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

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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’. 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 Eurasia\(^2\) as well as Himalaya\(^3\). 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 (SSHL). 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, chaperones, 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 LHCCI type III, chloride channel calcium activated 4, retroelements polypolyprotein, 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 CJHSKP26A); LEA proteins were classified into various groups depending upon the presence of specific sequence motifs or biased amino acid composition\(^4\) and those associated with growth and development (early light inducible protein (CJELIP), CJABA inducible, CJCDPK, indole acetic acid inducible protein (CJAA inducible), auxin responsive factor 7 (CJARF7), MYB transcription factor 133 (GMYB133), rare cold inducible 2A (CJRCI2A), cold acclimation responsive2 (CJCAR2), cold acclimation specific (CjCAS), mammalian cell entry family protein (CjMice family))\(^5\) 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\(^6\) (Arabidopsis thaliana; a cold-tolerant plant species), rice\(^7\) (Oryza sativa; a cold-sensitive plant species) and soybean\(^8\) (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 expressed 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).

**CJHSKP26A 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 CJHSKP26A 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...
Figure 1 | Functional analyses of expressed sequence tags (ESTs) obtained from suppression subtractive hybridization library (SSHL) 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.
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 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).

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 (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 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 (LT50, 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 μmol m⁻² s⁻¹ and 60–80% relative humidity. For cold acclimation, plants were exposed to 4°C for 3 h (×) and 12 h (α). 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 (PN) were measured in control (CO, ◆), non-acclimated (NA, ▲), and acclimated (ACC, ■) 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) up to 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 tolerance
dehydrin superfamily, enhanced freezing tolerance in tobacco25. Wheat dehydrin gene WCO4010 enhanced tolerance of strawberry leaves to freezing6. 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 LT50 (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 LT29.

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 LT30–32. 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 Ca2+-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 (NONPHOTOTROPHIC HYPOCOTYL4 (ARF7/NPH4) was up-regulated in Arabidopsis during LT41. Data (Fig. 2a,b and Supplementary Fig. S3) suggested a role of CjIAA inducible and the CjARF genes in maintaining plant growth and
Mycobacterium tuberculosis maintaining growth in gestive of their roles in cold acclimation, freezing tolerance and in the niche environment (Fig. 2a,b and Supplementary Fig. S3) is sug-

Fig. S9).

b a can be explained based upon down regulation of genes associated with cold acclimation, freezing tolerance in transgenic Arabidopsis. 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 tolerance and in maintenance of growth particularly at LT. Constitutive over-expression of COR1sa enhanced freezing tolerance in non-acclimated transgenic plants of Arabidopsis. 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 temperature. An early induction of these genes (GjRCI2A, GjCAR2 and GjCAS; up to 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. 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 acclimation. In M. truncatula, leaf LT50 was (−11)°C when cold acclimated at 2°C for 4 weeks. 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 conductance. LT mediated reduction in Pn in C. jubata can be explained based upon down regulation of genes associated with photosynthesis. These include those encoding for chlorophyll a/b-binding protein, chlorophyll a/b-binding protein of LHCCI 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 environments. 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 stress. 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)°C.

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 Arabi-
dopsis (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 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 excep-
tional 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 distribu-
ted naturally, experiences frequent fluctuations in temperature 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 envir-
onment of high altitude.

Method

Plant material and growth conditions. Seeds of C. jubata were collected from the plants growing in its niche in Kibber-Gete (32°18’11”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 (SSHLib) 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 SSHLib. SSHLib was constructed using PCR-select™ cDNA SSH Kit (Clontech, USA) essentially as previously described [15]. Total RNA was extracted 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 described [16]. Poly (A)+ RNA (mRNA) was purified from total RNA using GentraPure 200 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

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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 (3130xl Genetic Analyzer, Applied Biosystems, USA) with M13 forward primer (5’-GTAAAGCAGCCG-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 methods 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 previously53, 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 previously53. Details of RNA isolation, cDNA synthesis, PCR, and quantification of amplicons using integrated density value are given in Supplementary Fig S5. 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 genes 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 silico tool (http://jsp.weigelworld.org/expviz/expviz.jsp). Datasets obtained were Microarray data for Arabidopsis at LT was retrieved from AtGenExpress (http://jsp.weigelworld.org/expviz/expviz.jsp). Datasets obtained were also screened by slot blot hybridization (reverse northern analysis) as described previously56. Details of RNA isolation, cDNA synthesis, PCR, and electrophoresis 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. PSF 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

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