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RESEARCH ARTICLE

Identification and characterization of cold shock domain proteins (CSPs) for their response to cold stress in pigeonpea [*Cajanus cajan* (L.) Millsp.]

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Abstract

Chilling stress arrests plant metabolism, slowly perturbing homeostasis, which eventually leads to senescence. If imposed for a long time, it can cause plant death. Prokaryotes possess cold shock domain-containing proteins (CSPs) in their genomes that help them acclimate to chilling stress by acting as RNA chaperones, ensuring the maintenance of cellular functions. Considering this as an important class of genes in maintaining homeostasis during chilling stress, the present study was conducted to carry out a genome-wide survey of these genes in *Cajanus cajan*, which has not been studied to date. *In-silico* genome-wide characterization of the *Cajanus* genome identified 4 CSPs (*Cc*CSP1, *Cc*CSP2, *Cc*CSP3 and *Cc*CSP4) members. Domains prediction identified each containing at least one CSD (cold shock domain), Glycine-rich repeats and CCHC-type zinc fingers, suggesting their role as nucleic acid binding proteins and protein-protein interactions. All, except the CcCSP2 gene, were found to be intronless. Additionally, CcCSP2 was found to be divergent from the rest of the three genes and phylogenetically close to *Medicago Mt*CSP3. Spatiotemporal expression in genotypes contrasting for cold stress response showed the highest expression of the CcCSP2 gene (almost 6-fold) in flowering bud tissue of tolerant genotypes, suggesting it may be involved in the cold adaptation mechanism. The study establishes that less-explored cold acclimation proteins, such as CSPs, are transcriptionally regulated genes in *Cajanus* when it experiences chilling stress during extreme weather conditions. This suggests that this subtropical crop may have the potential to cold-acclimate. Further studies are needed to establish the details so that crop damage is mitigated in these times of climate change.

Keywords: Cold stress, Cold shock domain, chaperones, adaptation, *Cajanus cajan*.

Introduction

The yield of legumes has decreased by about 50% worldwide due to several abiotic factors, including drought, extremely high temperatures, salinity, and heavy metal stress (Jan et al. 2023). Extreme temperature is one of the most significant elements impacting the entire stages of plant and crop phenology, which reduces productivity and yield (Repo et al. 2008). India is the world's highest producer of pigeonpea, which is an important source of protein in tropical and subtropical regions.

Environmental temperatures as low as 4 to 10°C cause chilling stress in plants from tropical and subtropical regions, whereas temperate species have the ability to endure such chilling temperatures and sometimes even lower than 0°C, albeit at varying degrees (Guo et al. 2018; Liu et al. 2018). Low-temperature stress causes several morphological, physiological, and biochemical changes in plant cells. It leads to membrane rigidification, ultimately increasing membrane permeability and causing solute leakage, disruption of

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macromolecular structures, enzymatic activities, ROS generation, and translocation and transport systems, as well as ice-crystal formation in extreme cases (Pierce 2001; Guo et al. 2018). This ensues a state of dehydration stress in the plants, in response to which the tolerant plants accumulate several osmolytes, such as sugars, proline, and cold resistance proteins (Thomashow 1999). Temperate crops, such as wheat, possess a "cold acclimation" capacity that enables them to tolerate chilling stress while also preparing for freezing stress when the temperature falls below 0°C (Guy et al. 1990, 2019). A plant is called "tolerant" only under with respect to the duration and degree of temperature it can tolerate. Temperate and tropical or subtropical grown crops thus have different low temperature threshold. Only a few subtropical crops, viz. rice, tomato, and soybean, have been the subject of cold stress studies, all suggesting they lack acclimation potential (Ding et al. 2019). However, soybeans have recently been reported to cold acclimate, although at lower levels than temperate crops (Robinson et al. 2016).

Being a subtropical crop, it is severely affected by the low temperatures below 10°C encountered during its reproductive stage, particularly during the chilling months of December and January in India (Choudhary et al. 2011). Long duration varieties usually encounter low and/or freezing temperatures during their flowering stages causing drying and senescing branches ultimately turning into dead tissues (Kumar et al. 2019) and also blossom as well as bud drop causing further reduction in potential yield by 6 to 100% (Singh 1997). Global climatic changes have brought about abrupt changes in environmental variables, ultimately affecting pigeonpea productivity. One factor among these is the extreme low temperature, which is intermittent with frost incidents, during the pigeon pea growth cycle (Kumar et al. 2019), and is damaging. It is not known whether pigeonpea can acclimate, and this is an investigative research theme.

Genetic variability for cold tolerance has been reported earlier (Singh et al. 1997; Singh et al. 2010). However, information on underlying molecular players in cold tolerance in pigeonpea is lacking (Singh et al. 1997). Identification of such QTLs/genes would, therefore, be important for their utilization in future breeding programs of medium and late-duration pigeonpea. Kumar et al. (2019) screened 302 pigeonpea breeding lines and identified the genotypes with contrasting cold responses. In the experiment, while dissecting the inheritance pattern, they came to the conclusion that cold tolerance is governed by a single major dominant locus in pigeonpea.

Previous studies have revealed the molecular basis of cold acclimation, which is established through multiple interconnected regulatory pathways, including the canonical CBF pathway (Fowler and Thomashow, 2002). A study by Zhao et al. (2016) identified CBF2 as one of the major controlling hub genes that regulate the expression of multiple vital genes involved in acclimation to low temperatures, as well as dehydration responses, and forms a signaling cascade. ICE1 (Inducer of CBF Expression 1) is a low-temperature regulated transcription activator of these hub CBF genes that binds to the C-repeat (CRT) motif (5′-CCGAC-3′) in promoter regions and activates their expression (Chinnusamy et al. 2007). CBF controls multiple COR (Cold-regulated Genes) expressions that are involved in cold acclimation response and confers cold response (Shi et al. 2018). Over 3,000 COR genes have been identified in Arabidopsis through sequencing approaches, but only a few CORs, such as dehydrins and Late Embryogenesis Abundant (LEA), have been identified and characterized. A vast majority of these functional genes, which play a role in cold acclimation, are still unavailable. Identification of these genes and understanding their role in crops under different genotypes and cold stress conditions will thus enhance our understanding of cold acclimation response in plant systems.

