

The identification of *RCD1* orthologs in a variety of plant species and their importance have been utilized for screening of the *W. coagulans* transcriptome dataset for the identification of *SRO* gene family members. Since, *W. coagulans* have medicinal importance and now becoming endangered due to their overexploitation and various changing environmental factors. The plant is of commercial importance and is currently in use for treating diabetes, insomnia, nervous disorder, and various other diseases. It is also used in treating cancer and microbial infections and also acts as an immune suppressant (Tripathi *et al*., 2018). Its medicinal value is because of its active chemical component withanolides, withanones, and withaferine which are lactone steroids having the potential to act as immune boosters and anti-viral agents (Sharma *et al*., 2023; Tripathi *et al*., 2021).

In the present investigation, *WcRCD1* like *SRO* gene family member from *W. coagulans* has been identified using the SRA dataset for the first time. A computational approach was used to analyze subcellular localization, GO annotation, domains analysis, phylogenetic relationship and conserved motifs. This study provides a preliminary report and made a foundation for further research to decipher the molecular mechanism of the SRO gene mediating developmental, physiological, and stress-responsive processes for genetic improvement.

II. Methods and materials

A. *Identification of SRO gene of Withania coagulans*

Identification of *SRO* gene family members in *W. coagulans* was performed by using *Musa acuminateRCD1* gene as a reference (Zhang *et al*., 2019) and an extensive BLAST search (https://www.ncbi.nlm.nih.gov/sra) was made for the search of *WcSRO* gene family members utilizing NCBI SRA database of leaf transcriptome (SRR12516448) (Gupta *et al*., 2022). Which was further assembled via the Galaxy online assembly tool (https://usegalaxy.org/). It results in the selection of *RCD1*-like *SRO* gene family members from *W. coagulans* having PARP and RST domains. This was further validated by Expasy-PROSITE online server (https://prosite.expasy.org/) for domain confirmation (Castro *et al*., 2006). Then, the PROSITE profiles for the RST domain (PS51879) and PARP domain (PS51059) were checked in the *WcSRO* gene family members to remove the false selection of the *SRO* genes. Additionally, NCBI ORF FINDER (https://www.ncbi.nlm.nih.gov/orffinder/) was employed for the selection of the longest ORF for further analysis. Furthermore, the physicochemical properties of the selected RCD1-like *SRO* gene family members were performed by the application of Expasy-ProtParam online web server (https://web.expasy.org/protparam/) (Bendtsen *et al*., 2004).

B. *MSA, Motif and Phylogenetic relationship study*

To predict the homology of *WcRCD1* proteins in other *SRO* gene members in different closely related species, MSA analysis has been carried out by Clustalx.2.1 online software (https://clustalx.software.informer.com/2.1/) (Sievers & Higgins, 2014). Further, to understand the evolutionary relationship between the *WcSRO* genes, a Neighbour-Joining phylogenetic tree was constructed with a bootstrap manner having 1000 value using MEGA X online software (https://www.megasoftware.net/). Also, the allocation of

considerable Motifs in *WcRCD1* and other homologous members was investigated using MEME suite 4.1.1.2 (Multiple Expectation Maximization) for motif prediction (http://meme.nbcr.net/) (Bailey *et al*., 2015). In the motif analysis, we change the default parameter the optimum number of motifs selected to 20 with motif width between 10 to 30 residues. The sequential similarities and dissimilarities present between WcRCD1 protein and their homologues were visualized by the Circos visualization tool (http://circos.ca/) (Zhang *et al*., 2013) applying cut-off range up to 50%.

C. *Structural Modeling and Validation*

The putative 3D WcRCD1 protein structures were made based on homology modelling by the usage of the SWISS-MODEL server (https://swissmodel.expasy.org/). The WcRCD1 protein structure was verified and validated by saves online web server (https://saves.mbi.ucla.edu/). The protein model was further submitted to the online repository database (PMDB) to obtain the accession number (PM0084368).

D. *Cellular Localization and GO Analysis*

To know the cellular localization, GO annotation analysis has been carried out for the WcRCD1 protein utilizing CELLO2GO online tool. To investigate the role of WcRCD1 protein interactions with other proteins string online software (https://string-db.org/) was also functionalized and results were obtained (Yu *et al*., 2014).

E. *In-silico expression analysis*

The publicly available *A. thaliana SRO* gene family member's expression data under abiotic stress and the developmental map was assessed by an online Arabidopsis eFP browser 2.0 web server(http://bar.utoronto.ca/efp2/Arabidopsis/Arabidopsis_eFP Browser2.html)(Nitta *et al*., 2018). Further, the in-silico expression profile of the selected *AtSRO* gene family members involved in different abiotic stresses was visualized by RSAT software.

F. *Plant material and drought treatment*

In the present study, plant seeds were taken from Botanical Garden, BHU, Varanasi. The seeds were further subjected to sand: soil mixture (1:1) and water at regular intervals. After the seedlings attained four leaf stage, they were transferred to Hoagland half-strength media maintained at 25 °C with 16-hour day and 8-hour dark conditions (Hoagland & Arnon, 1950). The drought (PEG6000), osmotic stress (300 mM Mannitol), and Salt stress (150 mM NaCl) was given along with half-strength Hoagland media nutrients. After 0, 12, and 24 hr, leaves were harvested and stored at 80°C for further RNA extraction and expression analysis by RT-PCR.

