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Overexpression of soybean *GmCBL1* enhances abiotic stress tolerance and promotes hypocotyl elongation in *Arabidopsis*

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ABSTRACT

Although extensive studies and remarkable progress have been made with *Arabidopsis* calcineurin B-like proteins (CBLs), knowledge of their functions in other plant species is still limited. Here we isolated gene *GmCBL1* from soybean, a homolog of AtCBL1 in *Arabidopsis*. *GmCBL1* was differentially induced by multiple abiotic stress and plant hormones, and its transcripts were abundant in seedlings and mature roots. We over-expressed *GmCBL1* in *Arabidopsis* and found that it enhanced tolerances to both high salt and drought stresses in the transgenic plants. Overexpression of *GmCBL1* also promoted hypocotyl elongation under light conditions. *GmCBL1* may regulate stress tolerance through activation of stress-related genes, and may control hypocotyl development by altering the expression of gibberellin biosynthesis-related genes. This study identifies a putative soybean CBL gene that functions in both stress tolerance and light-dependent hypocotyl development.

1. Introduction

Plants are frequently exposed to stressful environmental stimuli that have enormous impacts on plant growth and development. As sessile organisms, plants have developed a broad range of defense strategies and a complex network of signal transduction pathways to cope with environment stresses. Drought stress is among the most common adverse environmental conditions limiting plant development [1]. High soil salinity causes both ionic and osmotic stress, which not only affects the morphology, but also modifies the metabolisms of plants [2]. To date, a large number of genes regulated by salt and drought stress have been identified and their overexpression enhances tolerance to abiotic stress; a feature that could be important for improvement of stress tolerance in plants by genetic manipulation [3]. This also implies the importance of identifying more genes involved in stress response.

Calcium (Ca^{2+}) serves as a ubiquitous and central link in a large number of signaling pathways. Multiple extracellular signals such as light, hormones, and biotic and abiotic stresses elicit changes in Ca^{2+} levels in the cell [4–6]. The level of Ca^{2+} in cells triggers a wide range of signal transduction pathways through various calcium sensors, suggesting multiple functions of the sensors. Plants have evolved a diversity of unique proteins that bind Ca^{2+}. Among them, the calcineurin B-like (CBL) protein family that is unique to plants, contain multiple binding domains for Ca^{2+} and transmit signals by interacting with target proteins [7]. Thus they are well known to be involved in various stresses signaling processes. At least 10 CBLs have been reported in *Arabidopsis* and several of them function in response to multiple abiotic stresses [8–10]. Ten CBLs were identified in rice and shown to respond to diversity stresses [11]. Eight CBLs were also identified in sweet sorghum (*Sorghum bicolor*) and confirmed to be involved in alkaline stress response [12]. In addition to physiological stress response, CBLs also have roles in plant development. For instance, *Arabidopsis* CBL9 is involved in regulation of ABA response in seed germination by interaction with the protein kinase CIPK3 [13]. Gibberellin (GA)-regulated OsCBL2 promotes vacuolation of plant cells [14].

In spite of the extensive studies and remarkable progress in understanding *Arabidopsis* CBLs-mediated calcium signaling pathways, information about these signaling modules in other plant species is still quite limited. Here we report the isolation and

Abbreviations: CaMV 35S promoter, *Cauliflower mosaic virus* 35S promoter; GA, gibberellin; GFP, green fluorescent protein; MS medium, Murashige and Skoog medium; RT-PCR, reverse transcription polymerase chain reaction.

