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Molecular Cloning, Subcellular Localization and Expression Analysis of a *BeCWINV1* Gene in Bamboo (*Bambusa emeiensis*)

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Abstract Cell wall invertase (CWINV) is one of the key enzymes in sucrose degradation, which plays a key role in assimilate partitioning, the regulation of sink strength, and plant development. In order to explore the subcellular localization and expression level of *CWINV* gene in bamboo speices, a *BeCWINV1* gene was cloned from the shoots of *B. emeiensis* based on the transcriptome database. The *BeCWINV1* was 1 758 bp in the length of open reading frame, encoding a predicted protein of 585 amino acid. Sequence alignment and phylogenetic analysis showed that BeCWINV1 contains a cell-wall invertase active site (WECPD) motif, and shared higher identity with green bamboo Boβfruct1, rice CIN2 and maize INCW2, all of them belonging to a CWINV-specific cluster with other known CWINVs. Transient expression of the *BeCWINV1*-GFP fusion protein in the onion epidermis cells suggested that BeCWINV1 was located in the cell wall, indicating its role on sucrose hydrolysis in extracellular space. qRT-PCR analysis showed that the expression of *BeCWINV1* was significantly higher in the stem than other tissues tested. The extra application of sucrose had no significant effect on the expression of *BeCWINV1*, but its expression was activated when the sugar supply was limited. These results suggested that BeCWINV1 might be involved in phloem unloading of *B. emeiensis* stem, and might also have an essential role in maintaining intracellular sugar concentration and sink activity under stress.

Keywords *Bambusa emeiensis*; Cell wall invertases; Gene cloning; Subcellular localization; Expression analysis

In higher plants, carbohydrates synthesized by prothallial cells through photosynthesis maintain the growth and metabolism of sink tissues. The transport of carbohydrates from 'source' to 'sink' is mainly in the form of sucrose (Koch, 2004). Sucrose is composed of fructose and glucose connected by O-glycosidic bond. Before participating in the physiological metabolism of plants, it should be decomposed into monosaccharides, which needs to be dependent on sucrase and invertase. The difference between the two is mainly that sucrose decomposition by sucrase is reversible(Sturm and Tang, 1999), while sucrose decomposition by invertase is irreversible (Webster et al., 2012). According to subcellular localization, invertases can be divided into cell wall invertase (CWINV), cytoplasm invertase (CINV) and vacuole invertase (VINV). CINV (optimum pH 6.8~8.0) belongs to neutral/alkaline invertase, which can only decompose sucrose specifically (Vargas and Salerno, 2010) and distribute in cytoplasm, chloroplast, mitochondria and nucleus (Maruta et al., 2010). CWINV (optimum pH 3.5~5.0) and VINV (optimum pH 3.5~5.5) belong to acidic invertase, which can catalyze the hydrolysis of some oligosaccharides (such as stachyose) in addition to sucrose hydrolysis (Roitsch and González, 2004).

Cell wall invertase (CWINV) mainly exists in the cell wall, and participates in the unloading of sugar in the extracellular transport, which plays an important role in many aspects of plant growth and development (Roitsch et al., 2003). In tomato (*Lycopersicon esculentum*), the increased CWINV activity could delay leaf senescence and increase seed weight and sugar content in fruit (Jin et al., 2009). Maize (*Zea mays*) cell wall invertase *Mn1* (*INCW2*) gene is specifically expressed in endosperm, which is an important factor in determining sink strength during grain development. The loss of Mn1 function leads to 70% yield reduction (Chourey et al., 2012). The overexpression of *OsGIF1* gene in rice (*Oryza sativa*) increased grain size and yield. On the contrary, *OsGIF1* deficiency resulted in a decrease in grain filling rate and a decrease in grain yield by 24% (Wang et al., 2008). At

the same time, the hexose produced by sucrose hydrolysis catalyzed by CWINVs is also involved in the transduction of sugar signals (Ruan et al., 2010) and plays an important role in biotic and abiotic stresses (Bi et al., 2018).

Bambusa emeiensis is one of the native large clump bamboos in Sichuan Province. It has small stem diameter with thin stem wall, excellent fiber content and length, which is a good raw material for papermaking. Sucrose is the main form of organic compound transport in *Bambusa emeiensis*. Sucrose catabolism not only provides energy substances, but also provides carbon sources for the synthesis of cellulose and starch, which plays a very important role in the growth and development of *Bambusa emeiensis* (Huang et al., 2016). However, there are few reports on the mechanism of carbohydrate transportation, metabolism and distribution during the growth of *Bambusa emeiensis*, and the cloning and function of *CWINV* have not been reported. In this study, a *CWINV* gene was cloned and its function was preliminarily predicted with the help of the phylogenetic analysis, subcellular localization and induced expression analysis, which provided a theoretical basis for revealing the unloading of photosynthetic assimilates in stem and sucrose metabolism pathway.