Escherichia coli Cold Shock Protein CspA (CSPs) accumulate as high as 10% of the entire cellular protein when a low-temperature shock is applied to the microbe. They harbor a single cold shock domain (CSD) and bind nucleic acids, both single- and double-stranded, and are characterized as functioning as RNA chaperones and transcription anti-terminators. These proteins bind RNA molecules and destabilize their secondary structures so as to aid ribosomal translation, rate of mRNA degradation and termination of transcription (Jiang et al. 1997; Bae et al. 2000).

A microarray study in *Arabidopsis* has identified several RNA-related proteins that are crucial for cellular homeostasis under cold stress (Hannah et al. 2005). A Wheat protein, CSP1, was found to be induced differentially during cold acclimation, which was the first plant CSP to be functionally characterized as having RNA chaperone activity similar to that of a prokaryotic one (Karlson et al., 2002). Later, *Arabidopsis* CSP1 was found to be an overexpressed protein that co-fractionated with cellular mRNAs when a temperature of 4°C was applied, indicating its role as an RNA chaperone. These mRNAs were shown to be stabilized under cold conditions and had evidence of improved translation, suggesting a role for CSPs in ensuring cellular homeostasis during low-temperature stress (Juntawong et al. 2013).

E. coli CspA genes have homologs in multiple eukaryotes, including plants and animals. Their amino acid sequence is 43% identical to the "cold-shock domain" of the eukaryotic Y-box protein family, which interacts with RNA and DNA to regulate their functions (Jiang et al. 1997). Monocot as well as dicot plant genomes harbor CSPs that have CSDs and additional domains like Glycine-rich repeats and Zinc Finger Domains accruing their nucleic acid binding property

(Karlson and Imai 2003). Comparative functional studies report the roles of these conserved small family genes in plant growth, development, and stress responses (Sasaki and Imai 2012).

Thus, cold shock proteins are an important class of genes that are engaged in maintaining cellular homeostasis during cold acclimation response. This gene family has rcently been identified to play role in *Prunus* sp. (Guo et al. 2024), cotton (Yang et al. 2024) and wheat (Wang et al. 2025) under low temperature stress but not been exlored in legumes. Therefore, this study is an attempt to identify them *in-silico* and estimate their expression in cold stressed pigeonpea genotypes. This would aid our understanding of a unifying cold shock response in plants. Later identification of allelic variation will help breeders develop molecular markers based on these genes, which will be of significant use in creating tolerant varieties.

Materials and methods

Plant material, growth and stress conditions

Two pigeonpea cultivars, showing contrasting responses to cold stress, i.e., Curly (cold susceptible) and UP17 (cold tolerant), identified by Kumar et al. (2019), were used to capture the differential expression pattern of genes in cold and control conditions using RNA-Seq. Pigeonpea (4-week-old seedlings) were raised in a controlled growth chamber in the National Phytotron facility at ICAR- Indian Agricultural Research Institute, New Delhi, with conditions of 16/8 hours photoperiod, 25ºC temperature and 60% RH. Cold stress treatment was provided in a separate chamber with a constant temperature of 10°C for 24 hours to the seedlings. Leaf samples were collected from both genotypes after the cold treatment and from control plants (untreated plants raised in the growth chamber). Flowering leaves and buds were collected from a field experiment in the month of January 2022 when the maximum temperature was below 10ºC. Three leaves and five buds from three different plants were pooled to create one biological replicate for both leaf and bud. Three such replicates were used for RNA isolation. All samples were immediately frozen in liquid nitrogen (-196°C) after collection and stored at -80°C in an ultralow freezer until further use.

Identification of CSPs in Cajanus cajan, its orthologs in other legumes and phylogenetic analysis

The *C. cajan* reference protein sequence was used as the query sequence against the local Pfam database using Hidden Markov Model in the HMMER tool in order to identify all pigeonpea proteins containing CSD domain. Protparam was used to predict the physicochemical properties of *Cajanus* CSPs (Gasteiger et al. 2005). Subcellular localization of these proteins was predicted by WoLF PSORT (Horton et al. 2007). The aforementioned procedure was used to mine the CSD proteins present in *Arabidopsis thaliana* and *Medicago truncatula*, utilizing their respective proteomes. The identified *C. cajan*, *A. thaliana*, *M. truncatula* proteins and *E.coli* CspA (NP_418012.1) protein retrieved from NCBI were aligned using ClustalW and a Neighbour-Joining (NJ) tree was constructed. The protein sequences harboring CSD in *Cicer arietinum*, *Glycine max, Vigna radiata, Phaseolus vulgaris, Arachis hypogea* and *M. truncatula* were retrieved from LIS Database using keyword search. OrthoFinder was used to find orthologous clusters

Gene structure and upstream cis-element analysis, Domain arrangement and Motif analysis

The exon-intron structure of CcCSPs was analyzed using the Gene Structure Display Server (GSDS) (version 2.0) (Hu et al. 2015). These upstream sequences were run through the PlantCARE web server (Lescot et al., 2002) to identify regulatory promoter elements. Proteins were verified to contain CSD by the simple modular architecture research tool (SMART), and the NCBI Batch CD-Search tool was used to identify the domain organization in the proteins as well. MEME was used to find the conserved domains present in these proteins with default parameters. Multiple sequence alignment was done to visualize conserved residues and motifs in protein sequences using ClustalOmega.