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characterization of *GmCBL1*, a novel CBL gene from soybean encoding a putative homolog of the *Arabidopsis* CBL1 with novel properties. Our molecular and physiological results suggest that GmCBL1 is involved in the regulation of multiple abiotic stress responses and plant development. Overproduction of *GmCBL1* in *Arabidopsis* improved salt and drought tolerance by regulating the expression of important stress-responsive genes. *GmCBL1* overexpression also promoted hypocotyl elongation through modulating GA biosynthesis.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of *Arabidopsis* (*Arabidopsis thaliana*, Columbia) were surface sterilized in a 10% sodium hypochlorite solution for 15 min, and then extensively washed five times with sterilized water. The surface-sterilized seeds were sown on plates containing ½ Murashige and Skoog (MS) medium, maintained at 4 °C for 3 days in darkness and then incubated in a growth chamber at 22 °C with a 16 h light/8 h dark photoperiod. After two weeks, the seedlings were transferred to soil and grown in a growth chamber. Soybean seeds were germinated on vermiculite in a light chamber at 25 °C for 10 days. The soybean seedlings were subjected to a range of stresses, viz. 200 mM NaCl, 20% PEG, 100 μM abscisic acid (ABA), 100 μM GA for various times. Seedlings were sampled for RNA extraction.

2.2. Database search and isolation of GmCBL1

The soybean genomic sequence database (http://www.phytozome.net/search.php) was searched to identify soybean CBL genes with entire *Arabidopsis* AtCBL1 amino acid sequences. The predictions of soybean CBL coding sequences were further verified with available expressed sequence tag (EST) (http://www.ncbi.nlm.nih.gov/dbEST) and mRNA sequences. Among the identified putative soybean CBL genes, one mRNA sequence (BT092219) encoding a predicted protein with the highest similarity to *Arabidopsis* CBL1, was chosen for further study and designated as *GmCBL1*. Specific primers (Supplementary Table 1) were designed to amplify the full coding region of the soybean *GmCBL1* by a PCR approach. The PCR products were purified and cloned into a pGEM T-easy vector (Promega) and sequenced.

2.3. RNA extraction and reverse transcription PCR (RT-PCR) assays

Isolation of total RNA from plant materials was performed using an RNA extraction kit (Takara) according to the manufacture's recommendations. The cDNA synthesis and RT-PCR were conducted as previously described [15]. Semi-quantitative RT-PCR was conducted based on prior RT-PCR using different cycles to ensure that the resulting band intensities were within the linear range. Real-time PCR for examination of *GmCBL1* expression was carried out with an ABI Prism 7300 sequence detection system (Applied Biosystems) as previously described [16]. Soybean *Actin* (U60506) and *UBQ10* (AT4G05320) were used as internal controls for normalization of the template cDNA. All primers used in the study are listed in Supplementary Table 1.

2.4. Subcellular localization

Expression vectors with green fluorescent protein (GFP) tags were constructed for subcellular localization analysis [15]. The coding region of *GmCBL1* was amplified by RT-PCR using specific primers (Supplementary Table 1) and fused to the N-terminal end of GFP under control of the CaMV 35S promoter as 35S::*GmCBL1*-GFP. Subcellular localization of GFP expression in *Arabidopsis* protoplasts was monitored by a confocal microscopy 16 h after polyethylene glycol mediated transformation as described [17].

2.5. Arabidopsis transformation and stress tolerance characterization

For *Arabidopsis* transformation, the full coding sequence of *GmCBL1* in sense orientation was amplified with gene specific primers (Supplementary Table 1) and cloned into the *BamH*I/*Sac*I restriction enzyme sites of vector pBI121 under the control of the CaMV 35S promoter. The pBI121-*GmCBL1* construct was introduced into *Agrobacterium tumefaciens* C58C1 strain cells, and transformation was carried out by the floral dip method as described previously [18]. T4 generation homozygous lines of *Arabidopsis* were used for the phenotype analysis.

For germination assays, seeds of wild-type, transgenic plants and *sos3* mutant were placed on ½ MS medium containing 100 mM NaCl. Germination rates were then measured daily for one week. The survival rate of seedlings grown on ½ MS medium was further analyzed after transfer to plates containing different concentrations of NaCl (50, 100, or 150 mM). Plants developing true leaves under these conditions were designated as survivors. For the plant fresh weight assay, four-week-old plants growing in soil supplemented with NaCl or without water for 15 days were weighed. To detect the rate of water loss under dehydration conditions, detached leaves from four-week-old plants were exposed to air at room temperature and weighed at designated times.

