

Cold tolerance of ScCBL6 is associated with photosynthesis and tonoplast transporters in Arabidopsis

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Research article

Keywords: Calcineurin B-like protein, cold tolerance, *Stipa capillacea*, transcriptome, antioxidant

Posted Date: October 31st, 2019

DOI: <https://doi.org/10.21203/rs.2.16643/v1>

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Abstract

Plant adapted in the fragile zone offer enormous opportunity to understand the stress biology under ecological scenario. *Stipa capillacea* is widely distributed in the frigid and arid region of Tibet Plateau, but its signal system has never been investigated yet. In this study, we isolated a signal transduction gene, ScCBL6, in *Stipa capillacea*, to characterize its cold tolerance capacity by ectopic expression in *Arabidopsis*. The results suggested that full length ScCBL6 encodes 227 amino acids, and phylogenetically clustered with CBL6 protein in *Stipa purpurea* and *Oryza sativa*. In comparison with wild type (WT) plants, ScCBL6 overexpressing plants (ScCBL6-OXP) are tolerant to cold stress but not drought stress, which attested by the higher photosynthetic capacity (Fv/Fm) and survival rate of ScCBL6-OXP under cold stress. We further compared their cold-responsive transcriptome profiles through RNA-Seq. Totally, 3931 genes were differentially expressed by introduction of ScCBL6. They are participated in multiple processes like immune system, lipid catabolic, secondary metabolic and mainly enriched in plant hormone signal transduction and biomacro-molecule metabolism as regard to KEGG pathway. Differential expressed genes (DEGs) were predicted to locate in chloroplast, mitochondrion, vacuole, and so on, suggesting multitudinous function of ScCBL6. Based on the integrated analysis of ScCBL6-OXP, we inferred that ScCBL6 improve plant cold stress tolerance via regulate photosynthesis redox and vacuole metabolites transport in *Arabidopsis*.

Background

Sessile plants are confronted with ubiquitous environmental stimulus which shaping their geographical distribution [1, 2]. In the long-term biology- environment interaction, plant signal system elaborately orchestrates with functional protein to confer stress defense. Ca^{2+} is regarded as life in cell and developmental biology for its constituent role and flexible oscillation within cell parts [3], thus act as central hubs of plant signaling networks to coordinate plant growth [4]. Under the natural condition, Ca^{2+} keep nanomole range in cytoplasm and millimole range in cellular compartment [5, 6]; while cell organelles supported by double membrane (such as chloroplasts) promoted Ca^{2+} wave upon stress initiation [7]. However, as a signaling messenger in plant cell, Ca^{2+} is decoded by several kinds of proteins like CDPKs, CaMs and CBLs [8, 9], so that well connected the stress signal with tolerance response.

Unlike CaMs and CDPKs exert regulations in nucleus [10], the CBLs protein could be membrane anchored by myristylation and acylation but gain flexible regulations roundly through interact with CBL-interacting protein kinase (shorted as CIPK). Structurally, CBLs proteins characterized by conical EF-hand motif and share sequence similarity with yeast calcineurin B subunit of yeast and animal neuronal calcium sensor [11–13]. There are 10 family members in *Arabidopsis* and *Oryza sativa*. AtCBL1 was differentially responsive to multiple stress including salt, drought and cold stress [14]. AtCBL7 was documented to modulates plant low nitrate response [15]. Other CBLs proteins of *Arabidopsis* were also supported to mediate physiological response like osmotic regulation and ion homeostasis [16, 17], and also seed

development [18]. However, gene clone and functional characterization of CBL6 and CBL8 are still at a juvenile stage, both for model and non-model plants.

Cold stress is one of the environmental factors that cause physiological disorder and impair plant growth [17]. Low temperature lead to mechanical strain on cell wall and membrane rigidification by the lamellar to hexagonal II phase transitions lesion [19–21]. As responses, plants on one hand accumulate solutes like malate, fumarate and proline to enhance freezing tolerance [22], and one the other hand adjust gene expression to reprogramme the cell structures and redress physiological metabolism [23]. Unraveling corresponding cold responsive gene and network are of benefits to crop improvement engineering. However, ICE-CBF is the best-know cold stress tolerance cascade [24], more other components need to be investigated.

Stipa capillacea is a constructive species and important forage that tolerant to multiple abiotic stressors. Because of the long-established domestication in alpine climate of Tibet Plateau, *S. capillacea* provided a well system to resolve cold stress tolerance mechanism. In the previous study, *SpCBL6* of its sibling species, *Stipa purpurea*, was found to enhance cold tolerance in *Arabidopsis*. In this study, we isolated its homologous CBL6-like protein from cold-hardy species *Stipa capillacea*, to study whether *ScCBL6* share the same functionality and explore the extent to which the integrated regulation and cold tolerance was conditioned by CBL6 protein. By ectopic transformation in *Arabidopsis*, comparative transcriptomic analysis was employed to unravel the molecular mechanism of *ScCBL6* at transcriptional level.

