



# Isolation and functional characterization of the *SpCBF1* gene from *Solanum pinnatisectum*

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Received: 21 April 2017/Revised: 23 October 2017/Accepted: 9 April 2018/Published online: 8 May 2018  
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**Abstract** Low temperature causes a negative impact on plant growth and development, but plants evolve a series of mechanisms to respond to chilling stress, and one of them is *CBF* [C-repeat (CRT)/dehydration-responsive element (DRE) binding factor] gene family which has been well studied in different crops. In this paper, a new *CBF1* gene, named as *SpCBF1*, was isolated from frost-tolerant *Solanum pinnatisectum* by PCR and analyzed for its function in cold-tolerance by over-expression technique. The ORF of *SpCBF1* was 666 bp long and encoded a protein of 221 amino acids with a predicted molecular mass 24.5821 kDa and theoretically isoelectric point 5.0. *SpCBF1* protein contained a highly conserved specific AP2/ERF domain. *SpCBF1* was expressed in all tested tissues with the highest level in tuber and the lowest in root, and induced by chilling stress (0 °C). Under natural low temperature condition (1–10 °C), plants over-expressing *SpCBF1* (OE) exhibited slighter necrotic lesion and lower necrotic injury, compared with untransformed *Solanum tuberosum* cv. Désirée (WT) and antisense-*StCBF1* control lines. Over-expression of *CBF1* increased the level of *COR* (cold-regulated) gene transcripts in OE lines, and the

physiological indexes related to cold tolerance like the contents of SOD, soluble protein, MDA, proline and soluble sugar were higher in OE lines than in WT except RWC which was lower. All these results indicated that *SpCBF1* gene plays a promoting role in potato responding to cold stress.

**Keywords** *Solanum pinnatisectum* · *SpCBF1* · Cloning · Cold tolerance · Functional analysis

## Abbreviations

OE	Over-expression
WT	Untransformed control
COR	Cold-regulated
<i>Sp</i>	<i>Solanum pinnatisectum</i>
<i>CBF</i>	C-repeat (CRT)/dehydration-responsive element (DRE) binding factor
SOD	Superoxide dismutase
MDA	Malonaldehyde
RWC	Relative water content

## Introduction

Low temperature occurring during the growth period often causes severe adverse effects on plant growth and development. Plants evolve a series of mechanisms to respond to chilling stress, so studies of these mechanisms can not only enrich our understanding of plant cold tolerance, but also provide a theoretical basis for cold-tolerance breeding.

Plants adapt to the chilling stress at molecular, cellular, physiological and biochemical levels by changing the expression of a variety of genes (Nakashima et al. 2009), which are thought to induce the accumulation of

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s12298-018-0536-1>) contains supplementary material, which is available to authorized users.

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superoxide, hydrogen peroxide, and hydroxyl radicals to elevate stress tolerance (Xiong et al. 2002).

Among these genes responding to chilling stress, *CBF* family is of much interest because of the broad regulation functions. *CBFs* encode transcriptional activators which play a key role in cold-acclimation process during plant growth and development. The *CBF* protein contain an AP2/ERF domain (Stockinger et al. 1997), which can be distinguished from other AP2 domain containing proteins by amino acid signature sequences that flank the AP2 domain (Jaglo et al. 2001). When plants are exposed to low non-freezing temperature, a kind of transcriptional activator ICE (inducer of *CBF* expression) is activated and then induces the expression of *CBFs* by combining with the ICE box in the promoter of *CBF* genes. Subsequently, transcription factors *CBFs* recognize the CRT/DRE sequences which are present in promoter regions of many *COR* genes and then induce a series of *COR* expressions (Thomashow 2001). Most of *COR* polypeptides have amphipathic  $\alpha$ -helical regions which may have roles in stabilizing membranes against freezing damage and thus improving freezing tolerance of plants (Thomashow 1999). For now, many crops like rice, tobacco, strawberry, tomato and cucumber overexpressing *Arabidopsis CBF1* showed strong cold tolerance (Lee et al. 2004; Wei et al. 2006; Jin et al. 2009; Hsieh et al. 2002; Singh et al. 2011; Gupta et al. 2012).