Gene expression analysis

Total RNA was isolated following the manufacturer's protocol from Sigma®Total Plant RNA kit. About 1000 ng of RNA from each sample was used in a reverse transcription reaction was carried out in a reaction volume of 20 µL using Thermo Scientific RevertAid First Strand cDNA synthesis kit as per the manufacturer's instructions using OligodT primers. Gene-specific primers were designed using the Primer3 Plus tool (https://www.primer3plus.com/). Real-time RT-PCR reaction was carried out using Kapa SYBR Fast qPCR reaction mix on a Biorad CFX96™ Real-Time PCR detection system. Relative expression values were calculated by $2^{-\Delta\Delta Ct}$ method using the average of two reference genes, 18S and Tubulin and normalized to control treatment for fold-changes. The log2 transformed FPKM value of these proteins was retrieved from the expression atlas developed by Pazhamala et al. (2017). The expression values of all the four proteins was obtained for all 21 tissues and heatmap was generated

Results and discussion

Identification, gene structure, chromosomal distribution and physico-chemical properties of Cajanus CSPs

Four proteins containing CSD were retrieved from *C. cajan* whole protein sequences, similar in number to those found in other crop species, *viz. Arabidopsis* (4) (Karlson and Imai

2003), rice (2) (Chaikam and Karlson 2008), *Glycine max* (7) (Sasaki et al. 2012). The genes were renamed *Cc*CSP1 to *Cc*CSP4 based on the ascending order of the chromosome number on which they are located. Details on their characteristic genomic features are enlisted in Table 1. The gene size of CSPs ranged from 1.1kb to 1.7 kb, which is similar to *Medicago* CSPs but is smaller in size to *Arabidopsis* CSPs. Most CSPs of *Cajanus*, *Arabidopsis* and both *Medicago* CSPs are intron-less therefore, the occurrence of intron seems to be rare in CSPs. *Arabidopsis* CSP1 and *Cc*CSP2 have introns in their gene structure (Fig. 1). These proteins have glycine-rich residue near C-terminus. Glycine-rich Proteins are a class of RNA-binding proteins validated to play a role in cold acclimation (Kim et al. 2007). *WCSP1,* the first plant RNA chaperone, also possess Glycine-rich regions along with the CSD, so are other plant CSPs (Sasaki and Imai 2012; Karlson and Imai 2003). Subcellular localization analysis identified all 4 to be nuclear-localized while CcCSP2 localizes additionally to cytosol. *WCSP1* is an RNA chaperone expressed exclusively in the crown tissue of cold-stressed wheat seedlings, which was found to express steadily after 10 h of cold stress. WCSP1 potentially binds ssDNA, RNA as well as dsDNA and plays a role in transcriptional or translational regulation of cold acclimation-associated genes (Karlson et al. 2002). *At*CSDP1 possesses DNA melting as well as RNAse activity and plays a key role in the cold-acclimation process in *Arabidopsis* (Kim et al. 2007). It has been reported that *AtCSDP2* also acts as an RNA chaperone in cold acclimation and might be involved in the vernalization response in *Arabidopsis* after exposure to low temperatures (Park et al. 2009).

Domain organisation and motif composition of Cajanus CSPs

CcCSPs encoded proteins of size 166-196 amino acids which are smaller than *Arabidopsis* CSPs that range in size from 201 to 301 amino acids. Each protein had one CSD and four additional auxiliary domains, *viz.* PTZ00368, AIR1, Znf and Zf-CCHC, towards C-terminus. Largest ones spanning the regions are PTZ00368 and AIR1. A 49 aa long motif conserved in all four genes at the N-terminus corresponds to CSD, which is an RNA-binding domain, with two RNA-binding motifs, *viz.* RNP1 and RNP2 located on β2 and β3 strands respectively (Fig. 2).

Similar domain organization of *Arabidopsis* and *Medicago* CSPs is also shown. as depicted in Fig. 3. Bacterial CSPs have 1 CSDs. In contrast, eukaryotic CSPs are multi-domain in nature with a CSD followed by variety of domains in varying numbers towards C-terminal such as basic aromatic islands, usually Glycine-rich repeats, RG repeats or retroviral-type CCHC zinc fingers (Nakaminami et al. 2006). CSPs in plants have variable numbers of CCHC-Zinc fingers ranging from 2-7 and this is a functional determinant (Karlson et al. 2002). Monocots, such as barley and wheat, also possess CSPs with a single CSD domain, suggesting that these proteins acquired additional domains during the course of evolution, leading to functional divergence.

PTZ00368 belongs to CCHC-zinc finger-type protein family that binds to ssDNA in lower organism Trypanosomes for kineoplast division (Tzfati et al. 1995). This indicates that *Cajanus* CSPs possess DNA binding domains. AIR1 proteins belong to the arginine methyltransferase-interacting protein family, which also contains RING Zn-finger domains. The presence of this domain suggests epigenetic regulation of the cold stress response in *Cajanus*. Presence of multiple domains

Glycine-rich domains possessing proteins (GRPs) have been reported to be widely distributed in plants playing important role in stress response mediated through protein-protein and other molecule interactions as well as signaling molecules (Sachetto-Martins et al. 2000). Several