Results

Bioinformatic character of ScCBL6

ScCBL6 was cloned from *S. capillacea* based on its homologous gene in *S. purpurea*. After sequencing, an ORF contains 681 bp was designated as *ScCBL6*. The full length encodes a putative protein with 226 aa residues. Blastp search against NCBI found that *ScCBL6* share highest similarity with calcineurin B-like protein 6 in *Hordeum vulgare subsp. vulgare* (accession no. BAJ86242.1), calcineurin B-like protein in *S. purpurea* (accession no. AML23198.1), calcineurin B-like protein 6 in *Aegilops tauschii ssp. tauschii* (accession no. XP_020176977.1), calcineurin B-like protein 6 in *Brachypodium distachyon* (accession no. XP_003563139.1), respectively (Fig. 1A). All these proteins share four classical EF-hand in the motif scan analysis. *ScCBL6* clustered with *OsCBL6* in the phylogenetic analysis (Fig. 1B). The subcellular location result found *ScCBL6* protein mainly resided at tonoplast and plasma membrane (Fig. 1C).

***ScCBL6* Overexpressed *Arabidopsis* enhanced cold tolerance**

To explore the biological function of *ScCBL6 in vivo*, we generated transgenic *Arabidopsis* carrying *ScCBL6* under the promoter of cauliflower mosaic virus 35S. Three independent transgenic lines were

obtained by gene-specific PCR (Fig. S1). Under the natural greenhouse condition, *ScCBL6* over-expressed plants (short as ScCBL6-OXP) did not show much growth difference from wild type (WT) *Arabidopsis*, which also applied to the drought treatment as revealed by survival rate and Fv/Fm in Fig. 2. Under freezing condition, ScCBL6-OXP outgrew WT in term of both survival rate and Fv/Fm. In subsequent recovery process of cold treated plants, WT get perish for losing of regeneration, while ScCBL6-OXP retrieve normal growth gradually, indicating the better tolerance of ScCBL6-OXP than WT in response to cold stress. We also characterized the water loss rate of cold treated plants, and found that there is no significant difference between ScCBL6-OXP than WT (Fig. 3).

Comparative transcriptome analysis revealed transcriptional regulation of ScCBL6

To further investigate the molecular regulation excited by *ScCBL6*, we compared transcriptome variation between ScCBL6-OXP and WT for both cold and normal condition. In total, we sought out 6916 different expressed genes (DEGs) based on $|\log_2FC| \geq 2$ and $FDR \leq 0.05$, which varied between ScCBL6-OXP and WT irrespective of stress treatment. 4582 DEGs were found to be regulated by cold treatment in Col background (WT), however, 2185 DEGs were purely modified by the over expression of ScCBL6. In terms of cold response, ScCBL6-OXP share 43.64% (1300) overlaps with WT responders, the remaining 1679 (933+746) showed ScCBL6 dependency (Fig. 4). Among the 1300 overlaps, only 23.08% DEGs could be activated without cold treatment, suggesting the capability of ScCBL6 in response to cold stress.

For all the ScCBL6 regulated DEGs, the metabolic process, membrane and transferase activity contributed to the largest part for biological process (BP), cellular component (CC) and molecular function (MF), respectively (Fig. 5A). Further GO enrichments found that these DEGs involved in immune system process (GO:0002376), lipid catabolic process (GO:0016042), secondary metabolic process (GO:0019748), small molecule catabolic process (GO:0044282), organonitrogen compound catabolic process (GO:1901565), cutin biosynthetic process (GO:0010143), fatty acid metabolic process (GO:000661), and response to karrikin, auxin and insect (GO:0080167, GO:0009733 and GO:0009625) (Fig. 5B). While in terms of pathways, these mapped DEGs are mainly enriched in plant hormone signal transduction, starch and sucrose metabolism, cysteine and methionine metabolism, glycerophospholipid metabolism and fatty acid degradation, and so on (Fig. 5C).

Prospective cold tolerance mechanism mediated by ScCBL6

As documented in Zhang et al [25] and results of subcellular location of ScCBL6 (Fig. 1C), we inferred that ScCBL6 mediated cold tolerance by targeting of organelle located proteins. Therefore, ScCBL6 regulated DEGs were sequence extracted and searched against signal peptide database. The prediction of subcellular location indicated that most of the annotated DEGs located in chloroplast (266) and

engaged in secretory pathway (472), only 12 proteins located at tonoplast (Fig. 6). However, most of the tonoplast resided genes showed down-regulated expression, only two protein, i.e., polyamine choline transporter CAT2 and carbohydrate transporter TMT1 up-regulated their expression in response to cold treatment (Table 1). On the other hand, better chlorophyll fluorescence was observed in Fig. 2. We then further ranked the chloroplast located DEGs by $|\log_2FC|$, and plotted their expression profiles, 70% of top chloroplast DEGs up-regulated their expression levels (Fig. 7). Two up-regulated genes among the top 20 coresets, vicinal oxygen chelate (VOC) superfamily member members AT1G80160 and glutathione transferase GSTU3, pertains to the redox regulation. As indicated by the GR enzyme activity assays, antioxidant ability of ScCBL6-OXP overexpression lines were indeed improved (Fig. 8).

