



Braving the attitude of altitude: *Caragana jubata* at work in cold desert of Himalaya

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MOLECULAR ECOLOGY
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The present work was conducted to understand the basis of adaptation in *Caragana jubata* in its niche environment at high altitude cold desert of Himalaya. Molecular data showed predominance of genes encoding chaperones and those involved in growth and development at low temperature (LT), a major cue operative at high altitude. Importantly, these genes expressed in *C. jubata* in its natural habitat. Their homologues in *Arabidopsis thaliana*, *Oryza sativa*, and *Glycine max* did not exhibit similar trend of gene expression at LT. Constitutive expression and a quick up-regulation of the above genes suggested the ability of *C. jubata* to adjust its cellular machinery to maintain growth and development in its niche. This was reflected in LT₅₀ (the temperature at which 50% injury occurred) and LT mediated photosynthetic acclimatory response. Such molecular and physiological plasticity enables *C. jubata* to thrive in the high altitude cold desert of Himalayas.

High altitude ecosystem has traditionally been characterized as ‘harsh’¹. Apart from the declining surface area², both, species richness³ and diversity⁴ are known to decline at high altitude. This highlights the adaptive capability of only fewer species to perform against the increasing magnitude of environmental extremes, wherein low temperature (LT) is considered to be one of the critical cues. LT induces many anatomical and physiological alterations including reduced rates of biochemical reactions, shift in temperature optima of several physiological processes⁵, altered membrane fluidity and protein conformation⁶. LT is well known to suppress photosynthesis during winter in most woody plants, but many herbaceous plants are able to acclimate to avoid down-regulation of photosynthesis⁷. Several plants are able to modulate their metabolites levels which are believed to have varied roles⁸. Accumulation of cryoprotectant molecules such as soluble sugars, sugar alcohols, and low molecular weight nitrogenous compounds has been reported in response to LT⁹. A large number of molecular and metabolic modulations, including production of secondary messengers, reactive oxygen species (ROS), calcium-dependent protein kinases (CDPKs), and mitogen activated protein kinase (MAPK) cascade have been reported after LT exposure¹⁰. LT also modulates a large number of genes encoding specific proteins, which can be divided into two broad groups. The first group includes genes that encode proteins, such as late embryogenesis abundance (LEA) proteins, osmotin, antifreeze proteins, mRNA binding proteins, enzymes of osmolyte biosynthesis, water channel proteins, and detoxification enzymes, which have a role in protecting cells from LT damage. The second group consists of genes encoding transcription factors that regulate transcriptional activity under conditions of stress¹¹. Although, these studies provide insight into the plant response to LT, their roles in imparting functional advantage under natural environment remains less understood.

Preponderance of a plant species in an ecological niche is a reflection of its adaptive suitability at varied level of plant organization, primarily controlled by gene expression. Hence, gene expression analysis is likely to provide important clues of adaptive advantage of a species over others. For example, down-regulation of genes involved in jasmonate cascade modulated herbivore attack, thereby modulating plant fitness in a given environment¹². Understanding genomic response of an organism to environment can provide a valuable insight to extrapolate the population and community response as well, provided the experiments are conducted within the ecological framework¹³.

The present work was carried out in a high altitude cold desert plant *Caragana jubata* (Pall.) Poir, to understand LT responsive molecular mechanism and its adaptive capacity and fitness in its niche environment on the pretext of cross-talk of commonality of gene response to varied environmental cues¹⁴. *C. jubata* is a perennial



shrub of family Fabaceae distributed widely in temperate/alpine environment of Eurasia¹⁵ as well as Himalaya¹⁶. The habitat of *C. jubata* in Himalaya is snow covered during winter (October to March/April), and the species has a period of only 5–6 months for active growth and development. Even during this later period, *C. jubata* experiences extreme diurnal variations in temperature ranging from about 5 to 25°C during the daytime and about 2 to (–) 10°C during night.

Results

Cloning, sequencing and annotation of suppression subtractive hybridization cDNA library (SSH). To understand the plant response to LT, SSHL was prepared using the RNA isolated from the leaf tissue of *C. jubata* exposed to 25°C (control, CO) and 4°C (LT). A total of 1116 expressed sequence tags (ESTs) were generated from forward (CjSF, 556 ESTs) and reverse (CjSR, 560 ESTs) SSHL. ESTs in CjSF represent the genes up-regulated in response to LT, whereas those in CjSR show the genes down-regulated in response to LT. Depending upon the quality of sequences in CjSF, 502 ESTs were analyzed for annotation and contigs assembly (Fig. 1a and Supplementary Table S1). Gene function analysis grouped ESTs into 256 singletons and 36 contigs (Fig. 1a and Supplementary Table S1), constituting a total of 292 unigenes. Out of these, 98 were hypothetical/unknown proteins (i.e. cDNA sequences without an analog in protein database), whereas 70 represented novel proteins i.e. with no significant homology in database (Supplementary Fig. S1a and Supplementary Table S2). ESTs were classified into 11 broad categories consisting of transcription factors, defense, signal transduction, oxidative stress, photosynthesis/energy metabolism, metabolism, protein synthesis/turnover, chaperones, membrane and cellular transport, hypothetical/unknown, and novel genes (Fig. 1b and Supplementary Table S2). ESTs for chaperones (e.g. LEA proteins) were the most abundant followed by those encoding early light inducible protein (ELIP), abscisic acid (ABA) inducible protein, seed maturation protein PM32 (PM32), ribulose-1,5-bisphosphate carboxylase (Rubisco), hypothetical chloroplast release factor 1, LEA-2, LEA-1 and RNA polymerase sigma subunit E (Fig. 1b and Supplementary Table S2).

In CjSR, 515 ESTs were selected for annotation and contigs assembly (Fig. 1a and Supplementary Table S1). These ESTs were clustered into 297 singletons and 34 contigs, constituting a total of 331 unigenes. All the ESTs were assigned putative function and categorized into functional categories consisting of transcription factors, signal transduction, oxidative stress, photosynthesis/energy metabolism, metabolism, protein synthesis/turnover, cytoskeleton, membrane and cellular transport, defense, chaperones, retroelements, unclassified (i.e. ESTs with homology in the database but could not be assigned any function), hypothetical/unknown, and novel genes (Supplementary Fig. S1b). ESTs for putative pathogenesis related protein 1 precursor were most abundant in CjSR (Fig. 1b and Supplementary Table S3). This was followed by ESTs encoding glycine-rich RNA-binding protein 2, retrotransposon protein, chlorophyll a/b binding protein, RNA polymerase beta' subunit, chlorophyll a/b binding protein of LHCII type III, chloride channel calcium activated 4, retroelements pol polyprotein, unclassified and putative ammonium transporters, respectively.