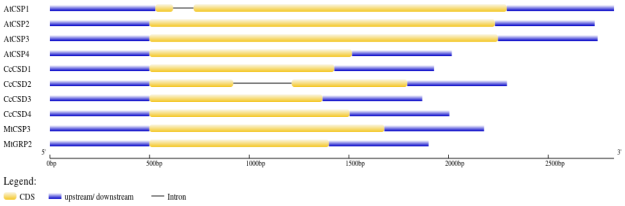
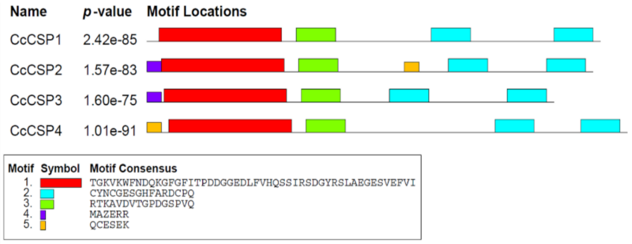


Fig. 1. *Exon-intron* gene structure of *Cajanus* CSPs. Blue bars indicate UTRs, Yellow are exons and black lines are intronic regions



Name	*p*-value
CcCSP1	2.42e-85
CcCSP2	1.57e-83
CcCSP3	1.60e-75
CcCSP4	1.01e-91

Motif	Symbol	Motif Consensus
1.	🟥	TGKVKWFNDQKGFGFITPDDGGEDLFVHQSSIRSDGYRSLAEGESVEFVI
2.	🟦	CYNCGESGHFARDCPQ
3.	🟩	RTKAVDVTGPDGSPVQ
4.	🟪	MAZERR
5.	🟧	QCESEK

Fig. 2. Discovered motifs in *Cajanus* CSPs by MEME suite

Table 1. Characteristics of *Cajanus* cold-shock proteins and their corresponding genes

Name	Protein NCBI ID	Locus ID	LIS ID	Pi/MW	Chr No.	Gene interval (bp)	Size (aa)
CcCSP1	XP_020215037.1	LOC109798986	C.cajan_21339	6.28/ 17727.71	Chr 4	4365357-4366284	185
CcCSP2	XP_020216872.1	LOC109800504	C.cajan_12733	7.55/ 18173.91	Chr 6	17887093-17888386	182
CcCSP3	XP_020228546.1	LOC109809607	C.cajan_01363	9.21/ 17060.53	Chr 11	14610874-14611742	166
CcCSP4	XP_020238008.1	LOC109817201	NA	6.29/ 18721.85	Sca000015	191526-192530	196

Fig. 3. Domain analysis of CSPs from *Arabidopsis, Medicago* and *C. cajan*

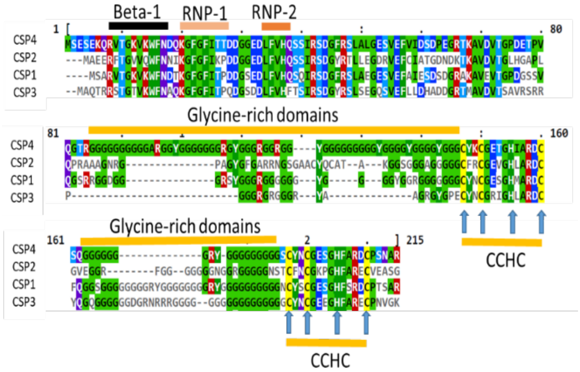


Fig. 4. Conserved Domains and Glycine-rich stretches in *Cajanus* CSPs

classes of GRPs are known to exist of which RNA-binding GRPs belonging to Class IV have been implicated their role in cold stress response in several studies Rice, Arabidopsis, *Nicotiana* and *Camellina* (Kim et al. 2007; Kim et al. 2005; Kim et al. 2010; Fusaro et al. 2007; Khan et al. 2013; Kwak et al. 2016). According to recent nomenclature, Plant CSPs belong to the Class IVc group of GRPs (Czolpinska and Rurek 2018).

Zinc fingers containing CSDs have RNA chaperonin function and have been reported to impart cold and freezing tolerance by mitigating mRNA export, like OsRZ2 in rice (Kim et al. 2010). Nuclear localization and the presence of C2H2-Zn finger domains in cold stress-induced proteins BrZAT12 suggest their additional role as Transcription Factors in cold response pathways (Ma et al. 2022).

The results above show that *Cajanus* CSPs possess RNA- as well as DNA-binding glycine-rich domains, known to be present in proteins involved in cold acclimation. This, along with the nuclear localization of CcCSP1-3, suggests that these might play a role as Transcription factors. The presence of an AIR1 domain suggests that they may be involved in interacting with epigenetic regulators to mediate the cold stress response. CCSP2's additional localization to the cytosol indicates its potential role as an RNA chaperone in *Cajanus* cold stress response. Thus, these can be potential transcriptional as well as post-transcriptional regulators in cold acclimation response in pigeonpea.

Multiple sequence alignment, phylogenetic analysis and orthologues groups with other legumes