2.6. Hypocotyl growth experiments

Surface-sterilized seeds of wild-type and transgenic plants were sown on plates containing ½ MS medium. For GA and GA biosynthetic inhibitor paclobutrazol (PAC) treatments, seeds were sown in agar medium supplemented with or without different concentrations of GA_3 and PAC and grown under the growth conditions mentioned above. Plates were stored at 4 °C for 3 days in darkness, and then exposed to white light to stimulate germination. Hypocotyl lengths were measured after 5 days on images by use of Image J software (http://rsb.info.nih.gov/ij/).

3. Results

3.1. Expression patterns of GmCBL1

Database searches together with bioinformatics analysis led to the identification of a putative unique soybean CBL mRNA sequence (BT092219) encoding a predicted protein with the highest sequence similarity (76%) to *Arabidopsis* AtCBL1. The sequence was designated as *GmCBL1* and was used in further study. Alignment with 10 CBLs in *Arabidopsis* showed that GmCBL1 was more similar to AtCBL1 than to others (Fig. 1A).

To gain insight into the possible function, we initially examined the expression patterns of *GmCBL1* under various stress treatments. As shown in Fig. 1B, *GmCBL1* transcripts increased in soybean seedlings treated with NaCl, PEG, ABA and GA. Expression pattern of *GmCBL1* in seedlings, young roots, cotyledons, mature leaves, green pods and mature roots of soybean was also investigated using real-time PCR. The relative expression levels were higher in seedlings and mature roots than in other organs examined (Fig. 1C). Subcellular localization analysis (Fig. 1D) indicated that GmCBL1 protein expression was mainly at the cell membrane. These findings suggest that GmCBL1 might exert a role in multiple stress responses.