Discussion

The signal transduction is of great importance to the well domestication of alpine plants. Ca^{2+} mediated signal transduction has been widely documented in plant kingdom [26]. CBL protein convey a broad range of stimulus signal via reversible Ca^{2+} docking in its EF-hand domain [27, 28], and expanded from one member in chlorophytes *Ostreococcus lucimarinus* to more ten members in higher plant like *Arabidopsis*[29]. *Stipa capillacea* has long distributed in Qinghai-tibet plateau, and its signal transduction has not been investigated ever. Based on transcriptome data, we use homology-based method to isolate one CBL gene from *Stipa capillacea* successfully. Sequence analysis found that ScCBL6 protein was very conservative, at least among grass types (Fig.1A). Phylogenetically, ScCBL6 showed paralogous relationship with CBL2 and CBL3 in both *Oryza sativa* and *Arabidopsis thaliana*, suggesting earlier gene divergence before speciation. However, CBL2 and CBL3 are well-known chloroplast located protein [18], lending support to the tonoplast target of CBL6 in *Arabidopsis* [25]. CBL2 and CBL3 proved to affect seed size and embryonic development by transgenic operation in *Arabidopsis* [18]. However, up to date, functional characterization of CBL6 merely reported in *Stipa purpurea* and *Triticum dicoccoides* [30], all of which provided novel insight but just catch a little glimpse of its talents. In this study, we enforced functional analysis for ScCBL6 by ectopic expression in *Arabidopsis*, followed by comparative transcriptome analysis between its wild type (WT) and transgenic lines as regard to cold stress response.

Gain-of-function mutants, ScCBL6-OXP, enhanced cold tolerance but not drought tolerance in *Arabidopsis* (Fig. 2), which is highly consistent with the performance in transgenic SpCBL6 plants [31]. ScCBL6 share 96.77% similarity with SpCBL6 at the nucleotide level, and only 2 aa different at protein level. The two sites were not thought to functional divergent, which supported by the KaKs calculator results ($Ka < 1$, $p < 0.05$). As cold stress impose damage on plant body mainly through membrane damage and dehydration [23, 32, 33], we speculated that ScCBL6 confer the cold tolerance mainly through membrane functions, as we did not find significant difference for water loss between WT and transgenic lines (Fig. 3).

In the transcriptome analysis, ScCBL6 totally regulated the expression levels of 3931 genes (Fig. 4). They are widely enriched in plant hormone signal transduction, secondary metabolism, fatty acid and protein regulation (Fig. 5C), indicating the miscellaneous role of ScCBL6 in plant *in vivo*. However, after cold

stress, 31.37% (1233) of 3931 differently expressed genes (DEGs) were further regulated and 746 DEGs act as the ScCBL6 specific cold-inductive responder, they should constitute the core repertoire of cold tolerance mechanism imparted by ScCBL6. They execute holistic regulation across tissues like chloroplast, mitochondrion and cytoplasm, and so on (Fig. 6). However, only 12 genes were thought as tonoplast resided, the up-regulated gene CAT2 could interact with glutathione metabolism protein (OXP1), and tyrosine catabolism protein (HGO) in STRING network (Fig. S2). Additionally, 266 genes were predicted to chloroplast resided, and they are involved in enzyme classification (carbon-oxygen lyase, transferase and oxidoreductase), solute transport (calcium cation-transporting ATPase), cell wall (xyloglucan synthesis, rhamnogalacturonan modification, APGs protein glycosylation, CCoA-OMT and lipid formation), protein modification (cyclophilin protein folding catalyst, LSF phosphoglucan phosphatase dephosphorylation, GSK kinase phosphorylation, TKL kinase phosphorylation), RNA biosynthesis (GRF-GIF transcriptional complex, transcription factor ERF, BBX, ARF, TAZ and NAC activation), DNA damage response (ZDP base excision repair), cell cycle (cohesin dissociation, RAD9, DNA helicase complex MCM5 preinitiation), chromatin organization (Rad54 chromatin remodeling, H2A-type histone), phytohormones (brassinosteroid signal, ethylene synthesis and GASA peptides), redox homeostasis (DHAR), secondary metabolism (mevalonate, methylerythritol and terpenoid), polyamine metabolism (spermine conjugation), lipid metabolism (campesterol synthesis and GIPC biosynthesis) and photosynthesis related (glycine cleavage, cytochrome c oxidase and amylose synthesis) (Fig. S3). As the photosynthetic nature of chloroplast, the cold responsive leave growth advantage of ScCBL6-OXP quantified by chlorophyll fluorescence could tenably ascribe to the photosynthetic protection of ScCBL6. Especially, GULLO2, AT1G80160, PCAP2, SAUR36, BT2, AT2G44010, AT2G14095, CCOAMT, IRX7 and AT5G25770 showed top 10 upregulation. GULLO2 encodes a homolog of rat L-gulono-1,4-lactone (L-Gull) oxidase, which involved in the biosynthesis of L-ascorbic acid. BT2 encode telomerase component, serve as scaffold protein and commonly regulated by a bZIP-mediated SnRK-dependent pathway [34]. IRX7 participate in secondary cell wall biosynthesis, is required for proper anchoring of seed coat mucilage to reduced xylan to form high molecular weight polymer [35]. Together with the membrane positioning of ScCBL6, it is reasonable to postulate that ScCBL6 enhanced cold tolerance through redox regulation and structure maintenance.