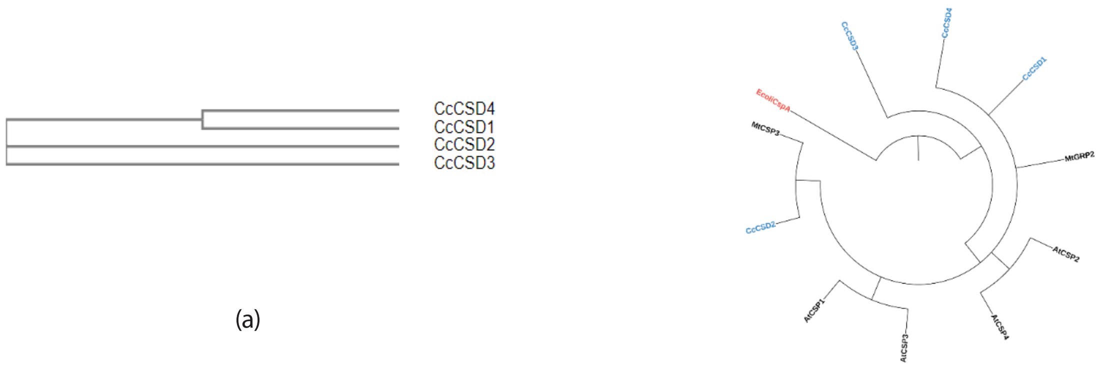
The divergence is due to the amino acid residues variation within the conserved RNP1 motif as depicted in Figure 4. CcCSP2 has a Lysine in place of Threonine, possibly converting it from an unpolar to a positively charged residue at the active site in the RNP1 motif. Sequence alignment showed that CcCSP homologies were in the range of 52 to 78%, with the maximum being CcCSP1 and CcCSP4, which showed a maximum identity of 78% (Fig. 5a). To determine the phylogenetic relationship with other plant model species, multiple sequence alignment of 4 *Arabidopsis* and 2 *Medicago* CSPs along with 4 *Cajanus* CSPs was done and then used to construct a phylogenetic tree along with *E. coli* CspA gene sequence (Fig. 5 b).

Based on distance, the tree can be categorized into three clades: I, II, and III (Fig. 5 b). Clade I have *the E. coli CspA* gene, which is quite obvious, as prokaryotic CSPs have only a single domain, as opposed to multiple functional domains in eukaryotic CSPs. *Cc*CSP1 and 4 belong to the same clade, while *Cc*CSP2 and *Cc*CSP3 belong to different clades, suggesting variation in their sequences leading to divergence. To gain insight into the homology within legumes, we carried out Orthologous clustering with other legumes. The CSPs from *Arachis, Glycine, Vigna* and *Phaseolus* were identified by homology searching with *Cajanus* CSPs. *Arachis* has the lowest number of CSP genes (2 in number), and *Glycine* has the maximum (7 in number), consistent with the results of Sasaki et al. (2012). This study indicates that cold acclimation proteins are also present in otherwise subtropical legumes, which have probably diversified their roles into multiple functions (Fusaro et al. 2007). Understanding their role in cold acclimation potential is an exciting area of research. Based on Orthology, *Cc*CSP genes fall into 4 separate orthologous clusters as depicted in Figure 6. *Cc*CSP4 belongs to cluster 4 and is orthologous to *Glycine* and *Phaseolus* CSP2 (Fig 6a).

Orthology analysis suggests the dynamic evolution of *Cc*CSPs in legumes that belong to different ortholog clusters. This indicates that all four CcCSPs paralogs have evolved independently, suggesting their specific roles and functions.

Promoter element analysis

To understand the transcriptional regulation of *Cc*CSPs, we carried out a scan of a 1.5 kb region upstream of the elements to search for regulatory regions for each gene. All CSP promoters have MYB/MYC, light, and phytohormone-



(a)

(b)

Fig. 5. (a) Phylogenetic tree showing homologies between CcCSPs and, (b) Phylogenetic relationship among *Arabidopsis, Medicago* and *Cajanus* CSP and *E.coli* CspA

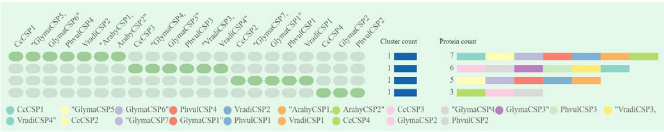


Fig. 6a. Orthologous groups identified by ClusterVenn2

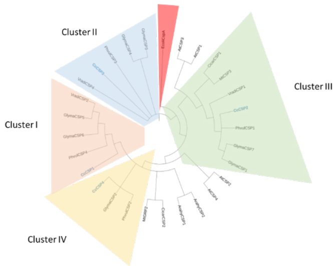


Fig. 6b. Orthologous clusters and their phylogenetic analysis

responsive elements, as well as EREs, in their upstream regions, as shown in Fig. 7b. Other phytohormone-responsive elements ABRE (for ABA), CGTCA, and TGACG-motif (MeJa-responsive) are present upstream to different CSPs, indicating the cross-signaling between cold stress and phytohormones (Fig. 7a). Largest number of putative elements in CcCSP2 and CcCSP3 indicates their complex regulation and involvement in different developmental pathways. Promoter analysis of cold-responsive genes in *Arabidopsis* revealed the presence of MYC/MYB elements in the upstream region and involved in cold-mediated signaling (Jiang et al. 2013). ICE1, a transcriptional activator active in early cold responses in plants, also binds to MYC elements of several genes, most notably CBF. They act in an ICE-CBF-COR regulon and activate the entire cascade, leading to cold acclimation (Chinnusamy et al. 2003). The presence of phytohormone-responsive elements, along with light-elements and wound-responsive elements, indicated the complex regulation of these genes and the functional divergence of this family in *C. cajan*. Fourteen tandem meristem activation elements in the promoter region of CcCSP3 suggested its role in meristematic tissues.

Gene expression analysis

Having known that *Cajanus* CSPs have domains that play a role in cold acclimation and have inducible cold-responsive promoter elements, a quantitative gene expression analysis was carried out for contrasting genotypes at the seedling and flowering stages by exposing them to cold stress.

Out of four genes, only three CcCSP1, 3 and 4 showed expression. A basal expression level of CcCSP1 and CcCSP2 in all tissues and both genotypes at all stress stages suggests that CSPs have pleiotropic roles, being involved in growth as well as developmental processes (Fusaro et al. 2007). Such expression is expected for genes having multiple pleiotropic roles has been reported earlier for this class of genes (Sasaki and Imai 2012).