In *Arabidopsis thaliana*, six of *CBF*-encoding genes have been found, and *CBF1*, *CBF2* and *CBF3* form a head-to-tail, tandemly-linked gene cluster on chromosome 4 (Gilmour et al. 1998; Medina et al. 1999). Over-expression of *CBF1* or *CBF3* in transgenic *Arabidopsis* induced the expression of multiple cold-responsive CRT/DRE-containing genes and then enhanced cold tolerance of plants, whereas *CBF2* mutants obviously showed stronger cold hardiness than wild-type whether cold acclimatized or not (Novillo et al. 2007), which demonstrate that *CBF1* and *CBF3* play positive regulation roles in inducing expression of *CORs*, but *CBF2* plays a negative role. The other *CBFs* disperse across the genome and are not responsive to low temperature, but are responsive to other stresses like drought (Haake et al. 2002; Sakuma et al. 2002).

Up to now, in addition to *Arabidopsis*, *CBFs* or their analogue have been identified in *Secale cereale* (Jaglo et al. 2001), *Brassica napus* (Jaglo et al. 2001; Gao et al. 2002), *Triticum aestivum* (Jaglo et al. 2001; Shen et al. 2003), *Hordeum vulgare* (Choi et al. 2002; Xue 2002, 2003), *Oryza sativa* (Dobouzet et al. 2003), *Solanum lycopersicum* (Jaglo et al. 2001; Zhang et al. 2004), *Zea mays* (Qin et al. 2004), *Capsicum annuum* (Kim et al. 2004), *Prunus avium* (Kitashiba et al. 2004), *Glycine max* (Li et al. 2005), *Avena sativa* (Bräutigam et al. 2005), *Populus* (Benedict et al. 2006), *Betula* (Welling and Palva 2008), *Hordeum spontaneum* (James et al. 2008), *Lolium perenne* (Xiong and

Fei, 2006; Zhao and Bughrara 2008), *Solanum commersonii* (Pennycooke et al. 2008), *Eucalyptus gunnii* (Navarro et al. 2009), *Solanum habrochaites* (Li et al. 2014) etc. In Solanaceae, the three *CBF* genes of many tomato cultivars, *Solanum habrochaites*, and two of the *Solanum pimpinellifolium* accessions are linked in series (Pennycooke et al. 2008), however, in contrast to *Arabidopsis*, not all three genes are responsive to cold. For example, only *CBF1* is low temperature responsive in *S. lycopersicum* and *S. habrochaites* (Zhang et al. 2004; Pennycooke et al. 2008).

Potato, a crop that is not resistant to both high temperature and low temperature, likes cool growth conditions. The majority of the potato cultivars were not resistant to chilling stress, which has become the limiting factor in potato production, greatly affecting the scope of use of potato varieties and their planting time. Therefore, improving the cold tolerance of potato varieties will be able to achieve the potato early planting or prolonged cultivation, increasing the yield of potatoes, which will need available excellent cold tolerance genes.

In potato, previous studies showed that *CBF1* and *CBF4* are cold-responsive in both *S. tuberosum* and *S. commersonii* (Pennycooke et al. 2008). Therefore, to further explore the cold-tolerant genes, in this study, we cloned a *SpCBF1* gene from *Solanum pinnatisectum*, a wild potato which shows strong tolerance to chilling stress and introduced it to cultivar Désirée. Further studies at the levels of phenotype, physiology, and biochemistry indicated that *SpCBF1* over-expression enhances cold tolerance in potato plants by modulating the expression of *COR* genes.

## Materials and methods

### Plant materials and preparation

In this study, *S. pinnatisectum* was used as *SpCBF1* gene donor and potato cultivar Désirée was employed as *SpCBF1* gene receptor. The in vitro plantlets of both materials were cultured under a 12 h light: 12 h dark regime at 25 °C on MS medium (Murashige and Skoog 1962) containing 3% sucrose and 0.8% agar at pH 5.8. The 4-week-old plantlets were used for gene cloning or as explants source for transformation experiments, or transplanted to pots stuffed with an 1:1:1 mixture of loam, vermiculite and perlite and grew in growth chamber for cold treatment experiments and expression analysis.