1 Results and Analysis

1.1 Cloning and phylogenetic tree analysis of *BeCWINV1* gene

In this study, the total RNA was extracted from young shoots of *B. emeiensis*, and reversely transcribed into cDNA as a template. PCR amplification was performed using gene-specific primers (*BeCWINV1*-F and *BeCWINV1*-R) (Table 1), and a specific *BeCWINV1* sequence was obtained. The sequencing results (Figure 1) showed that *BeCWINV1* had a complete open reading frame (ORF) with a full length of 1 758 bp encoding 585 amino acid residues.

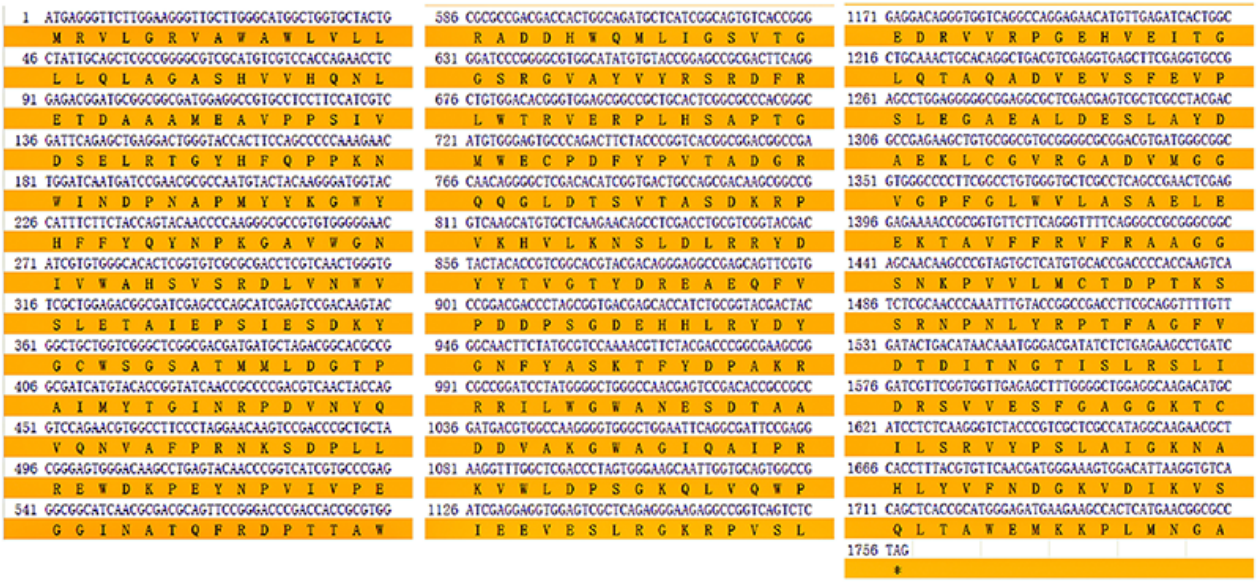

Figure 1 Full-length CDS sequence of *BeCWINV1* and putative amino acid sequence

The annotated acid invertase sequences of *Oryza sativa*, *Zea mays*, *Bambusa oldhamii*, *Vicia faba*, *Arabidopsis thaliana* and *Daucus carota* were retrieved from NCBI database to construct the phylogenetic tree. The results showed that the acid invertases of seven species, namely *B. emeiensis*, *Bambusa oldhamii*, *Oryza sativa*, *Zea mays*, *Arabidopsis thaliana*, *Vicia faba* and *Daucus carota*, can be divided into CWINVs group located in cell wall and VINVs group located in vacuole. The cell wall invertase of *B. emeiensis* (BeCWINV1) belongs to the monocotyledonous plant cluster of CWINVs group, and has close genetic relationship with *Bambusa oldhamii* FRUCT1, *Oryza sativa* CIN2 and *Zea mays* INCW2 (Figure 2).