G. *Analysis of stomatal ultra-structure*

SEM (FEI, quanta 200) was used to visualize the ultra-structure of stomata in drought conditions. Dry leaves under drought conditions and controls were cut into tiny discs and treated with 2.5% glutaraldehyde in phosphate buffer at pH 7.2 according to Hess, 1966. After that, dehydrate the leaves inanethanolic solution for 30 minutes at room temperature. BeforeSEM analysis, the leaves were coated with a thin layer of silver and viewed with a scanning electron microscope.

H. *RNA extraction and qRT-PCR*

Based on the MSA and the expression profile spectrum *WcRCD1* with the highest expression under abiotic stress was selected for the PCR analysis. The TRIzol reagent (GIBCO-BRL) was followed to obtain the total RNA using leaf tissues. Also, the primer for the *WcRCD1* designed using Primer 3 online tool(https://primer3.ut.ee/) taking 5'GGACGGAACCTGATTTCTGA3'and 3'CTGATGCCGAAGGTCATTTT5'as forward primer and reverse primer respectively (Untergasser *et al*., 2012). For QA/QC analysis RNA sample were analyzed by 260/280 absorption ratio using Nano-drop spectrophotometer (Thermo Scientific). Further, the selected *WcRCD1* primers were used in the PCR cycles using Thermo Cycler utilizing each template RNA sample (Bio-Rad). The amplified bands were verified via Gel- DOC EZ imager (Bio-Rad) by the application of Quantity One software (Bio-Rad).

III. Results

A. *Identification and Characterization of WcRCD1 gene*

The current bioinformatics analysis in *W. coagulans* was done by using *Musa acuminate RCD1* gene as a reference sequence (Zang *et al*. 2019). An extensive blast search was performed against SRA (Sequence Retrieve Archive) database from NCBI. We have also, examined the *W. coagulans* SRA dataset the closely related members of the *SRO* gene family using other plant species including other species of *Withania* i.e., *W. somnifera* by the NCBI blast program. After retrieving the sequences, we perform Nucleotide Blast for confirmation. The conserved sequence's similarity and differences in the multiple sequence alignment analysis were performed in a detailed profile given in (Fig.1B). Then, the conserved domain in WcRCD1 was identified by the Expasy-PROSITE scan online tool. The two distinctive domains have been identified, as the poly (ADP-ribose) polymerase (PARP) domain and C-terminal RST (RCD-SRO-TAF4) domain. The PARP domain was present in between 1 to 75 amino acid residues whereas the RST domain lies between 98 to 169 amino acid residues of the screened sequence. There is a gap of 23 amino acid residues between both domains (Fig.1A). Further, physio-chemical properties were studied by Expasy-ProtParum online tool. The WcRCD1 protein was composed of a total of 181 amino acids. The isoelectric point was found to be 7.0 along with instability index (54.21), molecular weight (20159.99), and hydropathy index (-0.375).

B. *Conserved Motif* analysis

The MEME motif analysis revealed a total of 14 motifs in the WcRCD1-like protein and its closely related family members in another genus. The motif analysis revealed that high similaritywas present in the sequence of *W. coagulans, W. somnifera, Vigna angularis, V. umbilata,* and *V. radiate* and *WcRCD1* have high similarity with *V. angularis*. There were separate groups present which diverged from the WcRCD1 protein due to the substitution of one or more amino acid residues in their sequences. From the result, out of 14 motifs, 7 conserved motifs are present in WcRCD1, like protein as represented in Fig.2.

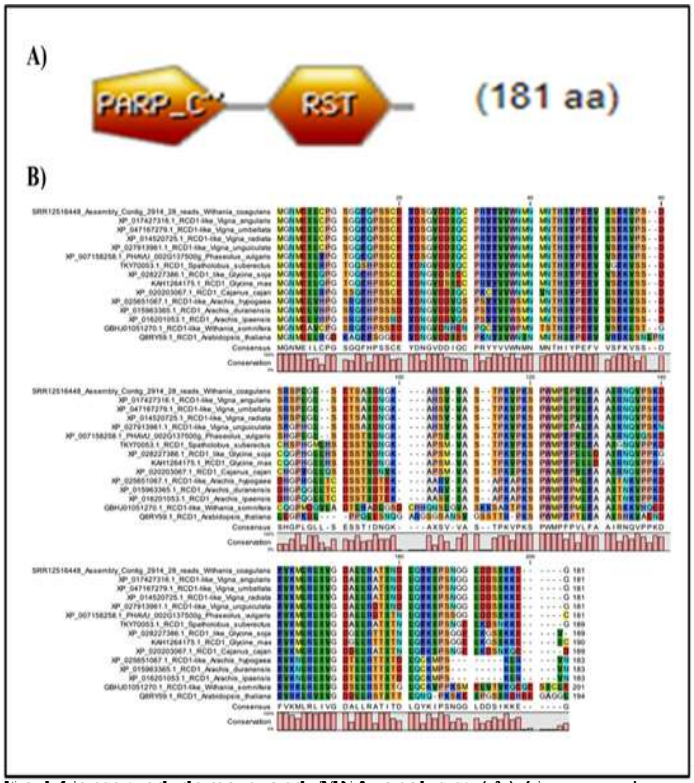

Fig.1.Conserved domain and MSA analysis: (A) Conserve domain analysis in WcRCD1 protein; The two-colored boxes indicate two domains of the *SRO* gene, N- terminal PARP catalytic domain and C-terminal RST domain. (B) MSA study in WcRCD1 protein; multiple sequence alignment of the conserved functional domain of *RCD1* with closely related orthologs within different plant species. center aligned, and having paragraph space before and after the heading.