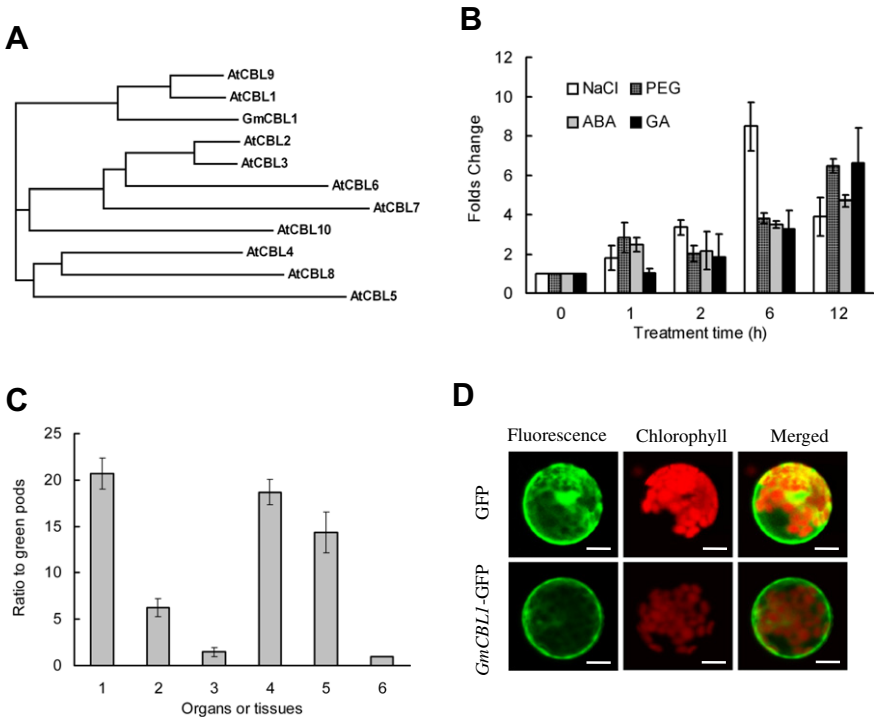

Fig. 1. Expression patterns of *GmCBL1*. (A) Phylogenetic relationship of GmCBL1 with 10 *Arabidopsis* CBLs. Multiple sequence alignment was performed using the ClustalX program, and the phylogenetic tree was constructed using the Neighbor-Joining method. The accession numbers of *Arabidopsis* are as follows: AtCBL1, AAC26008; AtCBL2, AAC26009; AtCBL3, AAC26010; AtCBL4, AAG28402; AtCBL5, AAG28401; AtCBL6, AAG28400; AtCBL7, AAG10059; AtCBL8, AAL10300; AtCBL9, AAL10301; AtCBL10, AAO72364. (B) Expression analysis of *GmCBL1* under different treatments. The transcript level at time 0 h was used as the control whose *GmCBL1* mRNA level was given as 1. (C) Expression of *GmCBL1* in various organs. 1, Seedlings; 2, young roots; 3, cotyledons; 4, mature leaves; 5, green pods; 6, mature roots. The transcript level in mature leaves was used for calibration and its *GmCBL1* mRNA level was given as 1. The soybean actin gene was used as the internal control for normalization of template cDNA. Each PCR was repeated at least three times and the error bars represent the SD in (B) and (C). (D) Subcellular localization of GmCBL1 protein. The 35S::*GmCBL1*-GFP and 35S::GFP control vectors were transiently expressed in *Arabidopsis* protoplasts. Results were visualized by a confocal microscopy. Bars = 10 μm.

3.2. Overexpression of GmCBL1 enhanced salt and drought tolerance

To examine the biological function of *GmCBL1*, transgenic *Arabidopsis* plants overexpressing *GmCBL1* were generated. RT-PCR analysis showed that *GmCBL1* mRNA accumulated in all transgenic lines, but not in wild-type (Col-0) plants (Fig. 2A). To examine whether *GmCBL1* mediates salt stress response in *Arabidopsis*, we compared wild-type and *GmCBL1* overexpressing plants with *sos3* mutant in a salt-tolerance assay. The salt hypersensitive mutant of *sos3* (salt-overly-sensitive 3) [19] and the untransformed wild type (ecotype Columbia) were used as the controls. In the germination assays under salt stress, transgenic plants were resistant to high salt stress (Fig. 2B). Transgenic seedlings were also more resistant to high-salt medium with higher survival ability following transfer from the normal medium (Fig. 2C). For example, in the presence of 150 mM NaCl, more than 60% of transgenic plants survived compared with a 30% survival rate in control plants and 6% survival rate in the *sos3* mutant. We also performed post-development assays using high concentration of NaCl and scored the fresh weight (FW) of plants as a growth indicator. As shown in Fig. 2D and E, transgenic seedlings were consistently more resistant to high salt than wild-type and *sos3* mutant plants when grown in soil supplemented 200 mM NaCl for 15 days. These analyses indicated that *GmCBL1* overexpressing plants were more tolerant to high-salt stress.

To evaluate drought stress tolerance of transgenic plants, we also scored the FW of plants under drought conditions as a growth indicator. Four-week-old wild-type and *GmCBL1* overexpressing plants grown in soil were withheld from water for 15 days. Rosette leaves of transgenic plants showed more resistance than the wild-type plants 2 days after rewatering (Fig. 2D). The FW of *GmCBL1* transgenic plants indicated that they were consistently more resistant than wild-type plants when grown under drought conditions (Fig. 2E). We also quantified the loss of water in detached leaves. The leaves from wild-type plants lost about 30% of their total water in 4 h, whereas leaves from transgenic plants had a much less lower water loss (Fig. 2F). These findings indicated that *GmCBL1* overexpression promoted drought tolerance in *Arabidopsis*.