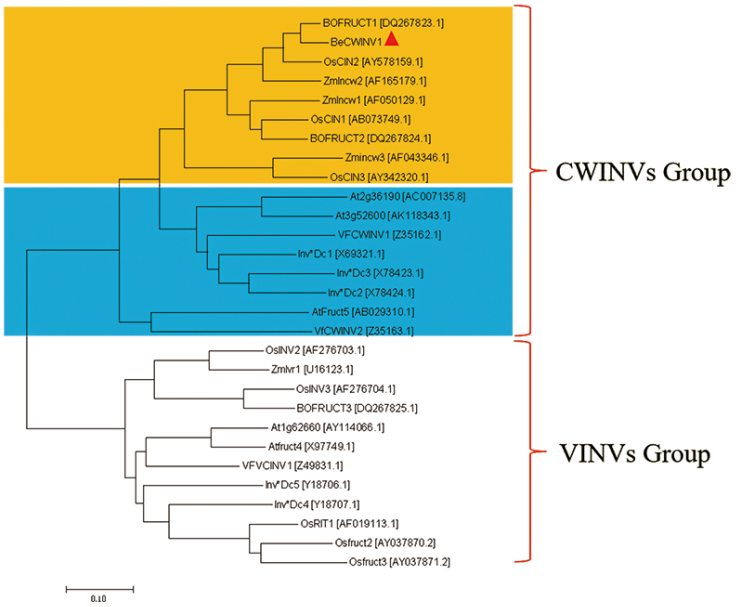

Figure 2 Phylogenetic analysis of INVs from different plant species

Note: Orange: Monocotyledons; Blue: Dicotyledons; BOFRUCT: Dendrocalamopsis invertase; OsCIN: Rice invertase; ZmIncw: Maize invertase; AtFruct: Arabidopsis invertase; VFCWINV: Broad bean invertase; Inv*Dc: Carrot invertase; Red triangle: The CWINV1 gene of *B. emeiensis*

1.2 Multiple sequence alignment of acidic invertase in *B. emeiensis*

DNAMAN analysis showed that BeCWINV1, FRUCT1, CIN2 and INCW2 squence contained typical motifs of plant acid invertase, including N-terminal β-fructosidase active site 'NWINDPNA', cell-wall invertase active site' WECPD' and C-terminal conserved 'SVVESF' sequence (Figure 3). Although fructosyltransferases in plant fructan metabolism also contain conserved sequences in acid invertases and they are highly homologous to each other, the amino acid triad (WIN) in the functional site of acid invertase activity can be distinguished from (WMN) in the functional site of fructosyltransferase activity (Hsieh et al., 2006). It was confirmed that *BeCWINV1* gene encoded acid invertase, rather than fructosyltransferase in fructan metabolism.

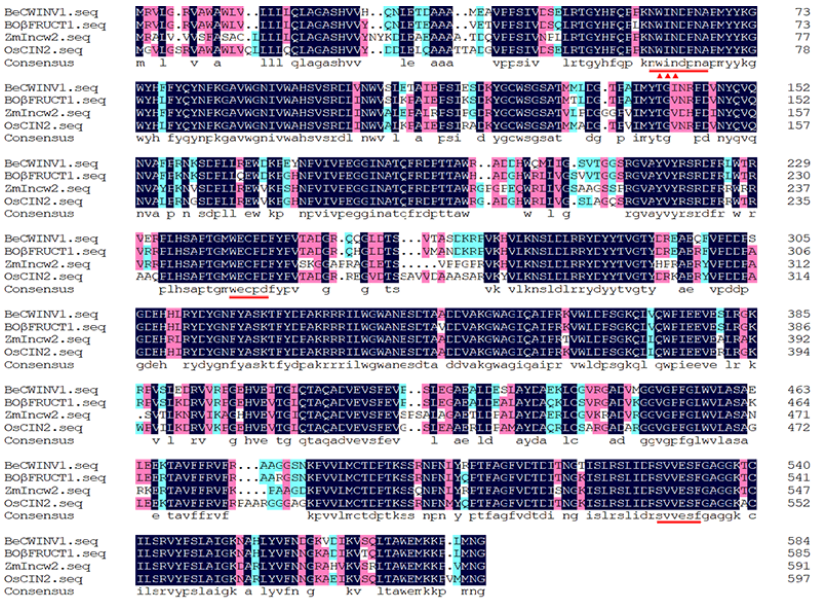

Figure 3 Amino acid alignment of BeCWINV1with other known CWINVs using DNAMAN

Note: N-treminal putative β-fructosidase (NDPN) and cell-wall invertase active site (WECPD) motifs and C-treminal conservative SVVESF-motif are underscored; BOβFRUCT1: Dendrocalamopsis acid invertase; ZmIncw2: Maize acid invertase; OsCIN2: Rice acid invertase