One of the interesting features of the SSHL analysis was the abundance of genes encoding chaperones [*CjLEA-1* (group 1), *CjLEA-2* (group 2), *CjLEA-14* (group 2), *CjDHN* (group 2), *CjLEA-Dc3* (group 3), *CjLEA group 3* (group 3), *LEA-cold inducible* (*CjLEA-CI*) (group 4), *CjLEA* (group 4), *CjLEA (Putative)* (group 5), *CjPM32* (group 6), *CjLEA-18* (group 6) and *CjHSP26A*; LEA proteins were classified into various groups depending upon the presence of specific sequence motifs or biased amino acid composition^{17,18} and those associated with growth and development [*early light inducible protein* (*CjELIP*), *CjABA inducible*, *CjCDPK*, *indole acetic acid inducible*

protein (*CjIAA inducible*), *auxin responsive factor 7* (*CjARF7*), *MYB transcription factor 133* (*CjMYB133*), *rare cold inducible 2A* (*CjRCI2A*), *cold acclimation responsive2* (*CjCAR2*), *cold acclimation specific* (*CjCAS*), *mammalian cell entry family protein* (*CjMce family*)^{19,20}] in CjSF (Supplementary Table S2). Since these genes could impart adaptive advantage, a detailed kinetic study was carried out at 13 time points spanning between 0–48 h, to identify the early and late responsive genes in response to LT (Fig. 2a and Supplementary Fig. S2a). Further, to understand the adaptive significance of these genes, their expression was also followed in two populations of *C. jubata* collected from the niche environment for two separate years (Fig. 2b, Supplementary Fig. S2b and Supplementary Fig. S3). A comparative expression analysis of gene homologues in *Arabidopsis thaliana*; a cold-tolerant plant species), rice²² (*Oryza sativa*; a cold-sensitive plant species) and soybean²³ (*Glycine max*; family Fabaceae), using publicly available microarray data, was also performed *in silico* (Supplementary Fig. S4, Supplementary Fig. S5 and Supplementary Table S6).

Genes encoding six groups of LEA proteins showed up-regulation in response to LT and in the niche environment. CjSF yielded 11 LEAs belonging to six groups. Differential expression of genes encoding different LEA proteins was confirmed by detailed kinetics of expression analysis at different time points (Fig. 2a and Supplementary Fig. S2a). Data showed that *CjLEA-Dc3* was constitutively expressed (Fig. 2a) whereas, *CjLEA*, *CjLEA-1*, *CjLEA-2*, *CjLEA (putative)* and *CjPM32*, exhibited a strong up-regulation at 6 h of exposure to LT (Fig. 2a). *CjLEA-18*, *CjLEA group 3*, *CjLEA-14*, *CjLEA-CI* and *CjDHN* exhibited constitutive expression in CO and were slightly down-regulated during early period of LT (upto 3 h) but up-regulation was recorded at 6 h of exposure to LT and maintained thereafter during the entire period of experiment (Fig. 2a). All the *CjLEAs* exhibited expression in the two populations of *C. jubata* at high altitude during both the years of analyses (Fig. 2b, Supplementary Fig. S2b and Supplementary Fig. S3).

It was important to note that in *Arabidopsis*, only three LEAs (homologues to *CjLEA*, *CjLEA-14*, *CjDHN*) exhibited up-regulation in response to LT (Supplementary Fig. S4 and Supplementary Table S4). In rice, expression of only one LEA (Os03g20680, a *CjLEA* homologue) was up-regulated under LT, while up-regulation of rest of the LEAs was not observed (Supplementary Fig. S5 and Supplementary Table S5). In soybean, only three LEAs (homologues to *CjLEA-18*, *CjLEA-14* and *CjLEA-CI*) exhibited up-regulation in response to LT (Supplementary Table S6).

CjHSP26A showed up- and down-regulation of expression during early period (upto 3 h) of exposure to LT. An evident up-regulation was observed at 6 h exposure to LT and maintained thereafter during the whole period of experiment (Fig. 2a and Supplementary Fig. S2a). Also, the gene was found to be up-regulated in populations of *C. jubata* growing in the high altitude (Fig. 2b, Supplementary Fig. S2b and Supplementary Fig. S3). In *Arabidopsis*, its homologue was induced within 30 min following the exposure to LT (Supplementary Fig. S4 and Supplementary Table S4). Expression analysis in soybean revealed up-regulation of *CjHSP26A* homologue under LT (Supplementary Table S6).

Genes involved in growth and development exhibited up-regulation in response to LT and in the niche environment. Gene encoding ELIP was present in abundance (5.2%) in CjSF during LT (Supplementary Fig. S1a). *CjELIP* exhibited both up- and down-regulation of expression pattern during early period (upto 3 h) followed by up-regulation at 6 h of exposure to LT and maintained thereafter during the entire period of experiment (Fig. 2a and Supplementary Fig. S2a). Up-regulation of *CjELIP* was also observed in populations growing at high altitude (Fig. 2b Supplementary Fig. S2b and Supplementary Fig. S3). *ELIP* homologue in *Arabidopsis* exhibited up-regulation at 3 h of cold treatment (Supplementary Fig. S4 and Supplementary