### *SpCBF1* cloning

*SpCBF1* gene was isolated from *S. pinnatisectum* plantlets with specific primers 5'-AGATCTTTAGCTCTCAACAACAATGAA-3' and 5'-ACTAGTTGTACTATTTAGA

TAGAATAA-3' designed based on potato *CBF1* in the Solanaceae genomics resource by RT-PCR. Total RNA was extracted from leaves by TRIzol reagent (Invitrogen, USA) and then pre-treated by DNase I. Purified RNA (3  $\mu$ l) was reversely transcribed to the first strand of cDNA using reverse transcriptase (TaKaRa) in a 20  $\mu$ l reaction volume. The PCR thermal cycling parameters for amplifying *SpCBF1* were 72 °C for 10 min followed by 95 °C for 4 min and 28 cycles of 95 °C for 45 s, 48.5 °C for 45 s, 72 °C for 45 s. PCR product was assessed by 1% agarose electrophoresis. Subsequently, the product of PCR was gel-purified with AxyPrep Gel DNA Extraction Kit (Axygen, China), and inserted into the pMD19-T vector (TaKaRa) for sequencing (Beijing Genomics Institute, China).

### Construction of phylogenetic tree

The comparative sequence analysis was performed using ClustalX program. The phylogenetic tree of plant CBF proteins was constructed with the MEGA program (v5.1) by the neighbor-joining method with 1000 bootstraps. The tertiary structure of *SpCBF1* was predicted by Swiss Model Server (<http://swissmodel.expasy.org/>).

### Tissue-specific and time-course expression pattern of *SpCBF1*

Tissue expression analysis and cold-induced expression analysis were conducted by semi-quantitative RT-PCR. For *SpCBF1* tissue expression analysis, total RNA was extracted from roots, stem, leaves, flowers, stolons and tubers of *S. pinnatisectum* plants using TRIzol reagent (Invitrogen, USA). For cold-induced expression analysis, total RNA was extracted from leaves of the plants exposed to 0 °C for 0, 1, 2, 4, 8, 16, 24 and 48 h, respectively, using TRIzol reagent. The RNAs were reversely transcribed to the first strand of cDNA using reverse transcriptase (TaKaRa) as described above. The conserved region of *SpCBF1* was specifically amplified with the primers: 5'-AACCTATTATTTCAGACCCAC-3' and 5'-TGACTTCAAACCCATT-3' under the following reaction conditions: 94 °C for 5 min, followed by 29 cycles (25 cycles for cold-induced expression analysis) of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 30 s and a final extension step of 72 °C for 10 min. *SpEF-1 $\alpha$*  (Elongation Factors-1 $\alpha$ ) was used as an inner control standard and its 150 bp fragment was amplified with the primers 5'-TGAAAGCGAGGAAA-GAATA-3' and 5'-TCCCATGAATGACCCGA-3' under the following reaction conditions: 94 °C for 5 min, followed by 18 cycles of 94 °C for 20 s, 54 °C for 20 s, 72 °C for 20 s and a final extension step of 72 °C for 10 min. PCR products were separated on 1% agarose gel and stained with ethidium bromide for UV visualization.

Quantity One software was used to analyze gray value of bands to help adjust the sample volume for the same brightness of the internal *EF-1 $\alpha$*  bands from different samples, and the brightness of the target gene bands showed the difference of expression levels.

### Potato transformation and identification

The ORF sequence of *SpCBF1* was inserted into the position behind CaMV 35S promoter of the vector pCAMBIA1304 designating as pCAMBIA1304-*SpCBF1* (Fig. 3a). The *StCBF1* antisense sequence was cloned from potato cultivar Désirée using primers (primers 5'-AGATCTCTAAAGCTGCCACGTCAT-3' and 5'-ACTAGTTTCACTATCATACTTTCCCACT-3') and also inserted in pCAMBIA1304 as pCAMBIA1304-*antisense-StCBF1*. The vectors were transferred into *Agrobacterium tumefaciens* (strain GV3101) by the method of freeze-thaw (Hofgen and Willmitzer 1998). Stem internodes of Désirée plantlets were infected with vector-harbored strain and shoots regenerated as described by Liu et al. (2012).