Direct interactions of ScCBL6 with these targets were not expected, because they are most probably subject to phosphorylation by CIPKs. In this study, CIPK12, CIPK16, CIPK17, CIPK22 and CIPK25 were documented to up-regulated by cold stress in WT, and all of them except CIPK12 are the same for cold-treated ScCBL6-OXP. Especially, CIPK16 and CIPK25 increased 27.33 and 17.02 times in cold-treated ScCBL6-OXP, indicating their potential interaction with ScCBL6. However, for the untreated comparison of WT and ScCBL6-OXP, no CIPK was significantly up-regulated, this result well correlated with the temperature dependence of CBL6/CIPK complex formation documented in Zhang et al [25]. In total, for cold stress response conferred by ScCBL6, many genes like GULLO2 and PCAP2 were potential targets to improve cold tolerance in other crop plant, however, for the lack of transformation system for *Stipa capillacea*, their underlying functional mechanism and pros and cons plasticity in other plant need more experimental investigation.

Conclusions

This study provided a well paradigm for the complexity and polyfunctionality of environmental signal transduction system. In this study, ScCBL6 isolated from *Stipa capillacea* caught variations in nearly whole cell part. In ScCBL6 overexpressing transgenic *Arabidopsis*, cold stress tolerance was verified by higher survival rate and better photosynthetic capacity. Further RNA-Seq revealed that genes related to metabolites transporter in tonoplast and genes related to redox regulation in chloroplast showed preferential up-regulation in cold treated ScCBL6-OXP. That is also confirmed by the enzyme of GR. Vacuole as the largest plant cell compartment, ScCBL6 as a membrane anchored protein, its controlled functions are important for the environmental adaptation.

Methods

Plant preparation and controlled stress

S. capillacea seeds used for experiment were collected from Qinghai-Tibet Plateau (33°26′56.5″ N, 79°48′58.4″ E). As *S. capillacea* is not endangered and distributed widely in semi-arid hillside the of alpine steppe (Fig. S4), collection of the samples does not need any necessary permission. The plants were taxonomic identified by Dr. Jiahui Chen, and corresponding voucher specimen number was 1270425. In this study, seeds were obtained from National Wild Seed Resource Center of Kunming Institute of Botany under the ID of 868710085206. After been picked visually, mature and plump seeds were surface sterilized with 2% HgCl₂ reagent, then rinsed with double distilled H₂O and sowed in pots filled with nutrient soil directly. Seeds were allowed to germinate in a transparent growth chamber with natural ventilation and illumination. One month-old seedlings were used for stress treatments, and the whole aboveground part was sampled for RNA extraction. Drought was done by withholding water for 7, 14 and 21 days. Cold was carried out by putting seedlings in cooled incubator at 4 °C for 0, 1, 3 and 12 h, respectively.

Isolation and sequence analysis of ScCBL6

ScCBL6 was isolated from *Stipa capillacea* by the homology-based cloning methods. Total RNA was extracted by kit as referenced in Zhou et al [36]. Primers of forward (5′-ATGGTGGATTTCCCGGAAGG-3′) and reverse (5′-TCAAGCGTCCTCAACCTGAG-3′) were used to amplify the complete coding sequence. The PCR reaction amplified with Q5 and recycled as follows: 94°C for 30s, 52°C for 30s and 1 min at 72°C for extension. PCR products was purified and ligated to pMD18-T vector (Takara) and transformed into *Escherichia coli* by heat shock at 42°C for 90s. After selected by kanamycin, positive clone was picked and confirmed by sequencing. Full length sequence of was aligned with CBL protein of several Poaceae species and the structural motif of ScCBL6 was predicted online by Motif Scan software (https://myhits.isb-sib.ch/cgi-bin/motif_scan). The phylogenetic relationship with their homologous genes in *Oryza sativa* and *Arabidopsis thaliana* was provided by MEGA6 with maximum likelihood (ML) method.

Plasmid construction and transgenic plants manipulation

To obtain overexpressing construct of *ScCBL6*, its coding sequence was amplified with extended primers: *ScCBL6-F* (5-GTCGACCCCGGGgtaccATGGTGGATTTCCCGGAAG-3) and *ScCBL6-R* (5-TCAGAAATTCGGATCCggtaccAGCGTCCTCAACCTGAGAG-3). The 5' end of primers are sticky to plant binary vector *pRI101-GFP*, which could initiate the expression of chimeric protein contains target gene fused with GFP sequence by *CaMV-35S* promoter. After *Kpn I* digestion of binary vector, PCR products was sub-cloned into vector *pRI101-GFP* by recombinase. Resultant construct was introduced into *Agrobacterium tumefaciens* strain *GV3101* and infected wild type *Arabidopsis* () by floral-dip method [37]. Transgenic seeds were screened by kanamycin and positive transformants were identified by gene-specific primers. Two lines of *ScCBL6* overexpressing *Arabidopsis* were used for further analysis.