3.3. GmCBL1 overexpression promoted hypocotyl elongation under light conditions

Fig. 3A shows the phenotypes of wild-type and T4 transgenic seedlings grown on a ½ MS plate for 5 days in light conditions. Hypocotyls were longer in *GmCBL1* overexpressing seedlings than in wild-type (Fig. 3B). However, there was no difference in hypocotyl length when plants were grown in darkness (Fig. 3C), suggesting that *GmCBL1* overexpression promotes hypocotyl elongation in a light-dependent manner. As shown in Fig. 3D, the distinct response to light was associated with expression of *GmCBL1*. RT-PCR analysis showed that *GmCBL1* was more highly expressed in light than in darkness. Moreover, several light-responsive cis-regulatory elements (G-box, I-box and T box) were detected in the 2 kb sequence upstream of the translation initiation site of *GmCBL1* using Plantcare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Fig. 3E), further implying an involvement in light response. These observations suggest that the light signaling pathway may be involved in regulation of GmCBL1 expression to modulate hypocotyl growth.

3.4. GmCBL1 overexpression altered GA response in hypocotyl elongation

GAs exert an opposite effect to light on photomorphogenesis [20]. To check whether GA was involved in *GmCBL1*-mediated

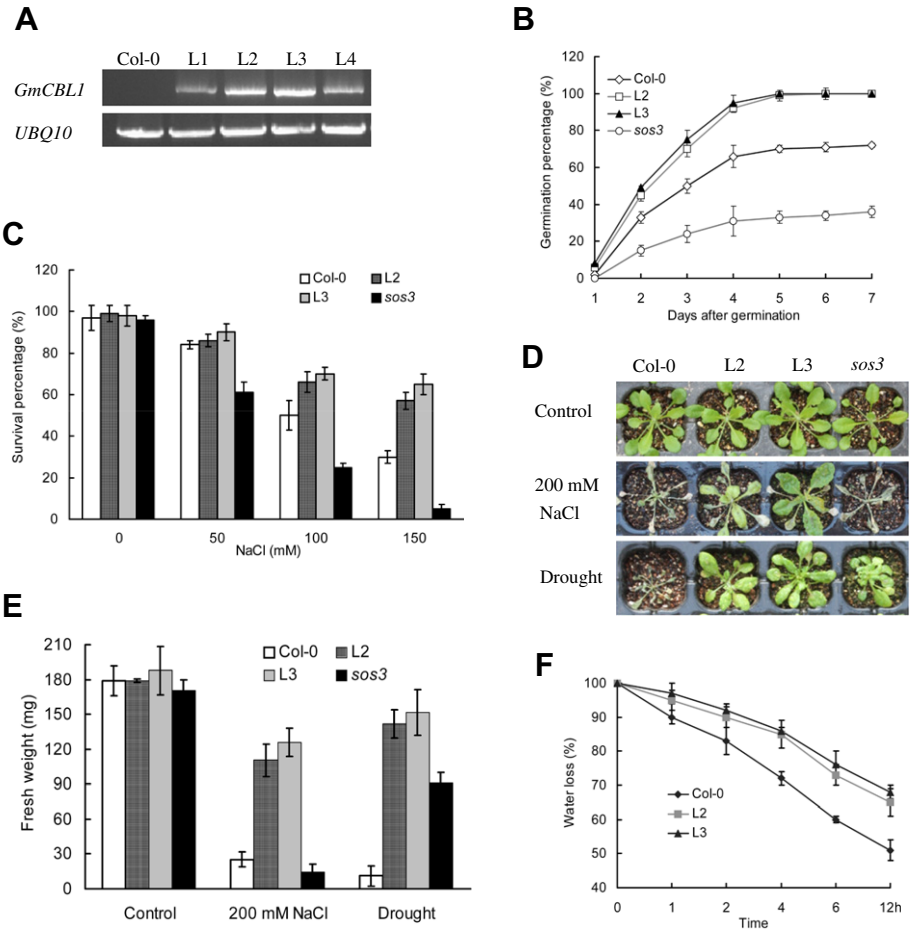

Fig. 2. Response of *GmCBL1* overexpressing plants to salt and drought stresses. (A) *GmCBL1* overexpression in *Arabidopsis*. Transcripts of *GmCBL1* in transgenic *Arabidopsis* lines (L1–4) were detected using RT-PCR. Wild-type (non-transformed) plants (Col-0) were used as control. (B) Seed germination in wild-type, *GmCBL1* overexpressing plants and *sos3* mutant. Seedlings were grown in the presence of 100 mM NaCl. Germination rates were determined daily after stratification. Data represent means ± SD (n = 90). (C) Relative survival rates of transgenic seedlings (L2 and L3) grown on media in the presence or absence of NaCl. Four-day-old seedlings were transferred to ½ MS medium with or without different concentrations of NaCl. Survival rates were measured 6 days after transfer. (D) Representative images of transgenic plants grown under salt and drought conditions. Four-week-old wild-type (Col-0), transgenic plants and *sos3* mutant were treated with 200 mM NaCl or withheld from water for 15 days. The photographs were taken 48 h after re-watering. Three independent experiments were conducted. (E) Fresh weight of *GmCBL1* transgenic plants after different treatments in D. Data represent means ± SD (n = 20). (F) Quantification of water loss in four-week-old wild-type, L2 and L3 transgenic plants. Leaves at the same developmental stages were excised and weighed at various time points after detachment. Data represent average values ± SD (n = 8).

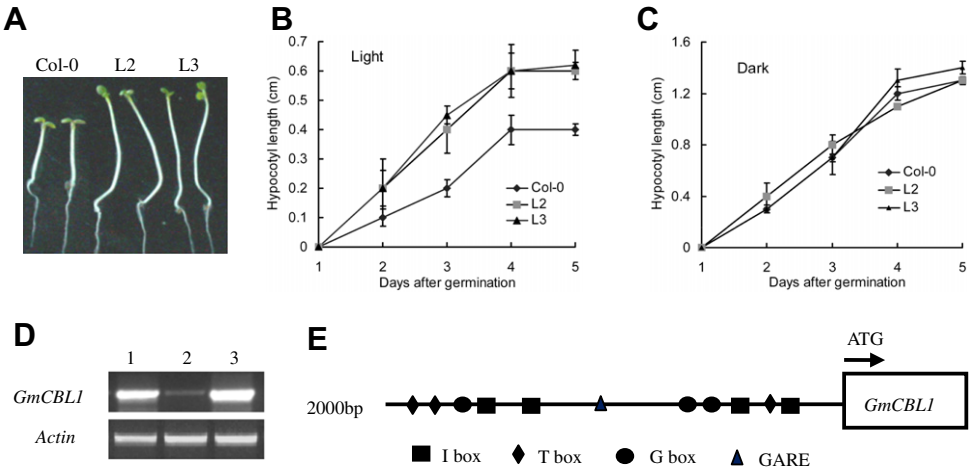

Fig. 3. Analysis of GmCBL1 mediated hypocotyl elongation. (A) Representative images of wild-type (Col-0) and *GmCBL1* overexpressing seedlings (L2 and L3) after 3 days of growth under light. (B and C) Quantification of hypocotyl length in wild-type and *GmCBL1* overexpressing seedlings grown in light and darkness conditions. Data represent average values ± SD (n = 20). (D) Analysis of *GmCBL1* transcripts levels by RT-PCR, which was carried out using total RNA prepared from soybean seedlings. Lane 1, seedlings before treatment; lane 2, 1 day in darkness; lane 3, 1 day in darkness followed by 1 day in light. *Actin* was used as an internal control. The numbers of reaction cycles for *GmCBL1* and *Actin* were 25 and 28, respectively. (E) Deduced cis-acting elements present in the promoter region of *GmCBL1*. *GARE*, gibberellin response element.