To identify transgenic plants, two pairs of specific primers (primers 5'-AACCTCCTCGGATTCCATTG-3' and 5'-GAAGCTAAAATAATTTCCCTCGTCAG-3' and primers 5'-AACCTCCTCGGATTCCATTG-3' and 5'-CTGCACGTATCCCTCAGGC-3') were designed for amplifying part of pCAMBIA1304-*SpCBF1* and pCAMBIA1304-*antisense-StCBF1*. PCR was conducted with the following conditions: 94 °C for 5 min, followed by 32 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 35 s or 94 °C for 5 min and 30 cycles of 94 °C for 50 s, 53 °C for 55 s, 72 °C for 60 s and a final extension step of 72 °C for 10 min, respectively. PCR products were separated on 1% agarose gel and stained with ethidium bromide for UV visualization.

### Expression analysis of *CBF1* in transgenic plants

To analyze the expression of *CBF1* in transgenic plants, 3 plants of each OE line (A2, A3 and A6), each antisense line (B2, B9) and non-transgenic plants (WT) were used. Four-week-old in vitro transgenic lines and WT plantlets were transferred to pots and cultured under a 12 h light: 12 h dark regime at 25 °C for four weeks. Then, they were exposed to 0 °C for 24 h. Quantitative RT-PCR (qRT-PCR) were carried out with total RNA derived from leaves of transgenic lines and WT plants using the same primers (5'-AACCTATTATTTCAGACCCAC-3' and 5'-TGACTTCAAACCCATT-3') for amplifying conserved fragment of *SpCBF1* and *StCBF1* with the same condition described above. *StEF-1 $\alpha$*  was used as an inner control standard and the primers and PCR reaction conditions were the same as described above. qRT-PCR was accomplished in ABI7500

Fast Real-Time PCR Systems machine (ABI, USA) according to the following SYBR Premix Ex Taq (TaKaRa) reaction system and steps: 94 °C for 1 min, (95 °C 15 s, 54 °C 20 s) × 40. The corresponding expression level ( $2^{-\Delta\Delta CT}$ ) of *CBF1*, *PI* and *DHN10* mRNA were calculated as follows:  $\Delta\Delta CT = (C_{T,Target} - C_{T,EF-1\alpha}) \times X - (C_{T,Target} - C_{T,EF-1\alpha}) \times T$ . X stands for different lines and T stands for one time expression of target gene calibrated by *StEF-1 $\alpha$* .

### Expression analysis of *COR* genes

*COR* genes *StPI* (Genbank: EU849681.1) and *StDHN10* (Genbank: EU849680.1) were examined for their expression level using the same cDNAs for analysis of *CBF1* in OE lines by qRT-PCR. Primers (5'-GACATTGC-GAGTCGTTGA-3' and 5'-CTCATTTAGCATGGCT-TAGTGC-3') were designed for amplifying the specific fragments of *StPI* and primers (5'-CATCACAAA GATGGAAAA-3' and 5'-GTTAGGTAAATAGGAAA TCA-3') were utilized for amplifying the specific fragments of *StDHN10*. PCR conditions: 94 °C for 4 min followed by 28 cycles of 94 °C for 25 s, 54 °C for 25 s, 72 °C for 25 s and a final extension step of 72 °C for 10 min. qRT-PCR was performed as described in expression analysis of *CBF1* above.

### Measure of physiological and biochemical indexes related to cold-tolerance

Twelve plants of OE lines (A2, A3 and A6) and WT were moved to growth chambers and exposed to 0 °C for 48 h. After the treatment, these plants were dedicated for the assay of superoxide dismutase (SOD), soluble proteins, malonaldehyde (MDA), relative water content (RWC), proline and soluble sugars contents.