1.3 Subcellular localization analysis of *BeCWINV1* gene in *B. emeiensis*

To further detect the subcellular localization of BeCWINV1, a *BeCWINV1*-GFP fusion expression vector was constructed and transformed into onion epidermal cells. The cells were cultured under hypertonic conditions (mannitol 0.2 mol/L+sorbitol 0.2 mol/L) for 16 h and then observed under confocal laser scanning microscope (CLSM). Onion epidermal cells transformed by GFP-control vector had obvious green fluorescence signals in cell wall and cytoplasm. Under hypertonic conditions, obvious plasmolysis occurred in some onion epidermis cells. The green fluorescence signal of *BeCWINV1*-GFP transformers was only observed in the cell wall (not in the cell membrane and cytoplasm), indicating that BeCWINV1 localized on the cell wall and participated in the hydrolysis of sucrose in the extracellular space (Figure 4).

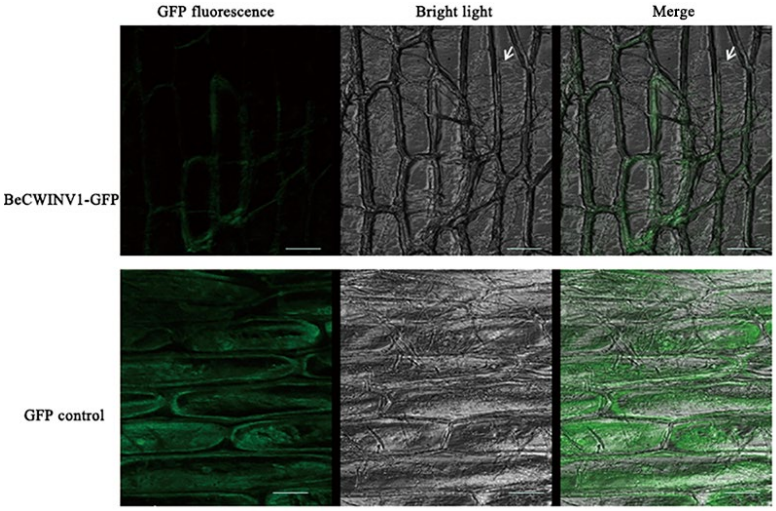

Figure 4 Localization pattern of *BeCWINV1*

Note: The white arrow shows plasmolysis; Scale: 50 μm

1.4 Tissue expression analysis of *BeCWINV1* gene in *B. emeiensis*

In order to study the expression level of *BeCWINV1* in the source and sink organs of *B. emeiensis*, total RNA was extracted from mature leaves (mesophyll and vein), young shoots (10 cm high above ground), and stem tissues in lateral branches. Real time PCR analysis was performed after reverse transcription. The relative expression of *BeCWINV1* gene in veins, lateral stems and 10 cm shoots of mature leaves was analyzed by using *Tublin* gene in *B. emeiensis* as an internal reference and the expression of *BeCWINV1* gene in mesophyll cells of mature leaves as a reference. Tissue expression analysis showed that *BeCWINV1* was expressed in all parts of *B. emeiensis*, but there were some differences in various tissues: the expression of *BeCWINV1* was lower in the veins of young shoots and mature leaves, followed by the mesophyll cells of mature leaves, and the highest expression was in the stem, which was 4~5 times higher than that of mesophyll cells and shoot tissues (Figure 5), indicating that *BeCWINV1* may play an important role in the unloading process of stem phloem.