Subcellular location of *ScCBL6*

Agrobacterium tumefaciens carrying the recombinant vector *pRI101-ScCBL6GFP* was inoculated into LB solution and propagated overnight. *Agrobacterium tumefaciens* were introduced into leaf abaxial epidermis of *Nicotiana tabacum* by syringe injection. Green fluorescence excited by GFP protein was imaged in Olympus FV1000 laser confocal microscopy (Olympus, Japan).

Transcriptome analysis of *ScCBL6* over-expressed transgenic plants in response to cold treatment

Soil cultured transgenic *Arabidopsis* were subjected to cold treatment, in comparison with the control of wild type *Arabidopsis* (*Columbia eco-type*). One-month old plants were acclimated at 4°C for 12 h and then suffered -6°C treatment for 2 h. All plants were recovered at room temperature for another 4 days. Samples were taken both before and after cold treatments, and frozen in liquid nitrogen for RNA isolation. Total RNA was quality controlled by Nanodrop and used for transcriptome analysis by Illumina HiSeq-PE150. The raw reads were filtered by Trimmomatic 3.6, and then clean reads were quality controlled by FastQC. Clean reads of each sample was pair-end assembled in TopHat2.1.1, and differential expressed genes (DEGs) were identified by Cufflinks pipeline with parameters of $FDR \leq 0.05$ and $\log_2FC \geq 2$. DEGs were functional annotated by gene ontology and enriched with clusterProfiler package in R. Subcellular locations of DEGs were predicted in TargetP (<https://omictools.com/targetp-tool>). Sequence extraction and formatting was handled in TBtools [38].

Water loss assay

To explore the water loss rate under dehydration conditions, leaves of 4-week-old wild type and transformant *Arabidopsis* plants were cut off and allowed to dry on Whatman paper at room temperature, and net weights were documented at the 0.5, 1, 2, 4, 6 h, respectively. Water loss rate was calculated as weight loss per unit time.

Antioxidative enzyme activity assays

The enzymatic activity was assayed according to [39] with small modifications. About 0.1 g fresh leaves were extracted in 50 mM Tris buffer of pH 7.0, which containing 1 mM AsA, 1 mM DTT, 1 mM GSH, 1 mM EDTA, 5 mM MgCl₂·6H₂O, 20% glycerol and 1% PVPP. Then the homogenate were centrifuged at 4 °C for 6 min (12000 g), with supernatents re-centrifuged at same condition for 16 min. The GR activity was characterized as the ability of crude extractant to reduce GSSG by the NADPH auxiliary, which quantified in the absorption at 340 nm per minute per gram fresh weight.

Abbreviations

ScCBL6-OXP: ScCBL6 gene overexpressing plants

CBL: Calcineurin B-like protein

DEGs: differential expressed genes

ORF: open reading frame

FDR: false discovery rate

AsA: Ascorbate acid

DTT: Dithiothreitol

GSH: Glutathione

EDTA: Ethylene Diamine Tetraacetic Acid

Declarations

Ethics approval and consent to participate

Not Applicable.

Consent to publish

Not Applicable.

Availability of data and materials

All data and resources in the manuscript are available upon reasonable request to the corresponding authors.

Competing interests

All authors declare that there is no financial competing interests and other non-financial conflicts.

Funding

This work was financially supported by the CAS “Light of West China” Program (No. 292017312D11022), and partly supported by the open funds of the National Key Laboratory of Crop Genetic Improvement (No. ZK201605). The funders had no role in the experimental design, data collection and analysis or writing the manuscript.

Authors' contributions

YPY and CJZ designed the idea; YLZ wrote the manuscript; CHZ and CLZ conducted the experiments; GQL guided some analysis; XDS and YQY guided some experiments. All authors have read and approved the manuscript.

Acknowledgments

We thank Elie Ntirenganya for the critical reading of this manuscript.

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Tables

Table 1 Information of tonoplast resided DEGs

Transcript id	Gene id	Locus	log ₂ FC	Description
XLOC_002346	AT1G07607	1:18517585-18521781	-1.52	Antisense long noncoding RNA
XLOC_004339	AT1G04830	1:1356083-1362019	-1.53	Ypt/Rab-GAP domain of gyp1p superfamily protein
XLOC_005225	TUB5	1:6937718-6940832	-2.13	Beta tubulin
XLOC_006801	CAT2	1:21464000-21468505	2.05	Encodes a member of the cationic amino acid transporter (CAT) subfamily of amino acid polyamine choline transporters
XLOC_005279	TMT1	1:7242895-7248588	2.10	Carbohydrate transporter
XLOC_007227	AT1G65920	1:24525101-24529362	-4.39	Regulator of chromosome condensation (RCC1) family with FYVE zinc finger domain-containing protein
XLOC_007791	TUB1	1:28449904-28453820	-4.99	Beta tubulin
XLOC_008966	AT2G21220	2:8987583-9198383	-4.22	SAUR-like auxin-responsive protein family
XLOC_011932	TUB7	2:12642502-12646057	-2.49	Beta tubulin
XLOC_016150	AT3G62570	3:23142484-23144747	-2.08	Tetratricopeptide repeat (TPR)-like superfamily protein
XLOC_019943	AT4G05275	4:4676542-4855814	-2.31	Long noncoding RNA
XLOC_023442	AT4G27270	4:13661200-13663371	-2.48	Quinone reductase family protein

Note: Transcript id were the assembly outputs in cufflinks, and corresponding gene id and locus were obtained from reads mapping against gff files. Log₂FC were calculated from FPKM ratio of before and after cold treated ScCBL6-OXP.