The tolerant genotype exhibits higher expression levels of CcCSP1, 3, and 4 during 6 hours of cold stress than the control, which later declines after 24 hours, similar to what is found in rice. Inducible CSPs expression immediately after cold shock is reported in *Arabidopsis AtCSP3,* and Wheat *WCSP1* and is responsible for inducing freezing tolerance in both (Kim et al. 2009; Karlson et al. 2002). Root and shoot tissues of rice seedlings exposed to cold temperatures showed a transient increase, up to 45 minutes, after which they slowly decreased to control levels over the course of 24 hours (Chaikham et al. 2008). Thus, a time-course study of expression is imperative to understand their regulation

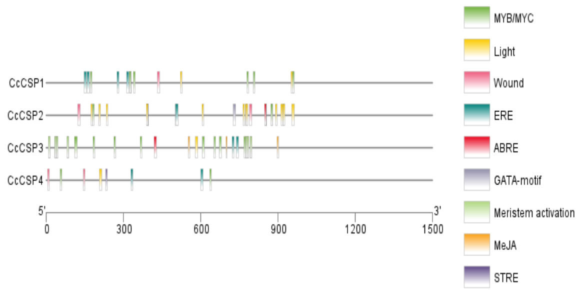


Fig. 7a. Distribution of promoter elements in *Cajanus* CSPs

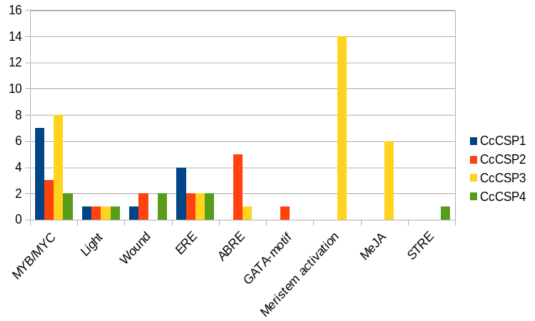
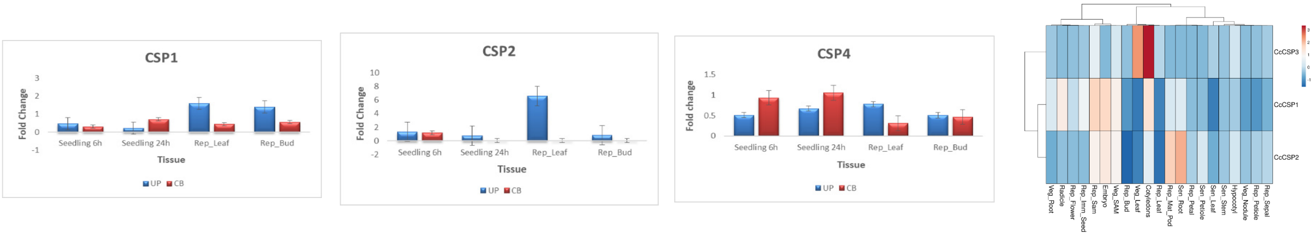


Fig. 7b. Enrichment of promoter elements in *Cajanus* CSPs



(a) (b)

Fig. 8. (a) qPCR analysis of CSP1, CSP2 and CSP4 in two growth stages (Seedling and Reproductive) of *Cajanus* under low temperatures and (b) Digital gene expression profile derived from *Cajanus* expression atlas data

in pigeonpea.

All CcCSPs showed higher expression during the reproductive stage than during their seedling stage, which is similar to results in studies on Wheat crown tissues that showed increased accumulation of transcripts up to 18 days (Karlson et al. 2002). However, a study to characterize the expression levels in control condition-grown plants over time since flowering would lead us to understand whether the expression level is transient or accumulates over time.

A high, almost 6-fold change with respect to the sensitive genotype was captured for the CcCSP2 gene in the reproductive leaf of the tolerant genotype, and a 2-fold change was observed in the reproductive stage bud. This suggests that these genes are indeed cold-inducible and may play a role in the stress response of the tolerant genotype. A controlled study of the reproductive stage of these plants is required to confirm the results further.

The heatmap generated from Gene Expression Atlas data serves as a proxy for tissue-specific expression derived from the curation of previous studies. Higher expression of CcCSP2 in reproductive leaf and bud in the heatmap is in congruence with the expression study.

It thus concludes that cold shock proteins in *Cajanus* exhibit differential expression upon cold stress exposure. However, a more detailed study, comprising diverse genotypes, controlled conditions, and a longer duration of exposure, will establish the expression profile of CcCSP genes. This will improve our understanding of the functional role of these cold-responsive genes in the acclimation potential of pigeonpea.

Although reports of Cold Shock Proteins playing a role in cold acclimation exist for crops like Wheat, Rice, Brassica, *Nicotiana* and model plant species like *Arabidopsis*, the same is missing in subtropical crops like pigeonpea. Plant acclimation or cold stress response is a manifestation of function of multiple genes and pathways, a major one being ICE-CBF-COR. Many COR genes are widely known to play a role in desiccation tolerance and cold acclimation; however, a vast majority of them are not well-known. Identification of such unexplored proteins and dissecting their specific role is needed. This is an attempt to identify such cold-acclimation responsive proteins in an otherwise subtropical crop. The results of the present study suggest that pigeonpea, being a subtropical crop, possesses cold acclimation proteins (CSPs) that are expressed differentially in various tissues and in response to stress. This, however, will be premature to conclude for their definitive role in cold stress response, but it opens up the possibility of carrying out an in-depth understanding of cold acclimation potential in these legume crops. Further investigation of cognate targets of CcCSP2 would provide insight into the cold-stress response and associated pathways involved in cold acclimation in pigeonpea. Also the genic information derived from this study can be utilized in breeding programs to develop cold-tolerant crops.