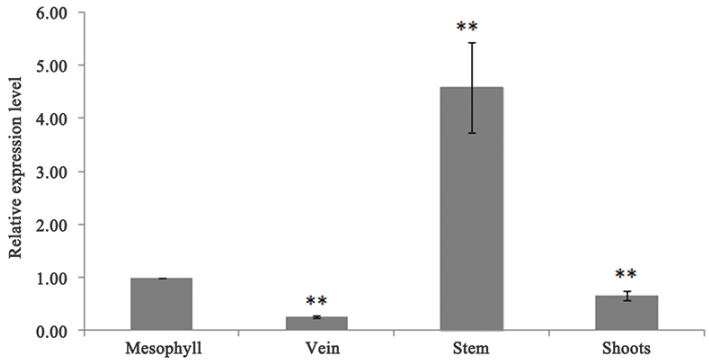

Figure 5 Tissue expression analysis of *BeCWINV1*

Note: Student-*t* test, $^{**}p<0.01$

1.5 Response of *BeCWINV1* gene in lateral branches of *B. emeiensis* to sugar signal

Previous studies have shown that CWINV is involved in sugar signal transduction (Ruan et al., 2010). In order to study the response of *BeCWINV1* gene to sugar signal, the newly sprouted branches were taken and inserted into Hoagland solution containing 100 mmol/L and 200 mmol/L sucrose for 24 h. The results showed that high concentration of sucrose (200 mmol/L) significantly inhibited the growth of lateral buds: tender buds began to fall off on the branches at 6 h of induction; the tender buds completely fell off after induction for 24 h. 100 mmol/L sucrose treatment had no significant effect on the growth of lateral buds within 24 h. Subsequently, gene expression analysis showed that glucose induction had no significant effect on the expression of *BeCWINV1*, but the expression of *BeCWINV1* increased significantly when cultured in Hoagland solution without glucose, which was 2.8~3.2 times higher than that of 0 h. Among them, the expression level was the highest after 6 h treatment, and decreased slightly at 12 h and 24 h (Figure 6), indicating that the expression of *BeCWINV1* was not activated by sugar, but was more sensitive to sugar consumption caused by its own metabolism.

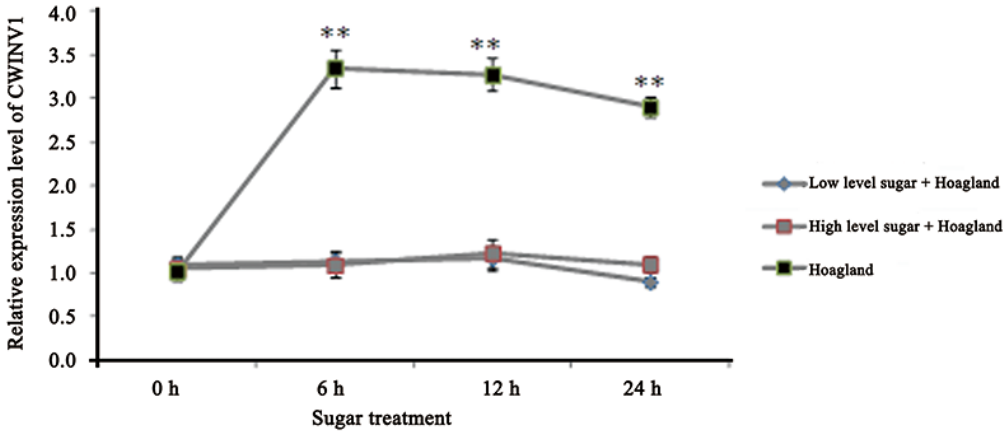

Figure 6 Effects of sugar treatment on the expression of BeCWINV1 in stems of the lateral branches
Note: Low level sugar refers to 100 mmol/L sucrose, High level sugar refers to 200 mmol/L sucrose; Student-*t* test, $^{**}p<0.01$

1.6 Effect of sugar deficiency on *BeCWINV1* gene expression in lateral branches of *B. emeiensis*

BeCWINV1 is located in the cell wall, which is mainly involved in the decomposition of sucrose in the extracellular space and the cleavage of sucrose into sugar and glucose. Monosaccharide transporter (Stp) is responsible for transporting monosaccharides in the apoplast space to cells, playing an important role in the transport, absorption, utilization and accumulation of monosaccharides, affecting the growth and development of plants, and participating in a variety of biological processes, such as the response of plants to various stresses (Rottmann et al., 2016). In order to study the effect of dark treatment on the apoplast unloading of BeCWINV1, the changes of sugar content in lateral branches and the expression of *BeCWINV1* and *Stp* genes were analyzed after dark treatment for 5~7 d.

The results showed that the soluble sugar content of lateral branch leaves decreased significantly and continuously under dark treatment. The soluble sugar content decreased from 64 mg/g FW to 55 mg/g FW at 2 d, and the leaf sugar content was only 51%~65% of the control (0 d) after 5~7 d. In the stem, the soluble sugar content increased slightly (5 d) after rapid decline (2 d), and then decreased significantly after 7 d, which decreased by 24% compared with 0 d (Figure 7A), indicating that dark treatment significantly reduced the available sugar content in lateral leaves and stems. At the same time, the sugar content in the extracellular space (extracellular free space) of the stem was analyzed by HPLC within 5 days after treatment. The results showed that sucrose content in extracellular space decreased rapidly after dark treatment for 2 d, which was only 41% of the control (0 d). Glucose and fructose content decreased slightly, but the difference was not obvious. After 5 days of treatment, the extracellular sugar content increased significantly, especially sucrose and glucose (Figure 7B).