#### RWC assay

RWC was measured by the method of Jiang and Zhang (2001)

#### MDA content

About 1 g fresh tissue of the plantlets above was grinded in 4 ml 0.6% 2-thiobarbituric acid (TBA) using a mortar and pestle. The homogenate was centrifuged at 4000 r/min for 10 min, and the 2 ml supernatant was then mixed with 2 ml 10% trichloroacetic acid (TCA). MDA was determined by Jin's method (Jin et al. 2013).

### SOD enzyme activity and soluble protein content

Fresh leaves (0.5 g) were grinded in a mortar and pestle with 8 ml of 50 mM cool phosphate buffer (pH 7.8) containing 1% (w/v) polyvinylpolypyrrolidone (PVP) for the detection of superoxide dismutase (SOD, EC 1.15.1.1). The homogenate was centrifuged at 10,000 g for 25 min at 4 °C, and supernatant was used for the assay of enzyme-activity detection and protein-content determination. Total SOD activity was assayed according to the method described before (Yang et al. 2013). One unit of SOD (U) was defined as the amount of crude enzyme extract required to inhibit the reduction rate of NBT by 50%. Soluble proteins were determined by Bradford's method (Bradford 1976) using bovine serum albumin (BSA) as a standard.

### Contents of proline and soluble sugars

Fresh leaves (0.5 g) were cut into chips and extracted in 5 mL of 1% boiled sulfosalicylic acid for proline assay. Freed proline was determined by acid ninhydrin method (Bates et al. 1973) using a spectrophotometer in a wavelength of 520 nm, using ninhydrin as specific reagent and proline as standard. Soluble sugars were determined by anthrone method (Li et al. 2000) using sucrose as standard.

### Cold treatment assay

To implement cold treatment, 15 plants of A3, antisense-*StCBF1* (a *StCBF1*-downregulated transgenic line constructed and preserved by our laboratory: B9, Fig. S1) and WT lines were placed in greenhouse with natural light and temperatures from Dec. 03, 2013. to Jan. 03, 2013 (the average daily minimum temperature is 1 °C and the average daily maximum temperature is 10 °C. Nanjing, 32°02'38" of North latitude). The number percentage of necrotic injuries ( $W2/W1 \times 100\%$ , W1 means the gross weight of 20 leaves from 5 plants which contained every four leaves from bottom to top; W2 means the weight of necrotic parts of blade including necrotic spots and water stained parts cut by scissors) was used as an index of the ability to resist low temperature and the estimation of necrotic injury was conducted by the method of Janowiak et al. (2003). The experiment was repeated three times.

### Statistical analysis

Data were means  $\pm$  standard errors of three independent experiments. Student's t test (two-tailed) was conducted where appropriate. The asterisk indicates a significant difference (\* means  $P < 0.05$ , \*\* means  $P < 0.01$ ) in two comparisons.

## Results

### *SpCBF1* cloning and expression pattern

Using the known related species tomato *CBF1* sequence as reference, we performed alignment using BLAST, the potato *CBF1* gene from the Solanaceae genomics resource (<http://solanaceae.plantbiology.msu.edu/>), and one *S. tuberosum* fragment (PGSC0003DMT400037121) having high homology with that in tomato was obtained, which was first named as *StCBF1* in our work. Then, *SpCBF1* was amplified from *S. pinnatisectum* with the sequence-specific primers designed according to *StCBF1* sequence by RT-PCR. The cDNA of *SpCBF1* consisted of an open reading fragment of 666 bp, encoding a protein of 221 amino acid residues with a predicted molecular mass 24.5821 kDa and theoretically isoelectric point 5.0. The *SpCBF1* had no intron when compared with the DNA sequence given in the Solanaceae genomics resource. SpCBF1 protein contained a highly conserved specific AP2/ERF domain like other CBF proteins and alignment analysis showed that the amino acid sequence of SpCBF1 was highly similar to its homologues derived from other 10 plant species (Fig. 1a). Three-dimensional structure analysis showed that the polypeptide contained one  $\alpha$ -helix and three  $\beta$ -pleated sheets (Fig. 1b). Phylogenetic analysis demonstrated that SpCBF1 was clustered in a clade with other *Solanum* plants and was most close with the CBF1 of *S. commersonii* and *S. tuberosum* (Fig. 1c).