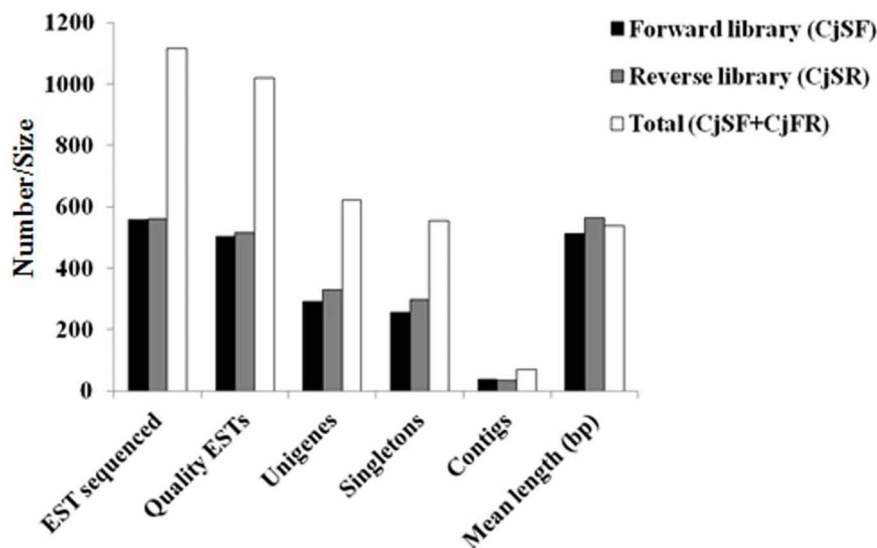
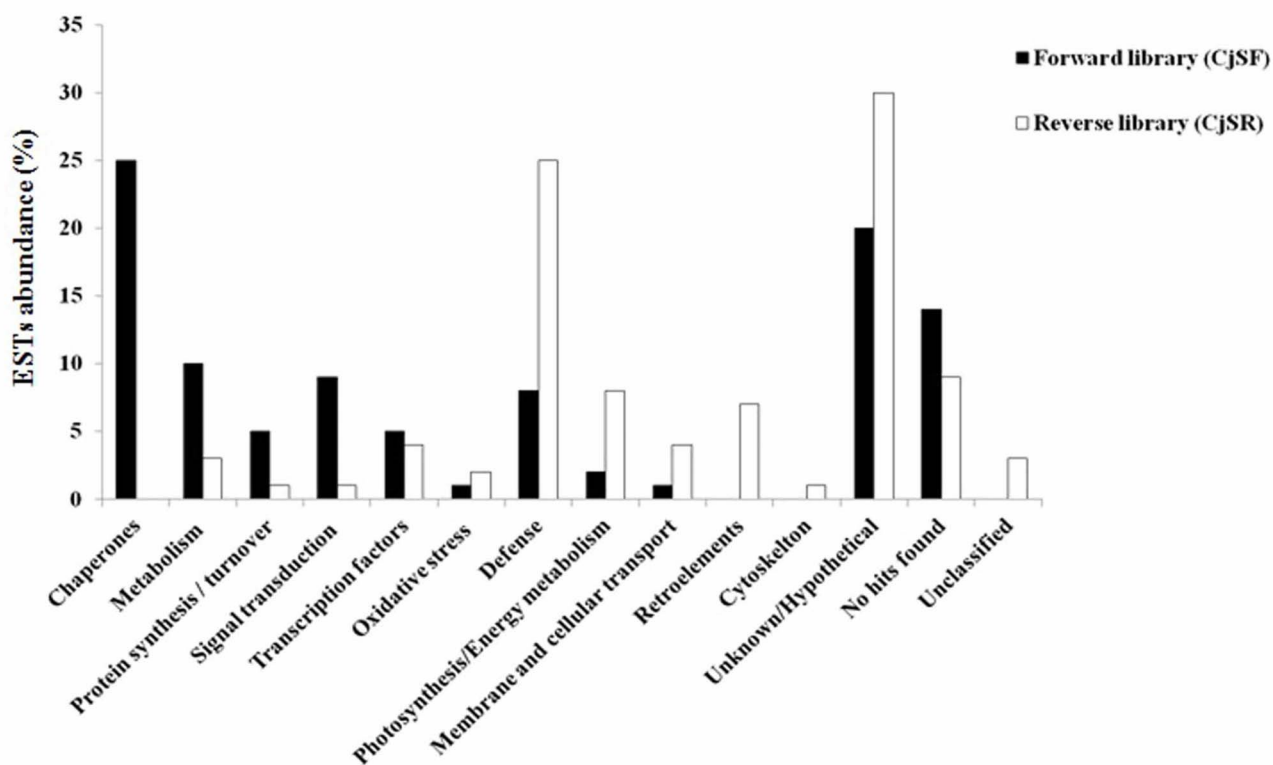
**a****b**

Figure 1 | Functional analyses of expressed sequence tags (ESTs) obtained from suppression subtractive hybridization library (SSH) prepared from leaf tissue of *Caragana jubata* exposed to 25°C (control, CO) and 4°C (low temperature, LT). Panel 'a' shows EST details of CO and LT SSHL. Major abundant transcripts in CO and LT SSHL are shown in panel 'b'. Transcripts were functionally classified according to Munich Information Center for Protein Sequences (MIPS, <http://www.helmholtz-muenchen.de/en/ibis>). Details on abundance of all the ESTs are shown in Supplementary Table S2 and Supplementary Table S3.