Compared with 0 d, the expression of *BeCWINV1* in dark treatment for 2~7 d was significantly increased, and reached the peak for 5 d, which was 7 times higher than that of 0 d (Figure 7C). Similar to *BeCWINV1*, the expression of *STP5* and *STP14* increased significantly at 2~7 d under dark treatment (Figure 7C), indicating that dark treatment significantly activated the expression of *BeCWINV1* and *STPs*, and its induced expression had a sustained effect, reaching its peak at 5 d. These results showed that the extracellular transport involved by *BeCWINV1* and *STPs* played an important role in regulating the sugar catabolism and maintaining energy supply in lateral branches of *B. emeiensis* under stress conditions.

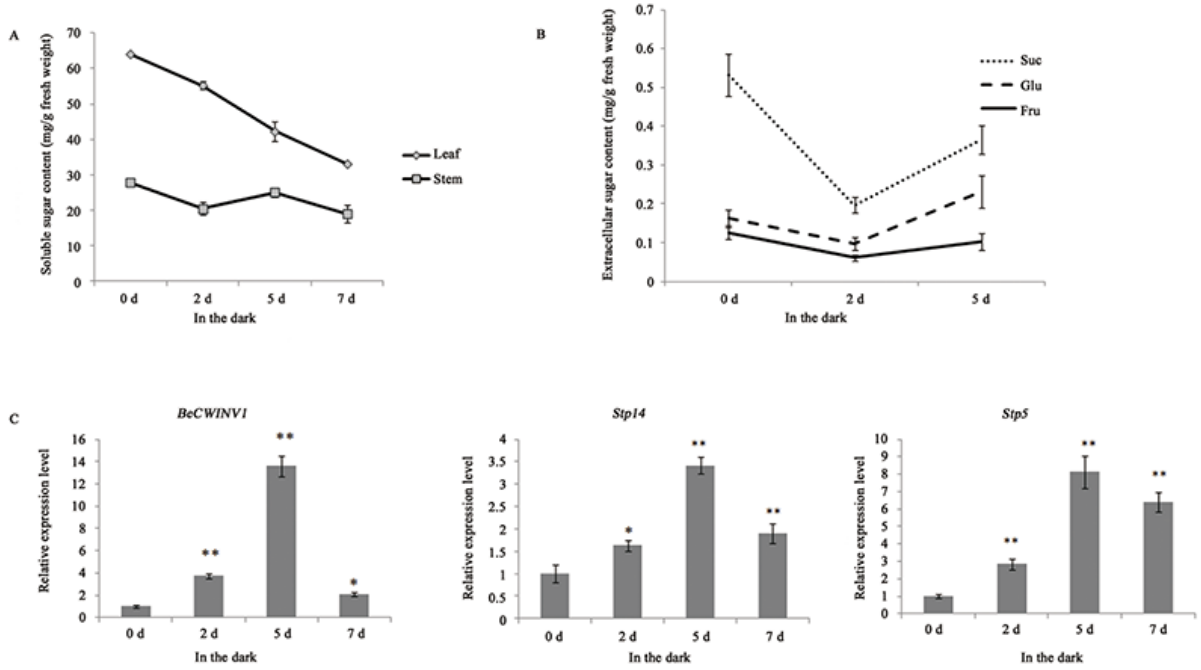

Figure 7 Effect of dark treatment on soluble sugar content (A), extracellular sugar content (B) and expression level of *BeCWINV1* and STPs (C)

Note: Student-*t* test, **p*<0.05, ***p*<0.01

2 Discussion

Sucrose invertase (INV) hydrolyzes the irreversible of sucrose into glucose and fructose, which plays an important role in regulating the content of monosaccharide and sucrose in plants, as well as the utilization and transport of sucrose. Different INVs play specific roles in plant growth and metabolism, which mainly depends on its subcellular localization. Vacuolar invertase (VINV) mainly exists in elongated tissues such as root tips and internodes, and participates in osmotic regulation, cell expansion and carbohydrate storage. Cell wall invertase (CWINV) is involved in the extracellular hydrolysis of sucrose, and its activity is closely related to the distribution of assimilates in sink cells, source-sink relationship, growth and development (Lara et al., 2004). The inhibition of cell wall invertase gene (*Lin8*) by RNAi in tomato reduced the photosynthesis of its leaves, which was mainly manifested by the significantly reduced starch content in leaves (Kocal et al., 2008). Inhibition of *CWINV* gene expression in *Litchi chinensis* resulted in abnormal seed development (Zhang et al., 2018). In this study, through subcellular localization and sequence analysis, we found that the protein encoded by *BeCWINV1* was located on the cell wall (Figure 4), which had a specific active functional site of cell wall invertase 'WECPD' and high homology with known cell wall invertases such as *Oryza sativa*, *Zea mays*, *Arabidopsis thaliana* and *Daucus carota* (Figure 2; Figure 3). This was consistent with previous research conclusions (Hsieh et al., 2006; Yan et al., 2014), indicating that *BeCWINV1* encoded protein belongs to the cell wall invertase family, which may be involved in the hydrolysis of sucrose in the extracellular space.