Figures

Fig. 1

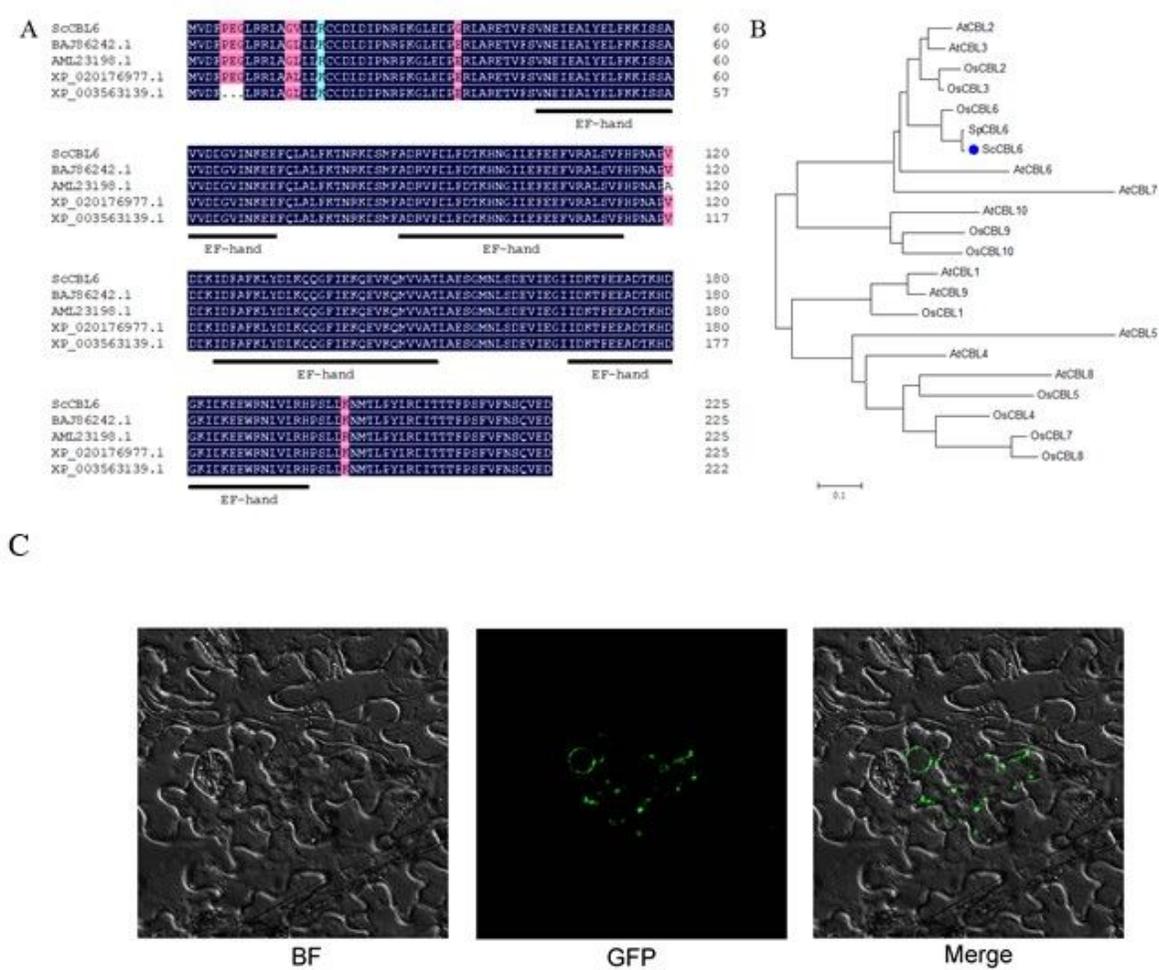


Figure 1

Biological property of ScCBL6. (A) Amino acid sequence alignment of CBL6 protein from *Sitpa capillacea* (ScCBL6), *Hordeum vulgare* subsp. *vulgare* (BAJ86242.1), *Stipa purpurea* (AML23198.1), *Aegilops tauschii* subsp. *tauschii* (XP_020176977.1), *Brachypodium distachyon* (XP_003563139.1). The cyan and pink color are bases with identity larger than 75% and 50%, respectively. (B) Phylogenetic tree of ScCBL6, SpCBL6 and CBL family member in *Arabidopsis thaliana* and *Oryza sativa*. Blue dot signifies the gene of interest for this paper. (C) Subcellular location of ScCBL6 protein. Green fluorescent protein excited at 488 nm was captured by Olympus FV1000 laser confocal microscopy.