### Tissue-specific and chilling-treated expression pattern of *SpCBF1*

To study the expression pattern of the *SpCBF1* gene, the level of this gene mRNA was measured in various tissues of *S. pinnatisectum* by semi-quantitative RT-PCR. The result showed that *SpCBF1* was expressed in all tested tissues and the highest level was observed in tuber then in stems and leaves, and the lowest level in roots (Fig. 2a). To know the response of *SpCBF1* to cold stress, *S. pinnatisectum* plants were subjected to 0 °C treatment at different times. The results showed that *SpCBF1* was induced by cold treatment and the highest level of the transcript appeared at 2 h treatment, then the content decreased gradually (Fig. 2b).

### Production and identification of potato transgenic lines

*SpCBF1*-overexpressing transgenic potato lines were constructed using potato cultivar Désirée as receptor (Fig. 3a). In total, 8 independent over-expression (OE) lines were obtained by screening of hygromycin B resistance and termed as A1–A8. Electrophoretic analysis identified an expected band in 5 OE transgenic lines (A2, A3, A4, A6, A8) (Fig. 3b). Further, the expression level of *CBF1* in three OE lines (A2, A3, A6) was detected by qRT-PCR. The results showed that the level of *CBF1* transcript was higher in the OE lines, compared with WT control (Fig. 3c), indicating that the transgenes had been integrated into potato genome and expressed normally.