C. *Phylogenetic, comparative relationship and protein interaction analysis*

To investigate the evolutionary relationship between *W. cogulans* and its closely related SRO protein members, full-length protein sequences were analyzed via Neighbour-joining phylogenetic tree using MEGA X software. It was observed that *W. coagulans* showed proximity with *V. angularis* (Fig.3a).Further sequential similarities and dissimilarities were visualized using Circos Visualization tool (Fig.3b). Protein-Protein interaction network of *WcRCD1* homologue in *A. thaliana* (*AtRCD1*) was also studied by string server. For the assessment of interacting partners of the STRING server opting for the default custom values of 10 integrators from the first and second shells.With the obtained results, we observed that *RCD1*interacted with the first shell DREB2A (Dehydration-responsive element-binding protein 2A) score value (0.992),DREB2B (Dehydration-responsive element-binding protein 2B) score value of 0.847, DREB2C (Dehydration-responsive element-binding protein 2C) score value of 0.853, NAC 13 (*Arabidopsis* NAC domain-containing protein 13), score value (0.948), NAC046 (NAC domain-containing protein 46), STO B-box zinc finger family protein, PARP 2 Poly [ADP-ribose] polymerase 2 score value (0.844), TAF 4 (Transcription initiation factor TF id subunit 4) score value (0.909), SOS1 (Sodium proton exchanger, putative (NHX7) (SOS1) score value (0.064), the interaction was also found which showed its involvement in oxidative stress and drought stress tolerance (Fig.3 C).

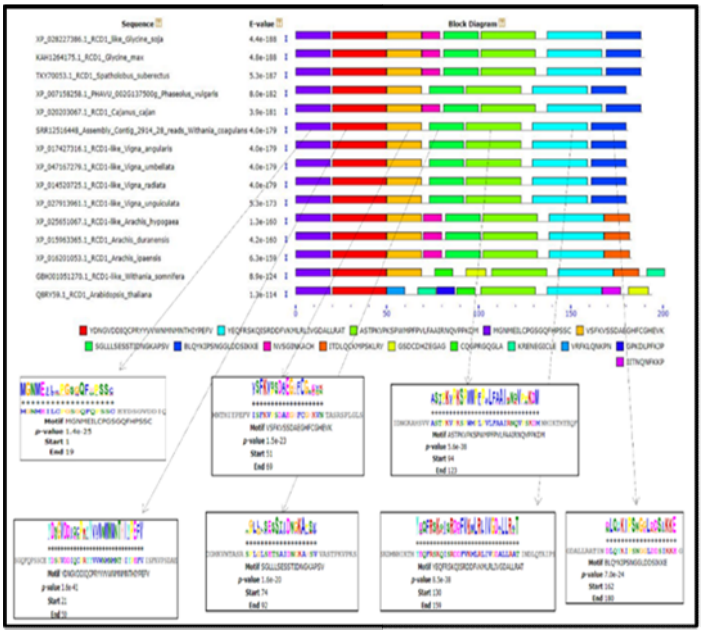

Fig.2. Conserved motif analysis: The Motifs of the conserved functional domain of WcRCD1 compared with closely related orthologs within different SRO gene family members; the dotted arrows depict the motif found in WcRCD1 identified gene.

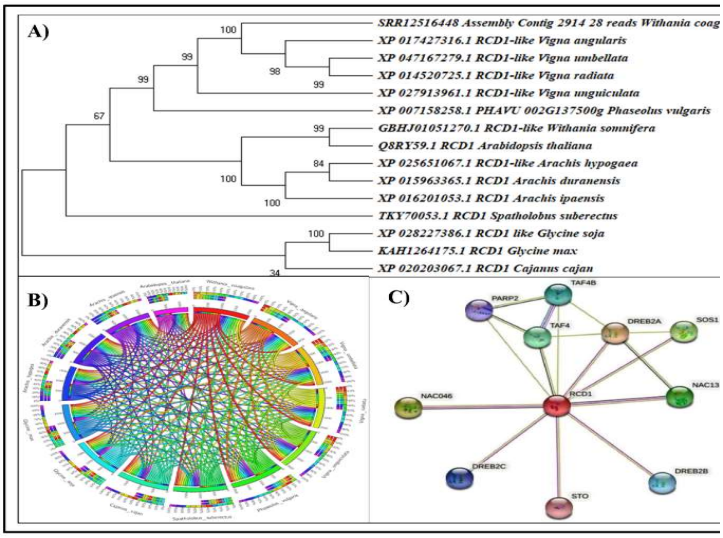

Fig.3. Phylogenetic, comparative relationship and protein-protein interaction studies: (A) The phylogenetic tree of *WcRCD1* like *SRO* gene family member with other orthologs is constructed by MEGA X software keeping 1000 bootstrap interactions. (B) The comparative similarity and differences between the *WcRCD1*-like *SRO* gene family member with the other orthologs are done via Circos online tool; the circular map is based on the similarity percentage matrices using Clustal W algorithm. (C) Protein-protein grouping interaction web of WcRCD1 protein obtained from String online web server.

D. Structural modelling and model validation of *WcRCD1*

The WcRCD1 protein structure has been also controlled by selecting an appropriate template which was based on sequence similarities, crystal resolution, and residue completeness. A total of four models were generated by SWISS-Modeller and visualized by Discovery Studio (DS) Client 3.0.Among them three structurally resolved templates viz 5n9q, 4fu3, and 5oao were selected and their qualitative analysis was done by PROCHEK and ProSa web server (Fig.5). Further, we refined these models by ModRefiner server. The refinement tool generated the best model which was also checked the model for reliability using the SAVES server on different parameters like Verify 3D, ERRAT score, and RMSD (Fig.4).Based on PROCHECK statistics, it was found that 98.3% of residues of our model were present in the most favored regions, while 1.7% of residues were in additional allowed regions, and 0.0% (no residue) were located in generously allowed and disallowed regions at the last, we have submitted the finalized protein model in PMDB (protein repository database) which was assigned by a unique sequence ID (PM0084368).