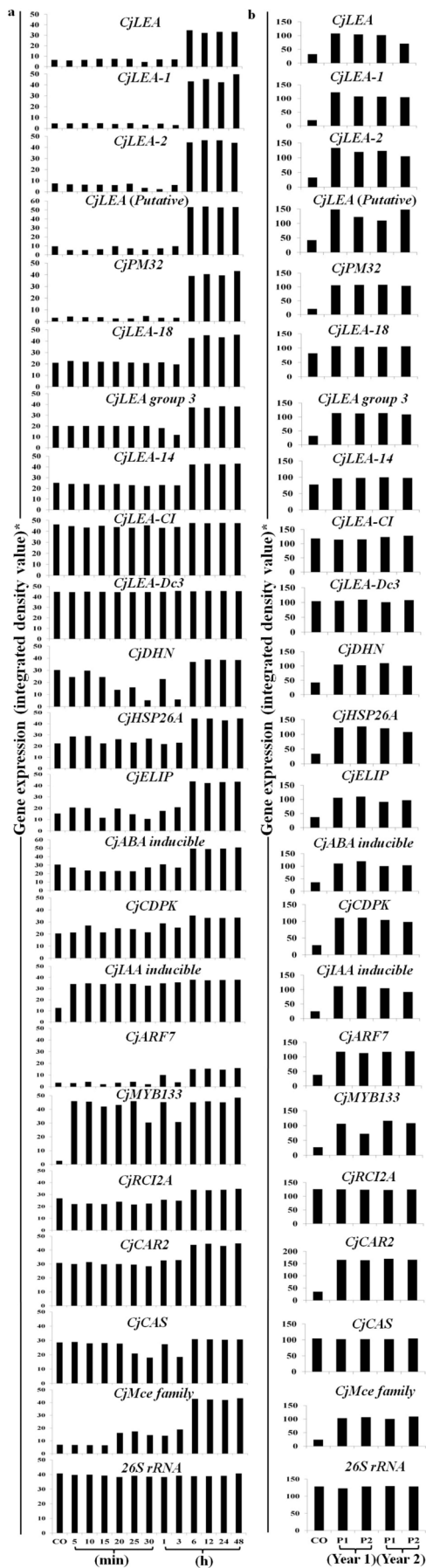


Figure 2 | Kinetics of gene expression in response to low temperature as a function of time, as well as at low and high altitude in the leaf tissue of *Caragana jubata*. For panel 'a', plants were shifted from $25 \pm 3^\circ\text{C}$ (control, CO) to $4 \pm 3^\circ\text{C}$ (low temperature, LT) and the samples were harvested at different time intervals as shown in the figure. Gene expression was studied by reverse transcriptase-polymerase chain reaction (RT-PCR) that was standardized to get amplification in the exponential phase. RT-PCR was also performed for the plants housed at 25°C at the same time intervals as performed for those kept at LT (data not shown since there was no change in gene expression over a span of 48 h). Name of the gene is shown in each panel and the corresponding accession number is shown in Supplementary Table S2. Details of primers and PCR conditions are mentioned in Supplementary Table S7. For panel 'b', leaves of two populations (P1 and P2) of *C. jubata* growing in niche environment of high altitude in Kibber-Gete area (Lahaul and Spiti district, Himachal Pradesh, India) were collected during September/October in two separate years (year 1 and year 2). Samples were also collected for the plants growing at $25 \pm 3^\circ\text{C}$ (CO) at low altitude in the institute (altitude, 1300 m; Palampur, Himachal Pradesh, India). Leaf tissues were harvested, stored immediately in liquid nitrogen on site and stored at (-80°C) till further use. *26S rRNA* served as internal control for both panel 'a' and 'b'. Gel figures for RT-PCR are shown in Supplementary Fig. S2a,b. *To calculate integrated density value (IDV), PCR products were separated on 1.2% agarose gel, stained with ethidium bromide and quantified using Alpha DigiDoc 1000 software supplied along with gel documentation system (Alpha DigiDocTM, Alpha Innotech, USA). IDV thus obtained was divided by 1,000 for ease of writing on the y axis; thus, the IDV needs to be multiplied by 1,000 to get the original value of gene expression.

Table S4), while there was no change in the expression of rice *ELIP* homologue (Supplementary Fig. S5 and Supplementary Table S5). In soybean, *ELIP* homologue exhibited down-regulation during LT (Supplementary Table S6).

CjCDPK also exhibited a pattern of up- and down-regulation of expression during early period (upto 3 h) of exposure to LT. Up-regulation was observed at 6 h and onwards of exposure to LT (Fig. 2a and Supplementary Fig. S2a). Up-regulation of *CjCDPK* was also observed in populations growing in high altitude (Fig. 2b, Supplementary Fig. S2b and Supplementary Fig. S3). Expression of *CDPK* homologue was unaltered in rice (Supplementary Fig. S5 and Supplementary Table S5) while in soybean, its homologue exhibited up-regulation at LT (Supplementary Table S6).

Gene encoding abscisic acid (ABA) inducible proteins was present to an extent of 4.9% in *CjSF* (Supplementary Fig. S1a). Expression analysis showed that *CjABA inducible* gene exhibited constitutive expression during early period of exposure to LT (upto 3 h) and was up-regulated at 6 h and onwards of exposure (Fig. 2a and Supplementary Fig. S2a). The gene also expressed under natural environment at high altitude (Fig. 2b, Supplementary Fig. S2b and Supplementary Fig. S3). Importantly, its homologue in Arabidopsis was down-regulated in response to LT (Supplementary Fig. S4 and Supplementary Table S4).

Expression of *CjIAA inducible* gene was not visible in CO, but at 5 min of exposure to LT, gene expression was noticeable. An evident up-regulation was observed at 6 h of exposure to LT and maintained thereafter (Fig. 2a and Supplementary Fig. S2a). Up-regulation of *CjIAA inducible* gene was also observed in populations growing in niche environment of high altitude (Fig. 2b, Supplementary Fig. S2b and Supplementary Fig. S3). Expression of its homologues in Arabidopsis and rice was unaltered (Supplementary Fig. S4 and S5 and Supplementary Table S4 and S5). In soybean, its homologue exhibited up-regulation during LT (Supplementary Table S6).

Auxin responsive factor 7 (CjARF7) was up-regulated at 6 h of exposure to LT in *C. jubata* (Fig. 2a and Supplementary Fig. S2a) and was expressing under natural conditions at high altitude (Fig. 2b, Supplementary Fig. S2b and Supplementary Fig. S3). Its homologue



in *Arabidopsis* exhibited down-regulation at 1 h and up-regulation at 12 h of LT treatment (Supplementary Fig. S4 and Supplementary Table S4). Also in soybean, its homologue exhibited down-regulation in response to LT (Supplementary Table S6).

Transcript of gene encoding MYB transcription factor (*CjMYB133*) was not detected under CO condition, but the expression was evident at 5 min of exposure to LT (Fig. 2a and Supplementary Fig. S2a). Also, the gene expression was evident in plants in its niche environment at high altitude (Fig. 2b, Supplementary Fig. S2b and Supplementary Fig. S3). In *Arabidopsis*, its homologue was up-regulated at 6 h of LT treatment (Supplementary Fig. S4 and Supplementary Table S4), while the expression of its homologue in rice was unaltered (Supplementary Fig. S5 and Supplementary Table S5).

Expression of *COR* gene encoding cold acclimation responsive (*CjCAR2*) protein was unaltered till 3 h of LT treatment, but up-regulated at 6 h and the up-regulation was maintained thereafter. Expression of genes encoding rare cold inducible (*CjRCI2A*) and cold acclimation sensitive (*CjCAS*) protein was down-regulated up to 3 h, but the expression regained to the constitutive level at 6 h of LT treatment (Fig. 2a and Supplementary Fig. S2a). Up-regulation of these *COR* genes was also observed in populations growing in the niche environment of high altitude (Fig. 2b, Supplementary Fig. S2b and Supplementary Fig. S3). In *Arabidopsis*, homologue of *CjCAR2* was up-regulated only at 12 h of LT treatment (Supplementary Fig. S4 and Supplementary Table S4), while homologue of *CjCAS* showed down-regulation at 30 min following LT treatment, and up-regulation was observed at 24 h of LT exposure (Supplementary Fig. S4 and Supplementary Table S4). In rice, homologues of both, *CjCAR2* and *CjCAS* did not show any change in expression at LT (Supplementary Fig. S5 and Supplementary Table S5) while, in soybean, homologues of both *CjCAR2* and *CjCAS* exhibited up-regulation at LT (Supplementary Table S6).

Putative *Mce family protein* (*CjMce family*) showed an early up-regulation at 5 min of LT treatment followed by a strong up-regulation at 6 h (Fig. 2a and Supplementary Fig. S2a). Also, the gene expressed in the samples of *C. jubata* collected from its niche environment (Fig. 2b, Supplementary Fig. S2b and Supplementary Fig. S3). Its homologue was not found in rice and soybean.

***C. jubata* acclimated quickly to LT.** Freezing tolerance (LT_{50} , the temperature at which 50% injury occurred) was evaluated through measurement of electrolyte leakage in *C. jubata*. Data showed that freezing tolerance of non-acclimated (NA) plant was (–) 1.8°C, which decreased to (–) 7°C and (–) 10°C at 3 h and 12 h, respectively upon exposure to 4°C (Fig. 3).