Authors' contribution

Conceptualization of research and designing of the experiments (SS, KG); Contribution of experimental materials (KD); Execution of field/lab experiments and data collection (SS, KK, PG, HS, RJ); Analysis of data and interpretation (SS, KK, PG, HS, RJ, KT); Preparation of the manuscript (SS,KT, SaS, KG).

References

Bae W., Xia B., Inouye M. and Severinov K. 2000. Escherichia coli CspA-family RNA chaperones are transcription antiterminators. Proc. National Acad. Sci., **97**(14): 7784-7789.

Chaikam V. and Karlson D. 2008. Functional characterization of two cold shock domain proteins from *Oryza sativa*. Plant Cell Env., **31**: 995-1006.

Chinnusamy V., Ohta M., Kanrar S., Lee B. H., Hong X., Agarwal M., and Zhu J. K. 2003. ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. Genes Dev., **17**:1043-1054. doi.org/101101/gad1077503.

Chinnusamy V., Zhu J. and Zhu J. K. 2007. Cold stress regulation of gene expression in plants. Trends Plant Sci., **12**(10): 444-51. doi: 10.1016/j.tplants.

Czolpinska, M. and Rurek, M., 2018. Plant glycine-rich proteins in stress response: an emerging, still prospective story. Front. Plant Sci., **9:** 302.

Ding Y., Shi Y. and Yang S. 2019. Advances and challenges in uncovering cold tolerance regulatory mechanisms in plants. New Phytologist, **222**: 1690-1704. doi.org/101111/nph15696.

Fusaro A. F., Bocca S. N., Ramos R. L. B., Barrôco R. M., Magioli C., Jorge V. C. and Sachetto-Martins G. 2007. AtGRP2 a cold-induced nucleo-cytoplasmic RNA-binding protein has a role in flower and seed development. Planta J., **225**: 1339-1351.

Guo P., Liu A., Qi Y., Wang X., Fan X., Guo X., Yu C. and Tian C. 2024. Genome-wide identification of cold shock proteins (CSPs) in sweet cherry (*Prunus avinus* L.) and exploring the differential responses PavCSP1 and PavCSP3 to low temperature and salt stress. Genes Genomics, **46**(9): 1023-1036.

Guo X., Liu D. and Chong K. 2018. Cold signaling in plants: Insights into mechanisms and regulation. J. Integ. Plant Bio., **60**: 745-756.

Guy CL. 1990. Cold acclimation and freezing stress tolerance: role of protein metabolism. Annual Rev. Plant Physiol. Plant Mol. Bio., **41**: 187–223.

Hannah M. A., Heyer A. G. and Hincha D. K. 2005. A global survey of gene regulation during cold acclimation in *Arabidopsis thaliana.* PLoS Genet., **1**: e26.

Horton P., Park K. J., Obayashi T., Fujita N., Harada H., Adams-Collier C. J. and Nakai K. 2007. WoLF PSORT: protein localization predictor. Nucleic Acids Res., **35**(Suppl_2): W585-W587.

Hu B., Jin J., Guo A. Y., Zhang H., Luo J., and Gao G. 2015. GSDS 20: an upgraded gene feature visualization server. Bioinformatics, **31**: 1296-1297.

Jan N., Rather A. M. U. D., John R., Chaturvedi P., Ghatak A., Weckwerth W. and Mir R. R. 2023. Proteomics for abiotic stresses in legumes: present status and future directions. Critical Reviews Biotechnol., 43**(2)**: 171-190

Jiang W., Hou Y. and Inouye M. 1997. CspA, the major cold-shock protein of Escherichia coli, is an RNA chaperone. J. Biol. Chem., 272**(1)**: 196-202.

Jiang Y., Peng D., Bai L. P., Ma H., Chen L. J., Zhao M. H. and Guo Z. F. 2013. Molecular switch for cold acclimation—anatomy of the cold-inducible promoter in plants. Biochemistry (Moscow), **78**: 342-354. doi.org/ 101134/S0006297913040032.

Juntawong P., Sorenson R. and Bailey-Serresz J., 2013. Cold shock protein 1 chaperones mRNA s during translation in A rabidopsis thaliana. The Plant J., 74**(6):**1016-1028.

Karlson D. and Imai R. 2003. Conservation of the cold shock domain protein family in plants. Plant Physiol., **131**: 12-15. doi.org/101104/pp014472.

Karlson D., Nakaminami K., Toyomasu T. and Imai R. 2002. A cold-regulated nucleic acid-binding protein of winter wheat shares a domain with bacterial cold shock proteins. J. Biol. Chem., **277**: 35248-35256.

Khan F., Sultana T., Deeba F. and Naqvi S. M. 2013. Dynamics of mRNA of glycine-rich RNA-binding protein during wounding, cold and salt stresses in *Nicotiana tabacum*. Pak. J. Bot. **45:** 297–300.

Kim Y. O., Kim J. S. and Kang H. 2005. Cold-inducible zinc finger-containing glycine-rich RNA-binding protein contributes to the enhancement of freezing tolerance in *Arabidopsis thaliana.* Plant J., **42**: 890–900. doi: 10.1111/j.1365-313X.2005.02420.x

Kim J. S., Park S. J., Kwak K. J., Kim Y. O., Kim J. Y., Song J. and Kang H. 2007. Cold shock domain proteins and glycine-rich RNA-binding proteins from *Arabidopsis thaliana* can promote the cold adaptation process in *Escherichia coli.* Nucleic Acids Res., **35**: 506-516.

Kim M.H., Sasaki K. and Imai R. 2009. Cold shock domain protein 3 regulates freezing tolerance in *Arabidopsis thaliana*. J. Biol. Chem., **284**(35): 23454-23460.