In general, *CWINV* is mainly highly expressed in sink tissues, while its activity in mature source leaves is usually low (Zhang et al., 2018). In this study, *BeCWINV1* was found to have low expression in the source organs of *B.*

emeiensis, including mesophyll cells synthesized by sucrose and veins loaded in phloem, but high expression in the stems rich in phloem (Figure 5), indicating that *BeCWINV1* may play an important role in the unloading of phloem assimilates in stems. Hsieh et al. (2006) also reported that the expression of *Bofruct1* and *Bofruct2* was related to development, and the expression was low in young shoots. However, with the elongation of shoots, the expression of *Bofruct1* and *Bofruct2* increased gradually, suggesting that they were involved in sucrose unloading at the shoot base. In particular, *Bofruct2* has high sucrose affinity, which plays an important role in extracellular sucrose hydrolysis and maintaining sucrose concentration gradient between source leaves and sinks. INV regulates sucrose metabolism in plants. As a signal molecule, sucrose content in plants also regulates INV expression. This study suggested that the addition of sucrose had no significant effect on the expression of *BeCWINV1*, but its expression was significantly activated when the sugar supply was limited.

CWINV catalyzes the conversion of sucrose in extracellular space into glucose and fructose. These hexose molecules are no longer transported through the phloem, but accumulate in the formation place or are transported to cells by monosaccharide transporters to participate in metabolism. Under the short-term dark treatment of mature lateral branches with leaves of *B. emeiensis*, the expression of *BeCWINV1* in stems was significantly activated, accelerating the conversion of sucrose in the extracellular space to glucose and fructose, and the sucrose content in the extracellular space was significantly decreased. At the same time, glucose and fructose in the extracellular space are rapidly transported to the cells to participate in metabolic activities, which is consistent with the up-regulation of *Stp5* and *Stp14* genes responsible for monosaccharide transport. It is suggested that the extracellular unloading process involved in BeCWINV1 plays an important role in maintaining intracellular glucose concentration and reservoir activity under stress.

Accumulation of assimilates during plant growth depends not only on the efficiency of photosynthesis, but also on the transport efficiency of photosynthetic products. Bamboo belongs to the subfamily Bambusoideae in the family of Poaceae, which grows rapidly and accumulates a large amount of organisms every year. However, the transport and distribution mechanism of assimilates during the growth and development of bamboo is still unclear. The functions of *BeCWINV1* gene in the phloem unloading of assimilates and resistance to stress remain to be further studied.

3 Materials and Methods

3.1 Plant materials

The young shoots (10 cm high above the ground), mature leaves (mesophyll and vein separation) and lateral branches of *Bambusa emeiensis* were collected for tissue expression analysis. The branches with young lateral buds (3~5 cm) were cultured in Hongland solution with different sucrose concentrations for 24 h to study the response of *BeCWINV1* gene to sugar signals. The lateral branches of *B. emeiensis* with fully unfolded leaves were taken from light for 7 days at room temperature to study the effect of dark treatment on phloem unloading with the participation of BeCWINV1. The materials were frozen in liquid nitrogen and stored in an ultra-low temperature refrigerator at -80°C. Plant materials were collected from Longshan Teaching Experimental Base of Southwest University of Science and Technology (31°32′16″N, 104°41′33″E).

3.2 Reagents and instruments

Plant RNA extraction kit, DNA purification and recovery kit, LA-Taq fidelity polymerase, *Xba*I, *Pst*I restriction endonuclease, pMD19-T vector, SuperReal PreMix Plus (SYBR Green) reagent were purchased from TaKaRa (Dalian) Biotech. Green View dye was purchased from BioBRK. Plasmid extraction kit, X-Gal, and IPTG were purchased from Tiangen Biotech (Beijing) Co. Ltd. *E. coli* DH5α was prepared by our laboratory.