Photosynthetic acclimation to LT. To study species acclimation response to LT, net photosynthetic rates (P_N) were studied in three sets of plants of *C. jubata* grown in growth chamber. In this experiment (lasting for 240 h), twelve weeks old plants were grouped into three sets, control (CO; 25°C), non-acclimated (NA) and acclimated (ACC) (Supplementary Fig. S6). NA and ACC plants were exposed to LT as shown in Supplementary Fig. S6. Each of the LT treatment (lasting for 72 h) was terminated by subjecting plants to 25°C for 48 h. P_N showed no significant change throughout the period of experiment in CO plants, but declined significantly during LT treatment (at 72 h in ACC plants and 192 h in NA plants; Fig. 4 and Supplementary Fig. S6). Exposing ACC plants to 25°C for 48 h restored P_N . At 120 h of experimentation, ACC plants were re-exposed to LT (for 72 h). P_N recorded at 192 h showed decline, but the percent decline was less than half to that recorded after the first LT treatment suggesting acclimation in P_N (Fig. 4 and Supplementary Fig. S6).

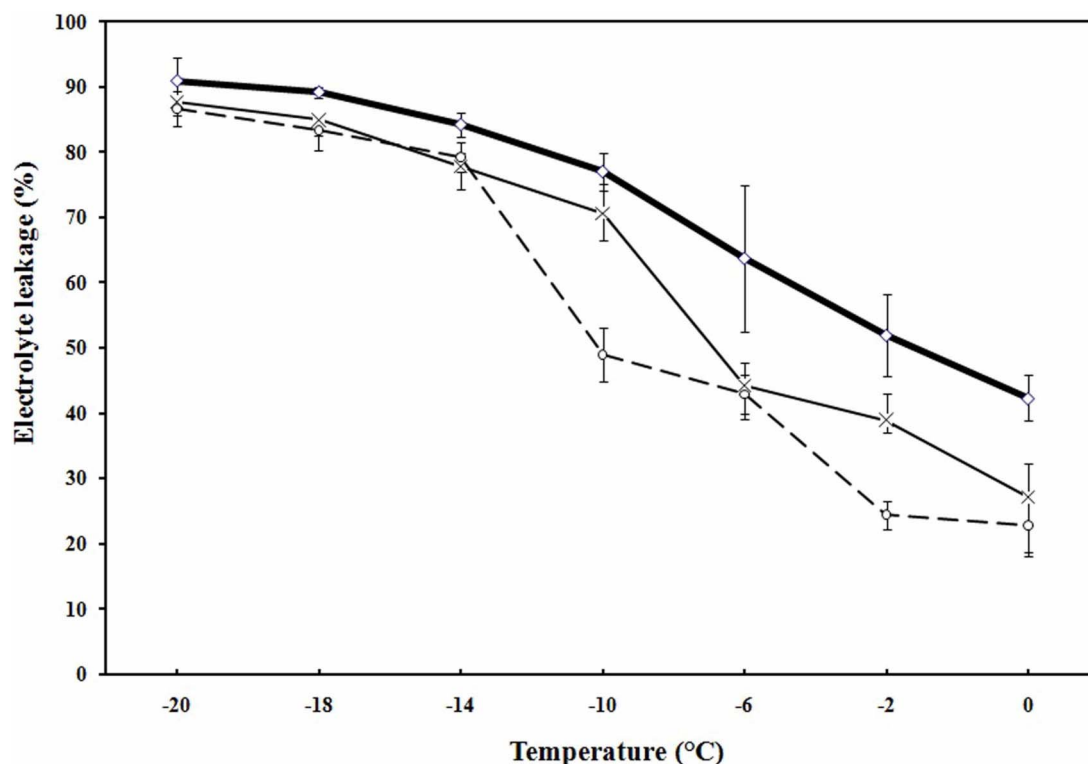


Figure 3 | Freezing tolerance of non-acclimated and acclimated leaf tissue of *Caragana jubata* as measured by electrolyte leakage. Control plants (◇, non-acclimated) were maintained in a plant growth chamber at 25°C, light intensity of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 60–80% relative humidity. For cold acclimation, plants were exposed to 4°C for 3 h (×) and 12 h (○). Each value is a mean of three separate biological replicates with standard error represented as error bar.

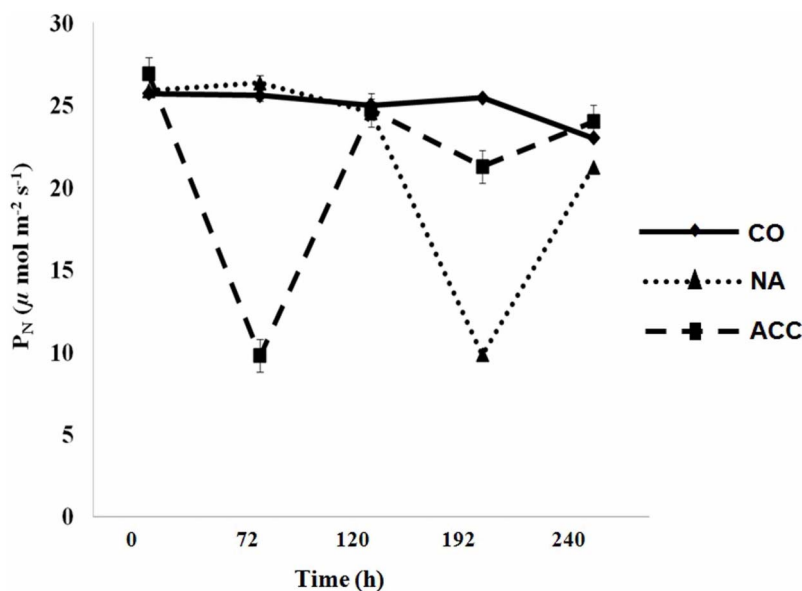


Figure 4 | Low temperature mediated photosynthetic acclimation in *Caragana jubata*. Net photosynthetic rates (P_N) were measured in control (CO, \blacklozenge), non-acclimated (NA, \blacktriangle), and acclimated (ACC, \blacksquare) plants. CO plants were exposed to 25°C throughout the experiment. NA plants were initially exposed to 25°C for 120 h followed by exposure to low temperature (4°C) upto 192 h for the next duration of 72 h; thereafter, plants were exposed to 25°C again (Supplementary Fig. S6). ACC plants were exposed to a cycle of 4°C, 25°C, 4°C, 25°C, at 0 h, 72 h, 120 h and 192 h, respectively (Supplementary Fig. S6). Each value is a mean of 45 observations on 5 different biological replicates with standard error shown as vertical bar.