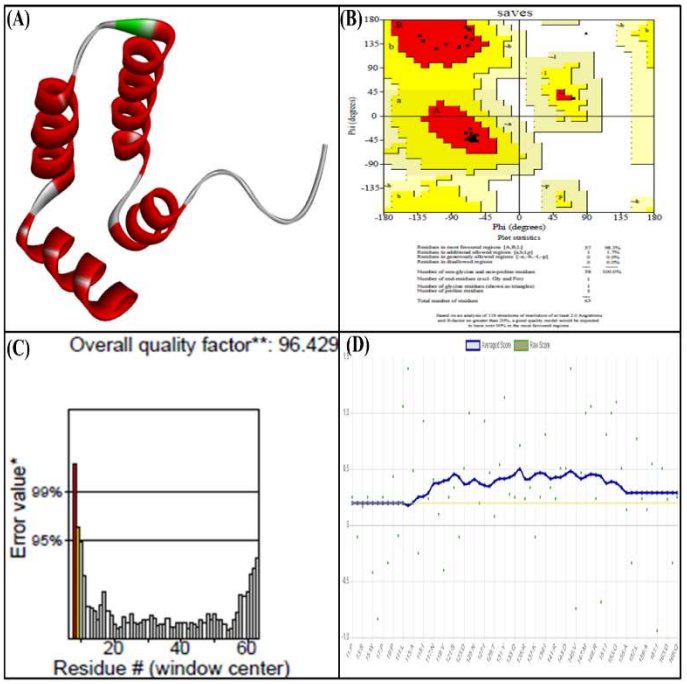

Fig.4. Protein modelling and structural validation analysis. (A) 3D modelling of WcRCD1 protein *via* online Swiss Modeller server (B) The Ramachandran plot showing the reliability of model by online saves server (C) Validation by ERRAT scoring online tool and (D) Validation by verify 3D online tool.

E. *Analysis of Gene Ontology enrichment and subcellular localization of WcRCD1 protein*

For the analysis of gene ontology, the WcRCD1 protein sequence was submitted on the CELLO2GO server to predict the different functions like molecular, cellular, and biological functions in the cell.The result showed that the WcRCD1 protein has involvement in several processes having its role at a cellular level.

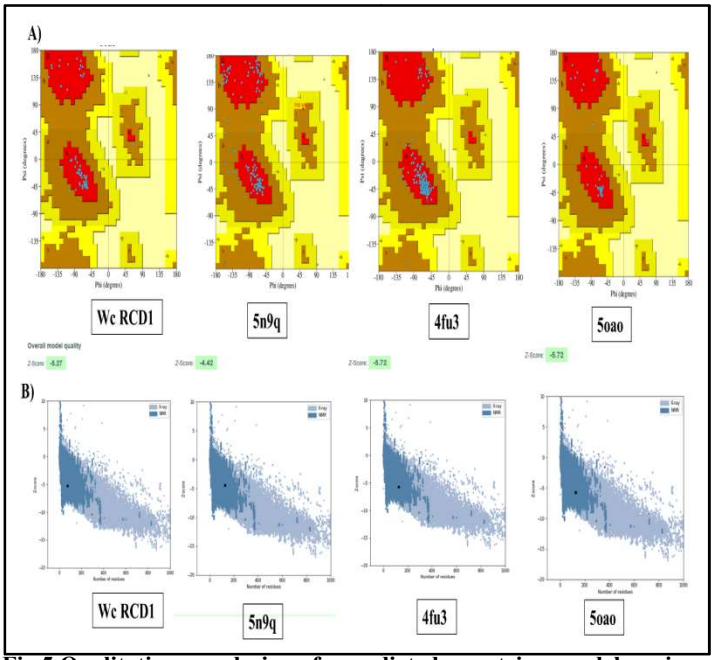

Fig.5.Qualitative analysis of predicted protein model using

PROCHECK and ProSA online tool. (A) The predicted model of *WcRCD1* was compared with available protein templates (5n9q, 4fu3, and 5oao). (B) Qualitative estimation of designed protein model by ProSA web server by measuring overall score, and structural error, ProSa score of *WcRCD1* was found near to their templates proteins.

In the biological processes, the localization probability of proteins based on their subcellular localization nature such as nuclear, cytoplasmic, extracellular, etc., based on localization percentage was also predicted. This protein shows 43% localization in the nucleus, 17.3% localization in the extracellular, and 24.8% localization in the cytoplasm, and the results were presented in Fig.6.