hypocotyl elongation under light, we examined the effects of exogenous GA_3 and the GA biosynthesis inhibitor PAC on hypocotyl elongation in *GmCBL1* overexpressing and wild-type seedlings. As shown in Fig. 4A, *GmCBL1* overexpressing lines slightly responded to bioactive GA_3, whereas PAC severely impaired hypocotyl elongation in *GmCBL1* overexpressing plants. These results indicated that GA is required for *GmCBL1*-mediated hypocotyl elongation. To investigate the role of *GmCBL1* in regulation of GA biosynthetic gene expression, we performed RT-PCR analyses on 6-day-old *GmCBL1* overexpressing and wild-type seedlings. Among these genes, *GA2ox2*, *GA3ox1* and *GA3ox2* were obviously upregulated (Fig. 4B). Thus GmCBL1 appears to increase bioactive GA levels for hypocotyl growth under light conditions.

4. Discussion

Ca^{2+} is widely known to play important roles in the regulation of plant cell responses to environmental cues [21]. As unique Ca^{2+} sensors in plants, CBLs have been identified in various signaling pathways involved in plant development and stress responses. Although a number of studies on plant CBLs have been reported, little knowledge was available for CBLs in soybean. In this study, we characterized and discussed the possible functions of *GmCBL1* from soybean based on the results of experiments using transgenic *Arabidopsis*.

Tissue specificity or signal responsiveness of gene expression often reflects the function of the corresponding gene products in plant development and signaling. As shown in Fig. 1B and C, *GmCBL1* responded to various stimuli and its transcripts were detected mainly in seedlings and mature roots, implying diverse roles in response to environmental signals at different developmental stages. *GmCBL1* overexpressing plants showed superior seed germination and seedling growth. Transgenic lines were more resistant than wild-type plants when grown under high-salt or drought conditions (Fig. 2D). To better understand the mechanisms of salt and drought tolerance conferred by *GmCBL1* overexpression, we investigated the expression of several known salt and drought induced genes in transgenic plants. As shown in Fig. 4C, all transgenic plants produced high levels of mRNA for *DREB1A*, *DREB2A*, *RD29A* and *KIN1*, indicating that overexpression of *GmCBL1* in transgenic plants activated expression of a number of stress-responsive genes under stress conditions. In addition, it has been reported that overexpression of stress-response genes, such as *DREB1A*, *KIN1*, and *RD29A*, enhances salt and drought tolerance in transgenic plants [22–24]. Thus, the dramatic induction of stress-response genes in *GmCBL1* overexpressing plants corresponded to the stress tolerance phenotypes. These results suggest that GmCBL1, like AtCBL1, may function as a positive regulator in salt and drought stress signaling pathways [8].

Light is the most important environmental factor for plant growth and development. In this study, we found that *GmCBL1* overexpression promoted hypocotyl elongation under light conditions (Fig. 3A and B), suggesting that GmCBL1 might be involved in light response. RT-PCR analysis showed that expression of *GmCBL1* was induced by light (Fig. 3D), indicating its role in light signaling. Cis-elements are important molecular switches involved in transcriptional regulation of a dynamic network of gene activities controlling various biological processes. As expected, several light-responsive cis-regulatory elements (G-box, T-box and I box) were identified in the promoter region of *GmCBL1* (Fig. 3E). These findings indicate that GmCBL1 might play a role in light-dependent hypocotyl growth. GA concentrations are partly responsible for light-regulated photomorphogenesis and bioactive GAs function as key mediators in the perception of light signals [25,26]. To examine whether GmCBL1 was associated with GA homeostasis,