Discussion

Simultaneous up-regulation of all the six groups of *LEA* protein genes in response to LT in *C. jubata* merits exceptional attention. Till date there is no report that describes LT related up-regulation of all the six groups of *LEA* protein genes simultaneously in any plant system. LEAs are typically highly hydrophilic proteins that provide stability to other proteins under dehydration, freezing, high salinity or drought and impart stress tolerance²⁴. Homologues of all the six groups of *LEAs* identified in the present work are reported to be associated with stress tolerance in plants¹⁷. For example, CAP85 and CAP160, the members of the *LEA/dehydrin* superfamily, enhanced freezing tolerance in tobacco²⁵. Wheat dehydrin gene *WCOR410* enhanced tolerance of strawberry leaves to freezing²⁶. Dehydrins contributed to freezing tolerance, partly due to their protective effect on membranes²⁷. Group 3 *LEA* protein gene *WCS19* improved freezing tolerance of the transgenic Arabidopsis, however only in the ACC plants suggesting a need of other endogenous components that only accumulate in response to low temperature²⁸. Thus, a role of *LEAs* can be attributed to acclimation in *C. jubata* leading to freezing tolerance as indicated by LT_{50} (Fig. 3).

Constitutive expression of *LEAs* (e.g. *CjLEA-Dc3*, *CjLEA-CI*, *CjLEA-14*) are suggestive of their role in taking care of initial shock (e.g. might be involved in imparting tolerance upon sudden exposure to LT, particularly for the period in which other chaperones were not induced in response to LT), and with increasing the protective demand at LT, inducible *LEAs* might function synergistically with other members. Since *C. jubata* in its niche environment is expected to be exposed to different environmental cues including LT, constitutively expressing *LEAs* could render a protective role. Similar protective role could be assigned to gene encoding low molecular weight heat shock protein *CjHSP26A*, which is up-regulated in response to LT as well as at high altitude (Fig. 2a,b and Supplementary Fig. S3). The protein has a protective effect through membrane protection, refolding of denatured proteins, and preventing protein aggregation at LT_{29} .

ELIPs are chloroplast proteins, the genes of which are transiently transcribed during the greening process of etiolated plants. Genes encoding ELIPs have been reported to be up-regulated in

Arabidopsis, pea (*Pisum sativum*), wheat (*Triticum aestivum*), tea (*Camellia sinensis*) and barley (*Hordeum vulgare*) in response to LT^{30-32} . By using an Arabidopsis mutant (*chaos*), a photo-protective role of *ELIP* was shown at LT in the presence of high light intensity³³. In *C. jubata*, up-regulation of *ELIP* in response to LT as well as at high altitude (Fig. 2a,b and Supplementary Fig. S3) is likely to provide protection against photo-oxidative damage and support plant growth.

CDPKs play a key role in Ca^{2+} -mediated signal transduction pathway. Genes encoding CDPKs are reported to be induced in other plant species including rice and maize (*Zea mays*) in response to LT and other abiotic stresses³⁴⁻³⁶. Over-expression of *CDPK* enhanced freezing tolerance in transgenic rice³⁷. An early up-regulation of *CjCDPK* in *C. jubata* (Fig. 2a,b and Supplementary Fig. S3) suggested its role in freezing tolerance, through signaling pathway.

ABA inducible proteins have been reported from various plant species including rice, wheat, and barley during different environmental stresses, with possible role in optimizing plant performance under stress conditions³⁸. Up-regulation of *CjABA inducible* (Fig. 2a,b and Supplementary Fig. S3) suggested its role in growth and development of *C. jubata* at high altitude.

Indole acetic acid (IAA) modulates plant growth and development through IAA inducible proteins that are short lived nuclear proteins and function as transcriptional regulators. These proteins do not interact with DNA but exert their regulatory activity through another group of proteins called auxin responsive factors. IAA inducible proteins have been reported in Arabidopsis (*AXR2/IAA17*, *AXR3/IAA17*) and pea (*IAA4*, *IAA6*), which play a role in auxin mediated signaling³⁹. Further, *ARF* gene family encodes transcriptional regulators that are involved in auxin signaling. These transcription factors bind to auxin-responsive *cis*-acting elements present in the promoter region of auxin-responsive genes through its DNA binding domain⁴⁰. *ARF7* and *ARF19* play essential roles in auxin-mediated plant development by regulating auxin responsive genes in Arabidopsis. *Auxin response factor 7/NONPHOTOTROPIC HYPOCOTYL4 (ARF7/NPH4)* was up-regulated in Arabidopsis during LT^{41} . Data (Fig. 2a,b and Supplementary Fig. S3) suggested a role of *CjIAA inducible* and the *CjARF* genes in maintaining plant growth and



development under LT as well as at high altitude through the mechanisms detailed above.

MYB proteins are a superfamily of transcription factors that play regulatory roles in developmental processes and defense responses. Plant MYB proteins are classified into three major groups: R2R3-MYB, R1R2R3-MYB and MYB related proteins⁴². Two R2R3-MYB transcription factors, *HOS10* in Arabidopsis and *Osmyb4* in rice imparted cold tolerance through CBF-independent pathway⁴³. Over expression of R1R2R3-MYB transcription factor, *OsMYB3R-2* from rice increased freezing tolerance in transgenic Arabidopsis⁴⁴. Arabidopsis MYB 15 (R2R3-MYB) is also involved in enhancing freeze tolerance⁴⁵. With this background on the importance of MYBs in imparting stress tolerance and maintenance of developmental processes, it is likely that up-regulated *CjMYB133* in *C. jubata* (Fig. 2a,b and Supplementary Fig. S3) has similar roles at LT and other stresses at high altitude.

The COR proteins are highly hydrophilic and have role in cold acclimation, freezing tolerance⁴⁶ and in maintenance of growth particularly at LT²⁰. Constitutive over-expression of *COR15a* enhanced freezing tolerance in non-acclimated transgenic plants of Arabidopsis⁴⁷. In *Medicago truncatula* and *M. falcata*, low expression of *COR* gene *CAS15* was reported in plants maintained at 25°C, but an evident up-regulation was observed at 6 h of exposure to low temperature⁴⁸. An early induction of these genes (*CjRCI2A*, *CjCAR2* and *CjCAS*; upto 3 h) at LT coupled with expression in the niche environment (Fig. 2a,b and Supplementary Fig. S3) is suggestive of their roles in cold acclimation, freezing tolerance and in maintaining growth in *C. jubata*.

Mce operon encodes a subfamily of ABC transporters that has a role in remodeling the cell envelop in *Mycobacterium tuberculosis*⁴⁹. This family of proteins is not well characterized in plant system. An evident up-regulation of *CjMce* family at LT and in the niche environment (Fig. 2a,b and Supplementary Fig. S3) requires detailed investigation to decipher its possible role in plants in response to environmental cue.

C. jubata in its natural habitat is exposed to a wide temperature range, from about 25°C during day to sub-zero temperature during night. Gene expression data showed the presence of the genes encoding chaperones and those associated with growth and development within 5 min to 3 h of exposure to LT (Fig. 2a). Importantly, all these genes also expressed in *C. jubata* in its natural habitat (Fig. 2b and Supplementary Fig. S3). Given the biological function of chaperones in cold acclimation and freezing tolerance, and the kinetics of their gene expression, data supported quick acclimation of the species to LT (Fig. 3). In Arabidopsis, LT₅₀ of (−) 7°C was achieved at day 7 of cold acclimation⁵⁰. In *M. truncatula*, leaf LT₅₀ was (−) 11°C when cold acclimated at 2°C for 4 weeks⁴⁸. *C. jubata*, thus quickly acclimated to LT as compared to Arabidopsis or *M. truncatula*. Similarly, expression of genes associated with growth and development would support *C. jubata* to thrive in its natural habitat.