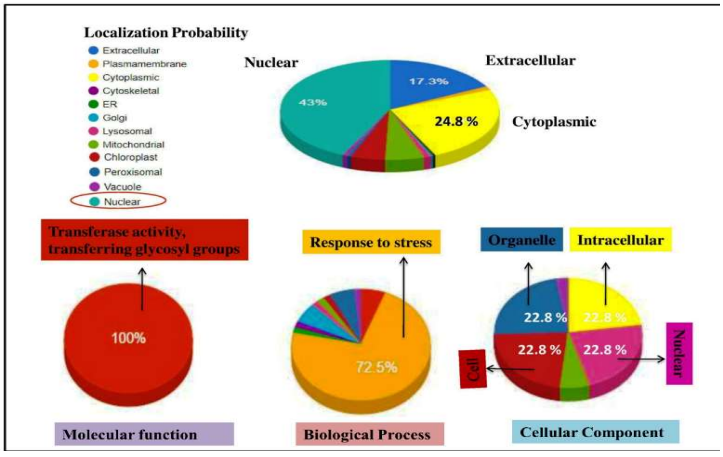

Fig.6. Analysis of sub-cellular localization and functional gene annotation by using CELLO2GO web server for *WcRCD1*. Thesignificant terms are represented in terms of their percentage contribution.

F. In-silico expression analysis and validation by RT-PCR

For the further assessment of the putative role of the *WcRCD1*-like *SRO* gene, the close homologues found via BLAST searches in *Arabidopsis* were selected. The SRO proteins found were named *SRO2* (At1g23550), *RCD1* (At1g32230), and *SRO5* (At5g62520) (Fig.7 A-C). It was observed that the *AtRCD1*-like *SRO* genes were highly expressed under abiotic stress specifically under drought stress, osmotic and salt stress. To further understand the relative expression of the above genes under various developmental stages, a heatmap was constructed. It was clear from the expression profile that the expression of *RCD1* was high in seed, stem, and stressed leaves.

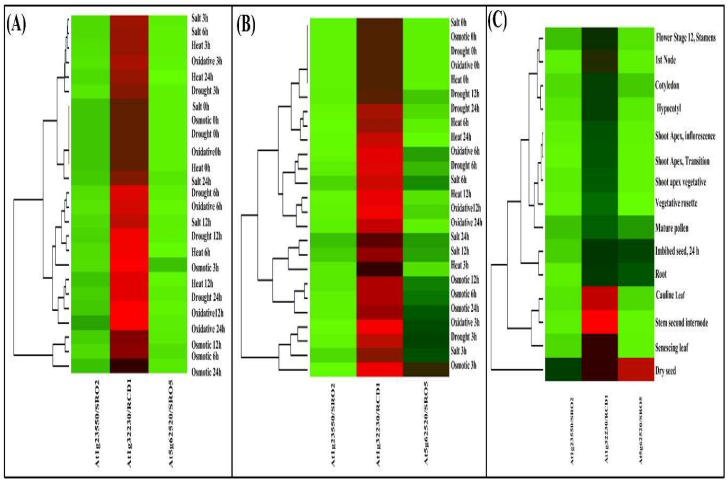

Fig.7. Heatmap profile of differential expression of Arabidopsis thaliana SRO genes in different tissues exposed to variable abiotic stress conditions and time intervals. (A) Expression profile of shoot tissues (B) Expression profile of root tissues (C) Expression response at different developmental stages. The Color bar ranges from light green to dark red colour in increasing order according to their expression response.

Therefore, to validate these results in *W. coagulans* the *RCD1* gene RT-PCR was carried out through osmotic stress, drought, and salt stress taking the root, stem, seed, leaf, and somatic meristem, callus, young shoot and somatic embryo tissues as samples under 0-, 12-, and 24-hour intervals the expression profile was investigated. The results indicated that *WcRCD1* has the highest expression profile in root, stem, and callus tissues under osmotic, salt, and drought stress. Also, in drought stress, it showed a prominent expression in young shoot tissues. In addition, on 24 hr stress treatment, *WcRCD1* was highly upregulated in comparison with 12 and 0 hr stress treatments, respectively. The results of real-timeexpression analysis indicated the putative role of *WcRCD1* in abiotic stress tolerance in *W. coagulans.* The detailed real-time expression analysis is presented in Fig.8.

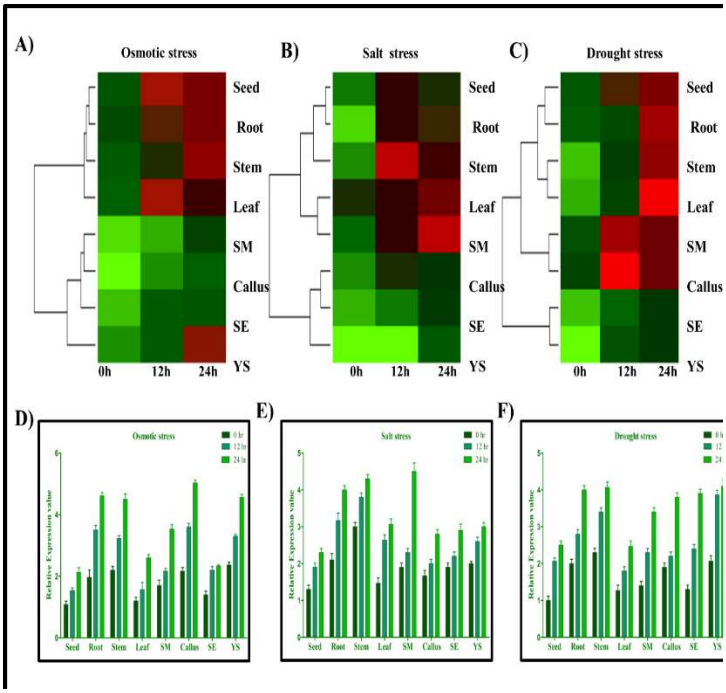

Fig.8. Heatmap profile and RT-PCR analysis of the differential expression of the *WcRCD1* gene in different tissues under abiotic stress conditions and time intervals.(A-C) Expression profile of seed, root, stem, leaf, somatic embryo (SE), callus, somatic meristem (SM) and young shoot (YS) after 0h, 12h and 24h, respectively under osmotic, salt and drought stress. The Color bar ranges from light green to dark red colour in increasing order according to their expression response (C-D). The graphical representation of the *WcRCD1* under osmotic stress, salt and drought stress at 0h, 12h and 24 h respectively.