Kim J. Y., Kim W. Y., Kwak K. J., Oh S. H., Han Y. S. and Kang H. 2010. Zinc finger-containing glycine-rich RNA-binding protein in *Oryza sativa* has an RNA chaperone activity under cold stress conditions. Plant Cell Env., **33**: 759-768.

Kumar D., Joshi R., Kumar K., Gaikwad K., Raje R. S., and Prashat G. R. 2019. Inheritance pattern of cold tolerance in pigeonpea [*Cajanus cajan* (L) Millsp]. Indian J. Genet. Plant Breed., **79**: 404-410.

Kumar S., Nayyar H., Bhanwara R. K. and Upadhyaya H. D. 2010. Chilling stress effects on reproductive biology of chickpea. J. SAT Agric. Res., **8**: 1-14.

Kwak, K. J., Kim, H.-S., Jang, H. Y., Kang, H., and Ahn, S.-J. 2016. Diverse roles of glycine-rich RNA-binding protein 7 in the response of camelina (Camelina sativa) to abiotic stress. Acta Physiol. Plant, **38**:129. doi: 10.1007/s11738-016-2144-4

Lescot M., Déhais P. and Thijs G. 2002. PlantCARE a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res., **30**: 325-327.

Liu J., Shi Y. and Yang S. 2018. Insights into the regulation of CBF cold signaling in plants. J. Integ. Plant Biol., **9**: 780– 795.

Nakaminami K., Karlson D. T. and Imai R. 2006. Functional conservation of cold shock domains in bacteria and higher plants. Proc. National Acad. Sci., **103**: 10122-10127.

Park S. J., Kwak K. J., Oh T. R., Kim Y. O. and Kang H. 2009. Cold shock domain proteins affect seed germination and growth of *Arabidopsis thaliana* under abiotic stress conditions. Plant Cell Physiol., **50**: 869-878.

Pazhamala L. T., Purohit S. and Saxena R. K. 2017. Gene expression atlas of pigeonpea and its application to gain insights into genes associated with pollen fertility implicated in seed formation. J. Exp. Bot., **68**: 2037-2054.

Phadtare S., Alsina J. and Inouye M. 1999. Cold-shock response and cold-shock proteins. Current Opinion Microbiol., **2**:175-180. doi.org/101016/S1369-5274(99)80031-9.

Repo T., Mononen K., Alvila L., Pakkanen T. T. and Hänninen H. 2008. Cold acclimation of pedunculate oak (*Quercus robur* L) at its northernmost distribution range. Env. Exp. Bot., **63**: 59-70

Robison J., Arora N., Yamasaki Y., Saito M., Boone J., Blacklock B. and Randall S. 2017. Glycine max and Glycine soja are capable of cold acclimation. J. Agron. Crop Sci., 203**(6):** 553-561.

Sachetto-Martins G., Franco L.O. and de Oliveira D.E., 2000. Plant glycine-rich proteins: a family or just proteins with a common motif?. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression, 1492**(1)**: 1-14.

Sasaki K. and Imai R. 2012. Pleiotropic roles of cold shock domain proteins in plants. Front. Plant Sci., **2**: 116. doi.org/103389/fpls201100116.

Shi Y., Ding Y. and Yang S. 2018. Molecular Regulation of CBF Signaling in Cold Acclimation. Trends Plant Sci., **23**: 623-637. doi.org/101016/jtplants201804002.

Singh B. B., Singh D. P. and Singh N. P. 1997. Genetic variability in pigeonpea germplasm for cold tolerance. Indian J. Genet. Plant Breed., **57**(4): 425-430.

Singh M. N. and Singh R. S. 2010. Inheritance of pod setting under low temperature in pigeonpea. Indian J. Genet. Plant Breed., **70**(3): 277-280.

Singh N. K., Gupta D. K., Jayaswal P. K., Mahato A. K., Dutta S., Singh S. and Sharma T. R. 2012. The first draft of the pigeonpea genome sequence. J. Plant Biochem. Biotechnol., **21**: 98-112.

Thomas S., Krishna. G. K., Yadav P. and Pal M. 2019. Cloning and abiotic stress responsive expression analysis of arginine decarboxylase genes in contrasting rice genotypes. Indian J. Genet. Plant Breed., **79(**02): 411-419.

Thomashow MF. 1999. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annual Rev. Plant Physiol. Plant Mol. Biol., **50**: 571–599.

Tzfati Y, Abeliovich H, Avrahami D, Shlomai J. 1995. Universal minicircle sequence binding protein, a CCHC-type zinc finger protein that binds the universal minicircle sequence of trypanosomatids. Purification and characterization. J Biol. Chem., **270**(36): 21339-45.

Wang K., Guo G., Bai S., Ma J., Zhang Z., Xing Z., Wang W., Li H., Ling H., Li Z. and Si X. 2025. Horizontally acquired CSP genes contribute to wheat adaptation and improvement. Nat. Plants, **11**: 761-774.

Yamasaki Y. and Randall S. K. 2016. Functionality of soybean CBF/DREB1 transcription factors. Plant Sci., **246**: 80-90.

Yang W., Yu M., Zou C., Lu C., Yu D., Cheng H. and Zhou Z. 2019. Genome-wide comparative analysis of RNA-binding Glycine-rich protein family genes between *Gossypium arboreum* and *Gossypium raimondii*. PLoS One, **14**: e0218938.

Yang Y., Zhou Y., Xu J., Wang Y., Pu Y., Qu Y. and Sun G. 2024. Genome-wide identification and expression analysis unveil the involvement of Cold Shock Protein (CSP) gene family in cotton hyporthermia stress. Plants, **13**(5): 643.