Photosynthesis is generally suppressed at LT, as was also observed in our study (Fig. 4). This suppression could be due to reduction in activities of enzymes of carbon metabolism, thylakoid electron transport rate, photochemical efficiency of the photosystem (PS) II, and stomatal conductance⁵¹. LT mediated reduction in P_N in *C. jubata* can be explained based upon down regulation of genes associated with photosynthesis. These include those encoding for chlorophyll *a/l*-binding protein, chlorophyll *a-b* binding protein of LHCII type III, PS I light-harvesting proteins, Rubisco subunits, NADH dehydrogenase subunits (Supplementary Table S3 and Supplementary Fig. S9).

Photosynthetic acclimation is an important attribute that enhances plant fitness in changing environments⁵². LT-mediated photosynthetic acclimation in *C. jubata* (Fig. 4) can be related to up-regulation of chaperones such as *CjLEA-18*, a group 6 of *LEA* protein gene (Fig. 2a,b and Supplementary Fig. S3). Over-expression

of *CaLEA6*, a group 6 of *LEA* protein gene of *Capsicum annuum*, in transgenic Arabidopsis conferred protection in maintaining photosynthetic capacity under stress⁵³. The functional advantage of photosynthetic acclimation to LT could be seen in *Saxifraga paniculata*, where plant achieved values around 4.0 μmol CO₂ m^{−2} s^{−1} even after night temperature dropped to (−)22°C⁷.

Comparative expression analyses amongst *C. jubata*, Arabidopsis, rice and soybean showed that most of the genes exhibited either constitutive expression or early induction following LT treatment in *C. jubata*, only 12 out of 22 genes showed induction in Arabidopsis (cold-tolerant), one in rice (cold-sensitive) and 8 in soybean (a species of the same family as that of *C. jubata*). Though, Arabidopsis is considered a cold tolerant species, it is not distributed naturally in extremes of the environment such as cold desert. The data suggests adaptive advantage of *C. jubata* to LT over other plants such as Arabidopsis, rice and soybean. The LT responsive genes encoding chaperones and CORs in the present work are also known to confer tolerance to plants under other abiotic stresses^{18,54} and are the likely candidates to protect the plants under the environment of high altitude.

To conclude, the study suggested that: (1) *C. jubata* has an exceptional capability to express genes encoding chaperones and those associated with growth and development. Some of these were constitutively expressed, whereas others were strongly up-regulated within 5 min to 3 h of exposure to LT. Also these genes were expressed in the species in its niche environment, suggesting the capability of *C. jubata* to thrive in the environment of high altitude; (2) The temperate/alpine environment, where *C. jubata* is distributed naturally, experiences frequent fluctuations in temperature¹⁶ and calls for quick acclimation. Indeed, the species exhibited a quick acclimation response to LT (in terms of LT₅₀) with concomitant expression of the relevant genes as compared to the other species reported so far; (3) Photosynthetic acclimation of the species to LT was an important trait for functional advantage in the cold environment of high altitude.

Methods

Plant material and growth conditions. Seeds of *C. jubata* were collected from the plants growing in its niche in Kibber-Gete (32° 18' 21" N, 78° 01' 33" E; altitude 4,300 m; Lahaul and Spiti district, Himachal Pradesh), India and brought to the institute at Palampur (32° 06' N, 76° 33' E; altitude 1300 m; Kangra district, Himachal Pradesh), India for further studies. Seeds were sown in pots and placed in a greenhouse maintained at a temperature of 25 ± 3°C and relative humidity of 60–80%. Light intensity ranged between 500–800 μmol m^{−2} s^{−1}. At twelve weeks after sowing, plants were placed in two separate growth chambers (light intensity, 200 μmol m^{−2} s^{−1}; RH, 70 ± 10%) maintained at 25 ± 3°C (control, CO) and 4 ± 3°C (low temperature, LT). Leaves were harvested for the preparation of suppression subtractive hybridization cDNA libraries (SSH) and also for gene expression analysis.

In a separate experiment, leaf tissue was harvested from the plants growing at Kibber-Gete where air temperature was 12 ± 3°C and light intensity was 2500 ± 100 μmol m^{−2} s^{−1} at the time of sampling. The air temperature was measured by a digital thermometer and light intensity was measured using quantum sensor housed on a portable photosynthesis system LI 6400 (Li-COR, Lincoln, USA). Two populations of *C. jubata* were identified which were present in different hillocks separated by a water stream of melting snow water. Plants growing under similar climate conditions in a patch of 100 m × 100 m were considered as one population. Sampling was done during the period of September/October for two separate years (Supplementary Fig. S7), frozen immediately in liquid nitrogen, and stored at (−) 80°C till further use. Leaf and apical buds of minimum 15 plants of *C. jubata* were pooled together.

Construction of SSH. SSH was constructed using PCR-select™ cDNA SSH Kit (Clontech, USA) essentially as previously described⁵⁵ except that forward subtraction was performed using the cDNA synthesized from the mRNA of LT treated tissue (4°C for 48 h) as tester and driver cDNA was synthesized from the mRNA of CO (25°C). For reverse subtraction, tester cDNA was synthesized from mRNA of CO and driver cDNA was produced from the mRNA of LT treated tissue. For the purpose, total RNA was isolated from the leaves of CO and LT treated plants as previously described⁵⁶. Poly (A)⁺ RNA (mRNA) was purified from total RNA using GenElute™ mRNA Miniprep Kit (Sigma, USA) as per the manufacturer's recommendations. Yield and quality of RNA was determined spectrophotometrically by recording absorbance at 260 and 280 nm. Also, RNA was run on a denaturing agarose/formaldehyde gel and



visualized using a gel documentation system (Alpha DigiDocTM, Alpha Innotech, USA) to verify the integrity.

Sequencing and analysis of cloned expressed sequence tags (ESTs). Plasmids isolated by GenElute™ Plasmid Miniprep Kit (Sigma, USA) were single pass sequenced using BigDye terminator (version 3.1) Cycle Sequencing Mix (Applied Biosystems, USA) on an automated DNA sequencer (3130_{XL}, Genetic Analyzer, Applied Biosystems, USA) with M13 forward primer (5'-GTAAACGACGG-CCAGTG-3').