Fig.2

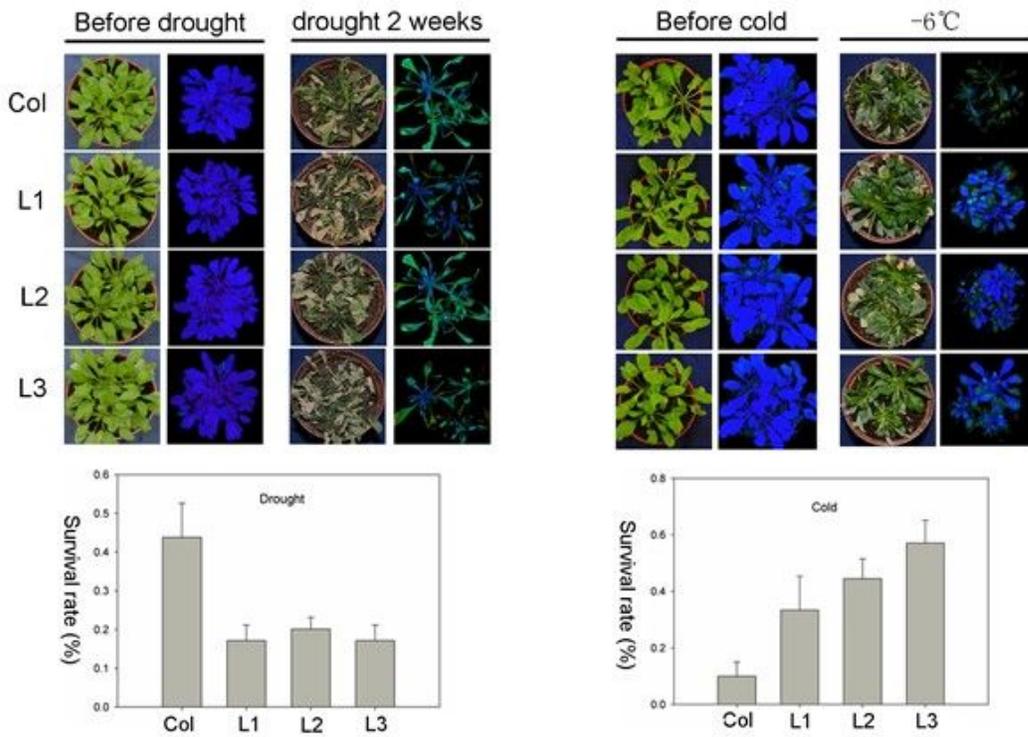


Figure 2

Seedling growth and survival rate comparison between ScCBL6-OXP and WT under drought and cold stress. Upper part indicated the morphology and photosynthetic efficiency (Fv/Fm) change after drought and cold treatment. Lower part showed the statistical survival rate of different lines after controlled drought and cold treatment, which based on at least seven biological replicates.

Fig. 3

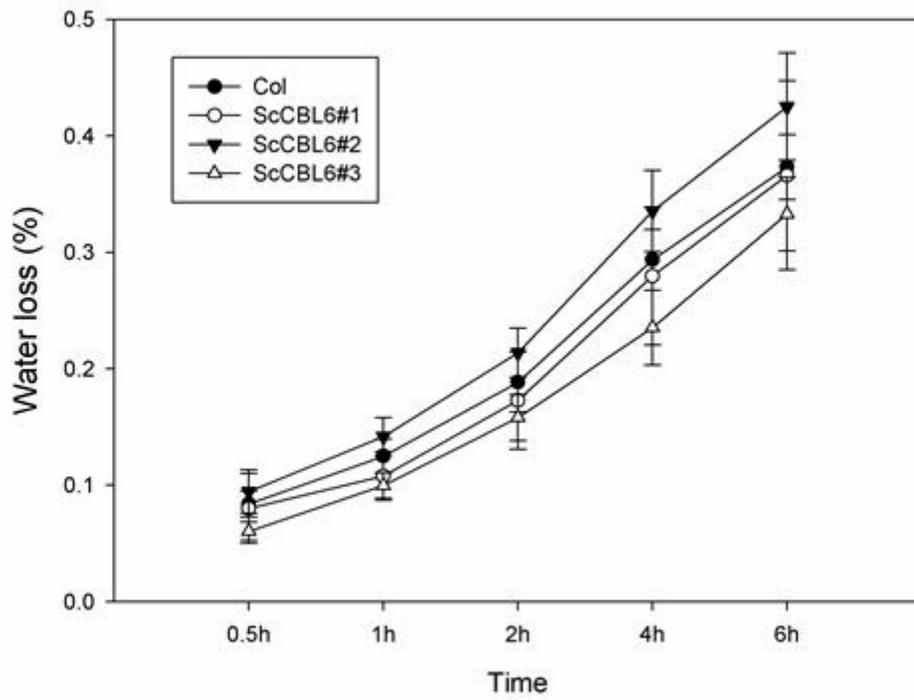


Figure 3

Water loss rate of ScCBL6-OXP and WT leaves. The water loss rate was calculated as weight difference between individual time point and original weight. Col, Colombia ecotype Arabidopsis; numbers followed # denoted different transgenic lines of ScCBL6-OXP.