G. Analysis of leaf surface by SEM

To analyze the change in the leaf surface cells and stomata under increasing drought stress in *W. coagulans*, leaf samples were also visualized by scanning electron microscopy. Narrowing of the stomatal aperture upon increment in drought exposure was observed in the leaf lamina surface observation. These observations can be correlated with the functions of *RCD1*-like genes. Also, EDX analysis showed a decrease in K^+ ion concentration in guard cells. The detailed analysis is given in Fig.9 A-B.

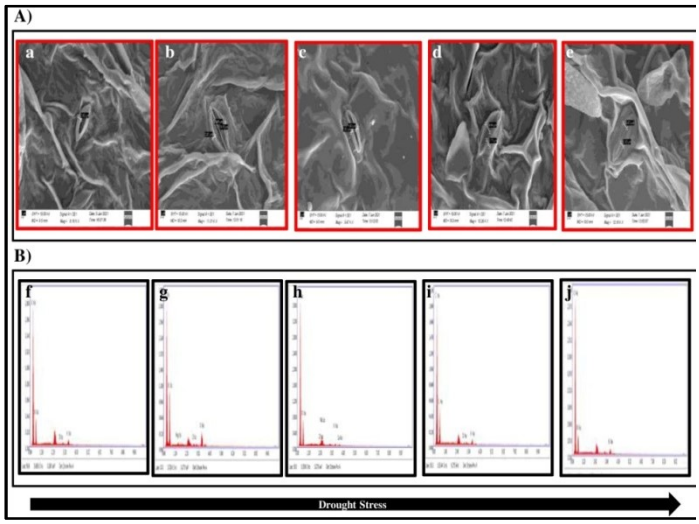

Fig.9. Scanning Electron Microscopic studies in *Withania coagulans* leaf tissue after increasing drought. (A) SEM microscopic images of stomata. (B) EDX analysis of the stomatal cells.

IV. Discussion

To survive in complex and adverse environmental conditions, plants have developed numerous metabolic and physiological mechanisms regulating stress-responsive regulatory and structural genes (Apoorva *et al*., 2021). The *SRO* gene family is a conserved small gene family specific to land plants and has been known as a major regulator in combating abiotic stress environments (Li *et al*., 2017).

The *SRO* gene family is already studied in many plant species including crop plants such as *Oryza sativa* (You *et al*., 2014), Zea mays (Jiang *et al*., 2018). *Vitis vinifera* (Gungor *et al*., 2019), *Brassica rapa* by Qiao *et al*., 2020, cucumber by Xiao *et al*., 2022, *Solanum lycopersicum* by Li *et al*., 2021, *Triticum aestivum* (Jiang *et al*., 2020), banana (*Musa* spp.), and *Malus domestica* (Li *et al*., 2017). In this study, *WcRCD1* is identified based on conserved domains (RST, PARP, and WWE) from the *W. coagulans* SRA dataset. The *SRO* genes are bifurcated into two types type A and type B based on WWE domain presence or absence of WWE domain. Type A contains RST, WWE and PARP domains on the other hand type B lacks the WWE domain. *WcRCD1* thus identified belongs to the type B category (Ma *et al*., 2022). The physical chemical properties of the WcRCD1 protein revealed that it has a total of 181 amino acids. The isoelectric point was found to be 7.0 along with instability index (54.21), molecular weight (20159.99), and hydropathy index (-0.375). Further, WcRCD1 protein was found to be involved in several biological processes at the molecular and cellular levels. Further, localization prediction inferred the probability of WcRCD1 protein occurrence in the nucleus, extracellular, and cytoplasmic levels.

In-silico tissue-specific expression profiling opens the window for analyzing these functions of genes involved in developmental processes. For example, in *Arabidopsis*, *RCD1*, and *SRO1* genes express in shoot meristems. Studies on apple plants have suggested tissue-specific differential expression under variable stress conditions. The expression level of *MdRCD1* was more in fruits than in other tissues (Li *et al*., 2017). But, in maize, the root tissues contain high expression levels of *ZmSRO*s than in other tissues (Jiang *et al*., 2018). The tissue-specific and hormone-responsive nature of *SRO*s were also seen in tomato (Li *et al*., 2021). Likewise, *in-silico* studies on *W. coagulans* suggested the *RCD1* expression in callus, leaf, seed, and root tissues.

Although *SRO* homologs widely exist in the plant kingdom, only a few members from rice and *Arabidopsis* have been characterized for the regulation of plant development and its

involvement in improving plant resistance against abiotic stress (Li *et al*., 2021). Various reports demonstrated that the *SRO* family genes can respond to abiotic stresses and stress-related hormones with a vital role in plant abiotic stress tolerance and hormone signaling pathways. For example, *Zea maysSRO* genes can be induced by different stress factors which are negatively involved in regulating anthocyanin production. In *Ipomea cairicaSRO* genes were upregulated to provide abiotic stress tolerance.

Further, the transgenic studies on *IcSRO* in *Arabidopsis* decipher its involvement in salt and water stress. In the present study, the expression levels of the *WcRCD1* gene under osmotic, salt, and drought stress were analyzed by qRT-PCR to detect its possible roles under abiotic stress. The results obtained from *WcRCD1* are associated with salt, osmotic, and drought stress responses. The *WcRCD1* expression results were also obtained, it was consistent with the results reported in banana, tomato, maize, and wheat as well. Our results suggest that upregulation of *WcRCD1* may be positive for the mitigation of osmotic responses.