The sequences were analysed and submitted at NCBI dbEST database [dbEST accession no. FE968693-FE968985 for forward library (CJSF) and dbEST accession no. GW327090-GW327420 for reverse library (CJSR)]. Details on the method of analyses are mentioned in the legend of Supplementary Table S2.

Differential screening of SSHL. Expressed sequence tags (ESTs) thus generated were screened by slot blot hybridization (reverse northern analysis) as described previously⁵⁶, except that labeled [α -³²P] probes were synthesized from the unsubtracted driver and tester cDNA of *C. jubata* (Supplementary Fig. S8 and Supplementary Fig. S9).

Gene expression analysis by reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR was performed using Superscript III (Invitrogen, USA) essentially as described previously⁵⁶. Details of RNA isolation, cDNA synthesis, PCR, and quantification of amplicons using integrated density value are given in Supplementary Fig. S2. Gene expression was also validated by quantitative real-time PCR (qRT-PCR; details mentioned in Supplementary Fig. S3) for the experiment on *C. jubata* growing at low and high altitudes.

In silico expression analysis of homologues of Arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*) and soybean (*Glycine max*) in response to LT. In silico expression analysis was carried out to study the response of *C. jubata* gene homologues in Arabidopsis, rice and soybean using the publicly available LT responsive expression data. Gene homologues were identified through BLASTX in TAIR 10 (www.arabidopsis.org) (Supplementary Table S4), RGAP 7⁵⁷ (Supplementary Table S5) and soybean genome (<http://www.phytozome.net/soybean.php>) (Supplementary Table S6). For soybean protein sequences, BLASTX with significant E-value of less than 0.01 was considered (Supplementary Table S6). Microarray data for genes of Arabidopsis at LT was retrieved from AtGenExpress (<http://jisp.weigelworld.org/expviz/expviz.jsp>). Datasets obtained were corresponding to LT (4°C) at different time points viz. 0.5, 1, 3, 6, 12, and 24 h for aerial tissues. Microarray data for Arabidopsis genes At4g04720 and At3g51290, homologues of *CjCDPK* and *CjMce* family, respectively were not available in database and hence could not be included in the analysis (Supplementary Fig. S4 and Supplementary Table S4).

Similarly, microarray data of rice genes at LT (experiment ID GSE6901) were retrieved from rice oligonucleotide array database (www.ricearray.org). In the experiment, seven-day-old light-grown rice seedlings were subjected to LT (4°C) for 3 h. The seedlings kept in water for 3 h at 28°C, served as control⁵⁸. The values were log₂ transformed and used to plot graphs for gene expression. Expression of each clone was tested in at least three independent LT treatment experiments. Microarray data for rice genes Os01g21250, Os05g38870, Os06g09660, Os05g04700, and Os11g05935, homologues of *CjLEA-CI*, *CjABA inducible*, *CjARF7*, *CjRCL2A*, and *CjMce* family, respectively were not available in the database and hence could not be included in the analysis (Supplementary Fig. S5 and Supplementary Table S5).

Microarray data of soybean under cold stress was available at <http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-3164>. In these experiments, soybean plants grown in soil-filled plastic pots were kept for 3 weeks under a 12 h light/12 h dark at 28°C. Thereafter these were transferred to 4°C and sampled at day 1 of the treatment²³. (Supplementary Table S6 shows various gene homologues in soybean that could be retrieved from the database).

Evaluation of freezing tolerance of *C. jubata* through measurement of electrolyte leakage. Non-acclimated (NA) plants were maintained in a plant growth chamber at 25°C at light intensity of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 60–80% relative humidity. For cold acclimation (ACC), plants were exposed to 4°C for 3 h and 12 h. Electrolyte leakage was measured essentially as previously described⁵⁹ with minor modifications. Leaf tissue (100 \pm 5 mg) was placed in a 15 ml plastic tube (Tarsons, India), which was capped and submerged in a refrigerated circulator bath (CoolTech 320, Thermo Scientific, Germany) filled with 60% glycerol. The bath was set at 0°C for 1 h, the temperature was lowered to (–) 2°C and held for 30 min. Small pieces of ice (4 crystals; each prepared with 200 μl of sterile demineralized water) were added in each tube to initiate nucleation. The temperature was maintained at (–) 2°C for 1 h and then lowered at a rate of 2°C h⁻¹. Samples were removed at different temperature points and allowed to thaw on ice for overnight⁵⁹. After thawing, sterile demineralized water was added to each tube to a final volume of 12 ml and the tubes were shaken for 4 h. Leaves were removed and frozen at (–) 80°C overnight in a deep freezer (Thermo Electron Corporation, USA). In the meantime, conductivity (C1) of the supernatant was measured using a conductivity meter (CyberScan PC 510, Eutech Instruments, Singapore) and the supernatant was stored in cold room (4°C) for further use. Leaf tissues placed in deep freezer were removed and transferred to their respective tubes containing the supernatant and shaken for another 4 h to measure

the conductivity (C2). Electrolyte leakage was measured using the equation: (C1/C2) \times 100.

Assessment of photosynthetic acclimation in response to low temperature. Twelve weeks old plants were grouped into three sets: control (CO), non-acclimated (NA) and acclimated (ACC) (details for CO, NA and ACC are mentioned in the legend of Fig. 4 and Supplementary Fig. S6). Net photosynthetic rate (P_N) was measured using a portable open gas exchange system LI 6400 (Li-COR, Lincoln, USA), as previously described⁶⁰. The gas exchange system was equipped with a programmable light, temperature and CO₂ control. For all measurements, irradiance of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ was maintained by a cool light source (6400-02 LED) fitted on top of the leaf chamber, and data was recorded after 3–4 minutes when a steady state was achieved. Temperature of the chamber was maintained at 25°C through a Peltier cooling and heating system. CO₂ within the chamber was maintained at 400 $\mu\text{mol mol}^{-1}$ using 6400-01 CO₂ injector and a CO₂ mixer supplied along with the instrument. Data were computed by one-way analysis of variance (ANOVA), using general linear model procedures in SPSS statistical package (SPSS, Chicago, USA). The differences were calculated at the significance level of $p < 0.05$. Significant results were compared using Gabriel *post hoc* comparison.

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Author contributions

PKB carried out the SSH cDNA library construction, expression analysis, data collection, data analysis, preliminary drafting of the manuscript. RK, DM performed cold acclimation experiment. GB performed expression analysis (RT-PCR and qRT-PCR) with samples obtained from the niche environment. AKS performed *in silico* analysis of gene expression in Arabidopsis and rice. VA performed *in silico* analysis of gene expression in soybean. SKV performed experiments on photosynthesis. RK, DM, AKS, VA, and SKV assisted in writing the manuscript. PSA supported the work. SK spotted the plant species and its niche, conceived the study, designed the experiments, analysed the data, wrote, integrated and finalised the manuscript including title. All authors read and approved the final manuscript.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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