3.3 Cloning and bioinformatics analysis of *BeCWINV1* gene

A *CWINV* gene of *B. emeiensis* (*BeCWINV1*) was screened from the transcriptome database, and the specific full-length primers were designed with Primer Premier 5.0 software (Table 1). The cDNA of *B. emeiensis* stems was used as the template for PCR amplification, and the products were recovered and ligated into the T vector to import into competent *E. coli* DH5α. After blue-white screening, the positive recombinant plasmid was selected

and sent to BGI for gene sequencing. A number of acid invertase amino acid sequences with confirmed functions were obtained from NCBI and Rice genome databases. The genetic distance was calculated as 0.476 (less than 1) using MEGA 7.0 software, and the phylogenetic tree was constructed by Neighbor-joining method. The amino acid sequence of BeCWINV1 was subjected to multiple sequence alignment using DNAMAN software after 1 000 self-inspection.

3.4 Construction of expression vector

Sequence analysis combined with pTEX vector to identify the appropriate double enzyme digestion sites and design primers (Table 1), and PCR amplification was performed using the previously sequenced plasmid as the template. After digestion and recovery with *XbaI* and *PstI*, it was directly constructed into the multi-clone site of the vector PTEX-GFP to form a plant expression vector containing the fusion of *BeCWINV1* gene and GFP.

3.5 Gene gun bombardment and subcellular localization observation

The inner epidermal cells of monolayer white onion were isolated and cultured in MS hypertonic medium (mannitol 0.2 mol/L+sorbitol 0.2 mol/L) for 4 h under dark. The *BeCWINV1*-GFP fusion expression vector was introduced into onion epidermis cells by PDS-1000/He gene gun (BIO-RAD of USA). After 16 hours of dark culture, GFP fluorescence was detected by laser scanning confocal microscope (Leica SP8). The excitation of GFP protein was 488 nm.

3.6 Analysis of gene expression

Tublin gene of *B. emeiensis* was used as internal standard to design the RT-PCR primers with the help of the Primer-Blast in NCBI database (Table 1). After using SuperReal PreMix Plus (SYBR Green) kit 20 μL, the PCR reaction was performed on IQ5 Multicolor RT-PCR automatic amplification instrument according to 'Three Steps'. After the reaction, the dissolution curve was analyzed to confirm the non-primer dimer and non-specific amplification. Each sample was repeated three times and the gene expression in different tissues was calculated by $2^{-\triangle\triangle Ct}$ method.

Table 1 Primers used in gene cloning and real-time PCR analysis in this study

Primer name	Primer sequence (5'-3')
BeCWINV1-F	ATGAGGGTTCTTGGAAGGGTTG
BeCWINV1-R	CTAGGCGCCGTTCATGAGTG
BeCWINV1-RT-F	TGGGACGATATCTCTGAGAAGC
BeCWINV1-RT-R	CGTTCATGAGTGGCTTCTTCAT
BeStp5-RT-F	CTTCTTCTCGCCGATACTGTTC
BeStp5-RT-R	ACCTGGCAAATGAACATGAGTG
BeStp14-RT-F	GTGTGCGTCAACCTCTTCTG
BeStp14-RT-R	AGTACCAGTGCTTGTCGAAGA
BeCWINV1-Xba Ⅰ-F	GCTCTAGAGCATGAGGGTTCTTGGAAGGGTTG
BeCWINV1-Pst Ⅰ-R	TGCACTGCAGTGCAGGCGCCGTTCATGAGTGGCTTC
Tublin-F	ATAGTGTTTGGATTGGAGGTTC
Tublin-R	GGATAGCCAAGATCGTATAAGG

3.7 Analysis of sugar content

Soluble sugar content in lateral branches, leaves and stems of *Bambusa emeiensis* was determined with the help of anthrone colorimetry (Wang and Huang, 2015, Higher Education Press, pp.171-173). The extracellular (intercellular space) sugars in stems were extracted according to the method of Wood et al. (1998), and centrifuged at low speed (6 000 rpm) for 15 min to collect extracellular free sugars. The contents of sucrose, glucose and fructose were analyzed by HPLC-ELSD. The sample was filtered by 0.22 μm filter membrane, the mobile phase was ultrapure water, the flow rate was 0.6 mL/min, the chromatographic column was Agilent Hi-plex Ca type, the column temperature was 80°C, and the detector was evaporative light-scattering detector.

Authors' Contributions

FJC is the the experimental designer and executor of this study, completing data analysis and writing the first draft of the paper. LTJ assisted in the experiment and data analysis. YQQ and WBW participated in the analysis of the experimental results. CY is the designer and director of the project, guiding experimental design, data analysis, paper writing and revision. All authors read and approved the final manuscript.

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