In the present state-of-art, we performed protein-protein interaction network analysis too using the STRING database and identified putativeWcRCD1-interacting proteins. Studies showed that *WcRCD1* also interacts with a large number of TFs and the RST domain always acts as the binding site. Probably, this domain is required for the co-expression and interaction of *SRO* genes with other TFs to participate in plant stress resistance. Further interacting proteins, such as DREB2B, NAC017, STO, and SOS1, are known interaction partners of *AtRCD1*. Many WcRCD1-interacting proteins as revealed by functional annotation are involved in abiotic stress responses. There are reports that the WcRCD1 protein could interact with the NAC transcription factor and lead to stomatal closure which was also demonstrated in the present study ultimately involved in drought tolerance. It was previously reported in *Arabidopsis*, that *SRO* genes can interact with 21 different transcription factors such as NAC, ERF, bHLH etc. to coordinate the stress responses via binding with the *RCD1* domain in rice using 29 AtTF's interaction network, it was possible to depict the involvement of *SRO* in leaf senescence, abiotic stress tolerance, and ROS signaling. In another study on wheat also it was found that six *SRO* genes interact with 14 TFs to regulate the stress responses (Jiang *et al*., 2020). Our results give a preliminary structural and functional analysis of the *WcRCD1* gene which can be further utilized for detailed investigation and its involvement in various stress responses.

V. Conclusion

Overall, for the first time, a *WcRCD1*-like *SRO* gene was identified using the NCBI SRA database along with protein characterization and validation have been carried out to assess its possible function. The protein structure was submitted to an online protein repository. The vital regulatory integrative network between proteins, specifically transcription factors has been depicted and can be further validated. Analysis of transcriptome data shows that *WcRCD1*gene shows its expression in different tissues of *W. coagulans* under different abiotic stresses, similar to other plants studied having its role in combating adverse environmental constraints. At the same time, qRT-PCR results of some *AtRCD1* and *WcRCD1*-like SRO family members showed that these genes responded significantly to salt, drought, and oxidative stress. Notably, the expression profile of *WcRCD1* was significantly upregulated in stem root and young shoot tissues under osmotic, salt, and drought stress. The results also decipher that, the WcRCD1 protein could interact with the NAC transcription factor and lead tostomatal closure for improving drought tolerance. Further, over-expression studies of *WcRCD1* can be designed for unveiling the true potential and molecular mechanisms of *RCD1*-like genes in combating the adverse effects of abiotic stresses. Additionally, the information related to*WcRCD1* can be usedfor developing stress-tolerant plants for theestablishment ofgrowthand development along with the production of important withanolides.

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References

Babajani, G., Effendy, J., & Plant, A. L. (2009). Sl-SROl1 increases salt tolerance and is a member of the radical-induced cell death 1—similar to *RCD1* gene family of tomato. Plant Science, 176 (2), 214-222.

Bailey, T. L., Johnson, J., Grant, C. E., & Noble, W. S. (2015). The MEME suite. Nucleic acids research, 43 (W1), W39-W49.

Bendtsen, J. D., Jensen, L. J., Blom, N., Von Heijne, G., & Brunak, S. (2004). Feature-based prediction of non-classical and leaderless protein secretion. Protein Engineering Design and Selection, 17 (4), 349-356.

Blue, Y. A., Kusumi, J., & Satake, A. (2021). Copy number analyses of DNA repair genes reveal the role of poly (ADP-

ribose) polymerase (PARP) in tree longevity. Iscience, 24 (7), 102779.

De Castro, E., Sigrist, C. J., Gattiker, A., Bulliard, V., Langendijk-Genevaux, P. S., Gasteiger, E., Hulo, N. (2006). ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. Nucleic acids research, 34 (suppl_2), W362-W365.

Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., & Shinozaki, K. (2006). Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Current Opinion in Plant Biology, 9 (4), 436-442.

Gungor, A. A., Nadaroglu, H., & Gultekin, D. D. (2019). Synthesis and characterization of nano-strontium oxide (SrO) Using erzincan cimin grape (*Vitis vinifera*, Cimin). Chem. Sci. Int. J, 26, 1-7.

Gupta, S., Bohra, M. K., Sharma, K., Kachhwaha, S., Kothari, S. L., & Jain, R. (2022). Leaves and roots of in vitro cultured *Withania coagulans* adopt different pathways for withanolide biosynthesis: a *comparative* transcriptome study.

Hess, W. (1966). Fixation and staining of fungus hyphae and host plant root tissues for electron microscopy. Stain Technology, 41(1), 27-35.

Hoagland, D. R., & Arnon, D. I. (1950). The water-culture method for growing plants without soil. Circular. California agricultural experiment station, 347(2nd edit).

Jaiswal, D., Pandey-Rai, S., & Agrawal, S. B. (2021). Untangling the UV-B radiation-induced transcriptional network regulating plant morphogenesis and secondary metabolite production. Environmental and Experimental Botany, 192, 104655.

Jaspers, P., & Kangasjärvi, J. (2010). Reactive oxygen species in abiotic stress signaling. Physiologia Plantarum, 138(4), 405-413.

Jiang, H., Xiao, Y., & Zhu, S. (2018). Genome-wide identification, systematic analysis and characterization of SRO family genes in maize (*Zea mays* L.). Acta Physiologiae Plantarum, 40, 1-12.