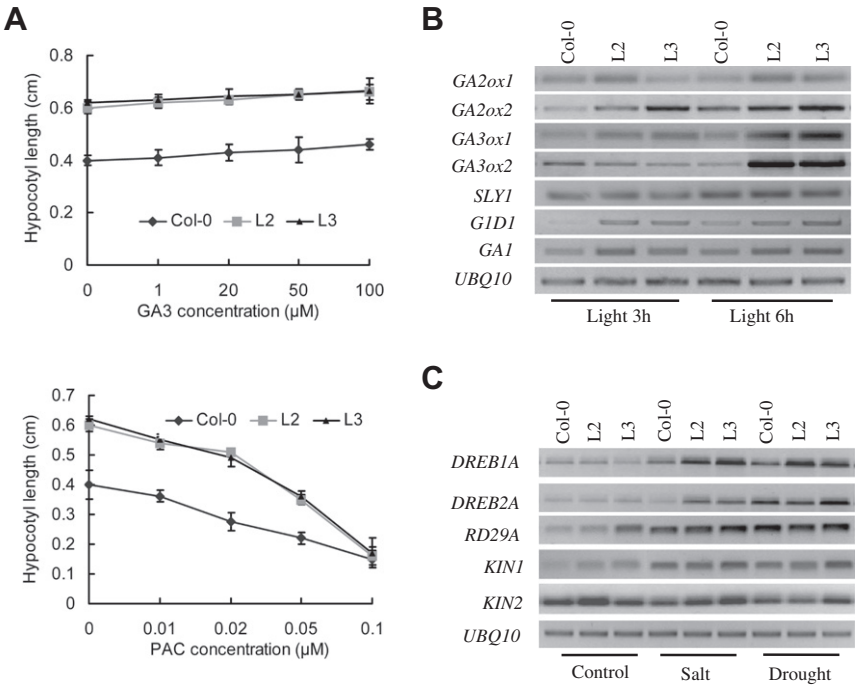

Fig. 4. (A) Effects of GA_3 or GA biosynthesis inhibitor PAC on GmCBL1-mediated enhancement of hypocotyl elongation. Seedlings were grown on ½ MS medium containing different concentrations of GA_3 or PAC. Hypocotyl lengths were measured after 5 days growth in light. Data represent average values ± SD (n = 20). (B) Alterations in transcriptional levels of GA biosynthetic genes in *GmCBL1* overexpressing plants. mRNA expression levels were determined by semi-quantitative RT-PCR. *UBQ10* was used as an internal control. The numbers of reaction cycles for *GA2ox1*, *GA2ox2*, *GA3ox1*, *GA2ox2*, *SLY1*, *G1D1*, *GA1* and *UBQ10* were 25, 28, 26, 30, 30, 28, 28 and 26, respectively. *SLY1*, *SLEEP1*; *G1D1*, *GA INSENSITIVE DWARF1A*; *GA1*, *GA INSENSITIVE*. (C) Expression of stress-responsive genes in wild-type and transgenic plants induced by 200 mM NaCl or drought treatments. *UBQ10* was used as an internal control. The numbers of reaction cycles for *DREB1A*, *DREB2A*, *RD29A*, *KIN1*, *KIN2* and *UBQ10* were 23, 23, 26, 25, 30 and 26, respectively.

we examined the effects of exogenous GA and GA biosynthesis inhibitor PAC on hypocotyl growth in *GmCBL1* overexpressing plants under light conditions. As shown in Fig. 4A, hypocotyl elongation in transgenic plants was clearly affected by the presence of PAC, suggesting GA-dependent hypocotyl growth in transgenic plants. Moreover, the expression of GA biosynthetic genes *GA2ox2*, *GA3ox1* and *GA3ox2* were obviously promoted in transgenic plants (Fig. 4B), which were highly elevated in a GA-deficient background and were responsible for GA synthesis and homeostasis of bioactive GA levels [27,28]. These findings indicate that light-dependent hypocotyl elongation in *GmCBL1* overexpressing plants is, at least a part, due to alteration in GA levels, and that GmCBL1 might serve as a molecular link in cross-talk of GA- and light-dependent hypocotyl growth.

In conclusion, GmCBL1 was shown to play diverse roles in stress response and plant development. This study provides further insight into the complex roles of CBLs in plants.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.128.

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