Fig. 4

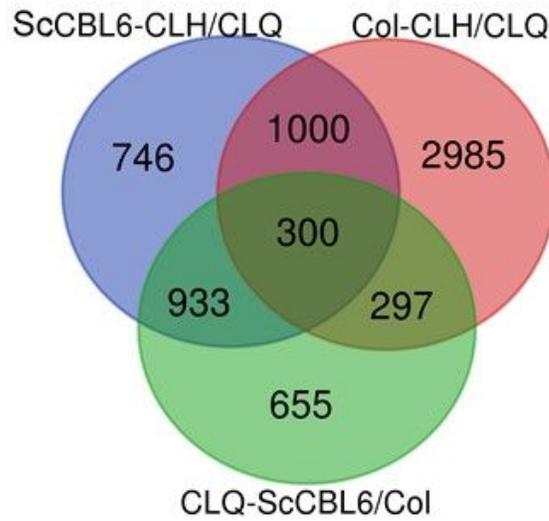


Figure 4

Venn diagram showed the overlap and specialty of DEGs affiliated to different condition. CLQ, before cold treatment; CLH, after cold treatment; ScCBL6 means the overexpressing Arabidopsis.

Fig. 6

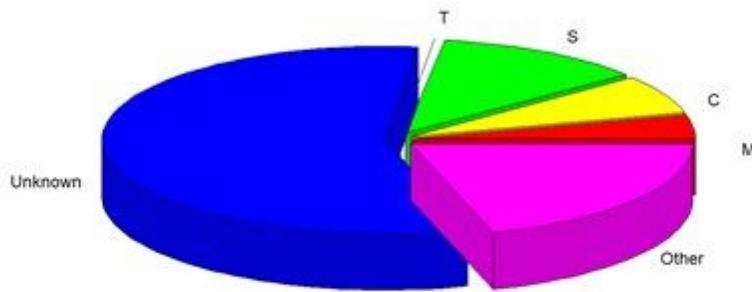


Figure 6

Subcellular location prediction of differential expressed genes. M, Mitochondrion (158); S, Secretory pathway (472); C, Chloroplast (266); T, tonoplast (12); Other (799); Unknown (2226). Tonoplast resided protein were predicted by blastx based search of proteins identified in Endler et al (2009) and Szponarski et al (2004), the other locations were predicted in targetP.

Fig. 7

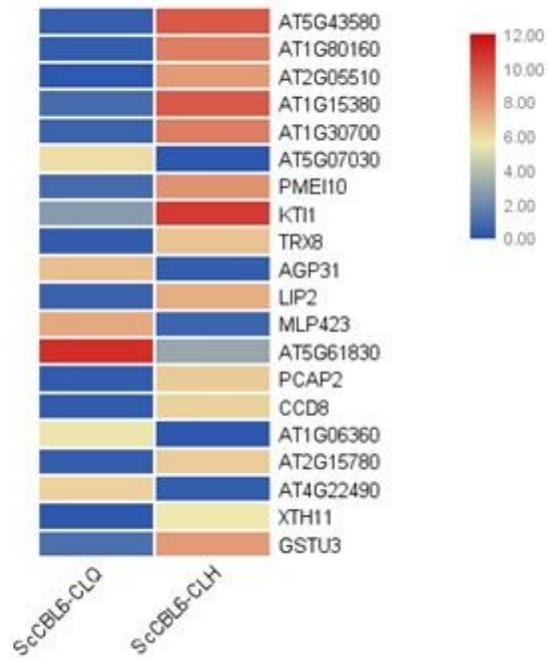


Figure 7

Expression levels of top 20 chloroplast-resident DEGs. The FPKM of ScCBL6-OXP were normalized by log scale and plotted in TBtools. CLQ, before cold treatment; CLH, after cold treatment.

Fig. 8

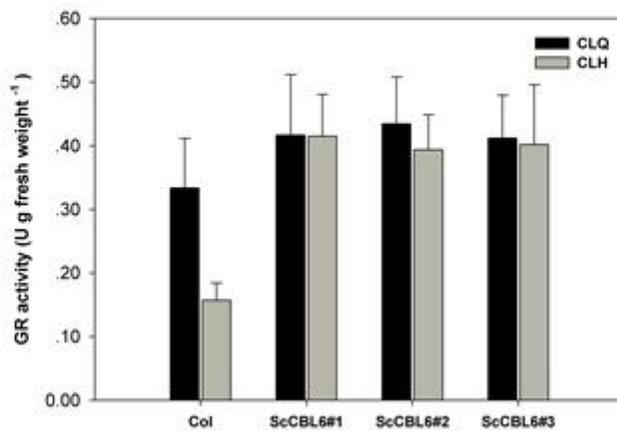


Figure 8

GR activities of Arabidopsis leaf in response to cold treatment.

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