Jiang, W., Geng, Y., Liu, Y., Chen, S., Cao, S., Li, W., Yin, J. (2020). Genome-wide identification and characterization of *SRO* gene family in wheat: Molecular evolution and expression profiles during different stresses. Plant Physiology and Biochemistry, 154, 590-611.

Li, H., Li, R., Qu, F., Yao, J., Hao, Y., Wang, X., & You, C. (2017). Identification of the *SRO* gene family in apples (Malus× domestica) with a functional characterization of *MdRCD1*. Tree Genetics & Genomes, 13, 1-14.

Li, N., Xu, R., Wang, B., Wang, J., Huang, S., Yu, Q., & Gao, J. (2021). Genome-wide identification and evolutionary analysis of the *SRO* gene family in tomato. Frontiers in genetics, 12, 753638.

Liu, A., Wei, M., Zhou, Y., Li, D., Zhou, R., Zhang, Y., You, J. (2021). Comprehensive analysis of *SRO* gene family in *Sesamum indicum* (L.) reveals its association with abiotic stress responses. International journal of molecular sciences, 22(23), 13048.

Ma, Y., Zhou, X., Liu, Z., & Wu, B. (2022). Comprehensive Analysis of *StSRO* Gene Family and Its Expression in Response to Different Abiotic Stresses in Potato. International journal of molecular sciences, 23(21), 13518.

Nitta, H., Tomita, H., Zhang, Y., Zhou, X., & Yamada, Y. (2018). Disgust and the rubber hand illusion: A registered replication report of Jalal, Krishnakumar, and Ramachandran (2015). Cognitive Research: Principles and Implications, 3(1), 1-11.

Qiao, Y., Gao, X., Liu, Z., Wu, Y., Hu, L., & Yu, J. (2020). Genome-wide identification and analysis of SRO gene family in Chinese Cabbage (*Brassica rapa* L.). Plants, 9(9), 1235.

Shapiguzov, A., Vainonen, J. P., Hunter, K., Tossavainen, H., Tiwari, A., Järvi, S., . . . Wybouw, B. (2019). Arabidopsis *RCD1* coordinates chloroplast and mitochondrial functions through interaction with ANAC transcription factors. Elife, 8, e43284.

Sharma, L., Maurya, B., & Rai, S. P. (2023). An overview of biotechnological interventions and abiotic elicitors on biomass and withanolide biosynthesis in *Withania somnifera* (L.) Dunal. Industrial Crops and Products, 193, 116238.

Shinozaki, K., & Yamaguchi-Shinozaki, K. (1997). Gene expression and signal transduction in water-stress response. Plant physiology, 115(2), 327.

Sievers, F., & Higgins, D. G. (2014). Clustal Omega, accurate alignment of very large numbers of sequences. Multiple sequence alignment methods, 105-116.

Tripathi, D., Meena, R. P., & Pandey-Rai, S. (2021). Short term UV-B radiation mediated modulation of physiological traits and withanolides production in *Withania coagulans* (L.) Dunal under in-vitro condition. Physiology and Molecular Biology of Plants, 27, 1823-1835.

Tripathi, D., Rai, K. K., Rai, S. K., & Rai, S. P. (2018). An improved thin cell layer culture system for efficient clonal propagation and in vitro withanolide production in a medicinal plant *Withania coagulans* Dunal. Industrial Crops and Products,119, 172-182.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., & Rozen, S. G. (2012). Primer3-new capabilities and interfaces. Nucleic acids research, 40(15), e115-e115.

Vainonen, J. P., Jaspers, P., Wrzaczek, M., Lamminmäki, A., Reddy, R. A., Vaahtera, L., . . . Kangasjärvi, J. (2012). RCD1–DREB2A interaction in leaf senescence and stress responses in *Arabidopsis thaliana*. Biochemical Journal, 442(3), 573-581.

Xiao, L., Zhou, Z., Zhu, C., Zhao, J., Hu, Z., Liu, S., & Zhou, Y. (2022). Molecular Cloning, Characterization, and Expression Analysis of SIMILAR TO RCD-ONE (*SRO*) Family Genes

Responding to Abiotic and Biotic Stress in Cucumber. Horticulturae, 8(7), 634.

You, J., Zong, W., Du, H., Hu, H., & Xiong, L. (2014). A special member of the rice SRO family, *OsSRO1c*, mediates responses to multiple abiotic stresses through interaction with various transcription factors. Plant Molecular Biology, 84, 693-705.

You, J., Zong, W., Li, X., Ning, J., Hu, H., Li, X., Xiong, L. (2013). The SNAC1-targeted gene OsSRO1c modulates stomatal closure and oxidative stress tolerance by regulating hydrogen peroxide in rice. Journal of experimental botany, 64(2), 569-583.

Yu, C.-S., Cheng, C.-W., Su, W.-C., Chang, K.-C., Huang, S.-W., Hwang, J.-K., & Lu, C.-H. (2014). CELLO2GO: a web server for protein subCELlular LOcalization prediction with functional gene ontology annotation. PLoS One, 9(6), e99368.

Zhang, H., Meltzer, P., & Davis, S. (2013). RCircos: an R package for Circos 2D track plots. BMC bioinformatics, 14, 1-5.

Zhang, L., Zhou, D., Hu, H., Li, W., Hu, Y., Xie, J., Wang, W. (2019). Genome-wide characterization of an SRO gene family involved in response to biotic and abiotic stresses in banana (Musa spp.). BMC plant biology, 19(1), 1-14.