### *CBF1* regulates *COR* expression in transgenic lines

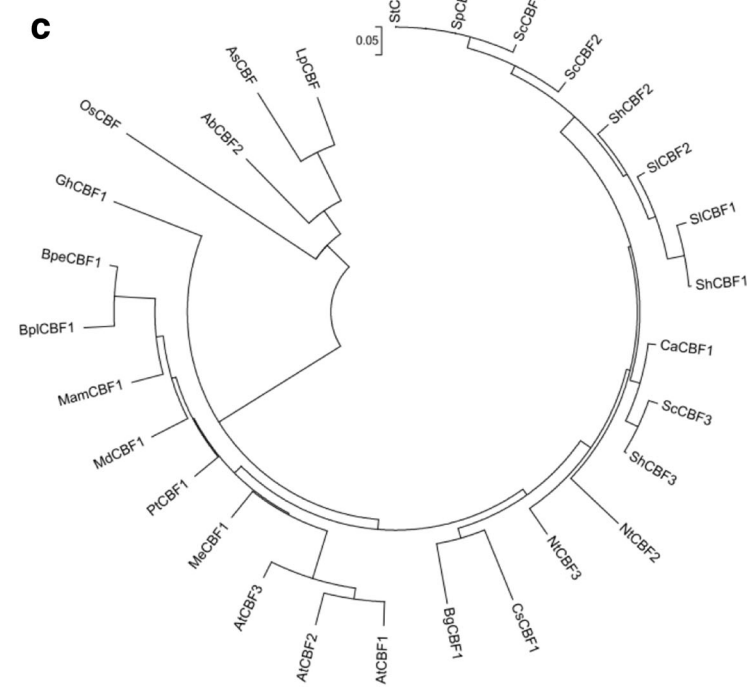
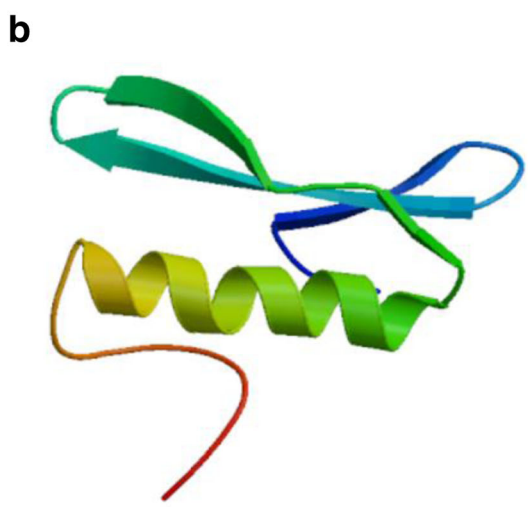
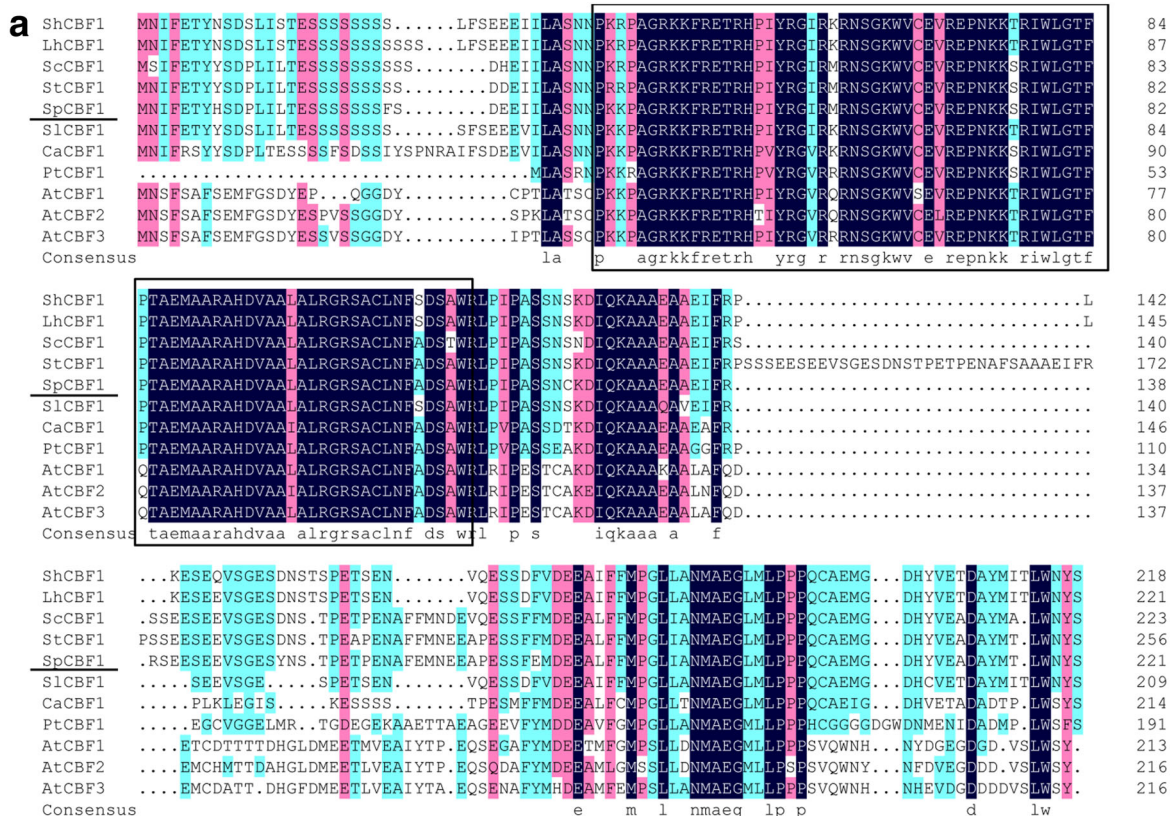
*COR* gene is a class of genes that are located downstream of the *CBF* gene, which respond to low temperature. To understand how *SpCBF1* regulates plant cold tolerance, the expression of *CBF1*, *StPI* (Genbank: EU849681.1) and *StDHN10* (Genbank: EU849680.1) in OE lines (A2, A3, A6) exposed to 0 °C condition were analyzed (Fig. 4). The result showed that the expression of *CBF1*, *StPI* and *StDHN10* in OE lines increased compared with WT and they had the same expression pattern, suggesting that *CBF1* in potato under cold stress regulates the expression of *PI* and *DHN10*.

### Change of physiological and biochemical indexes in transgenic lines

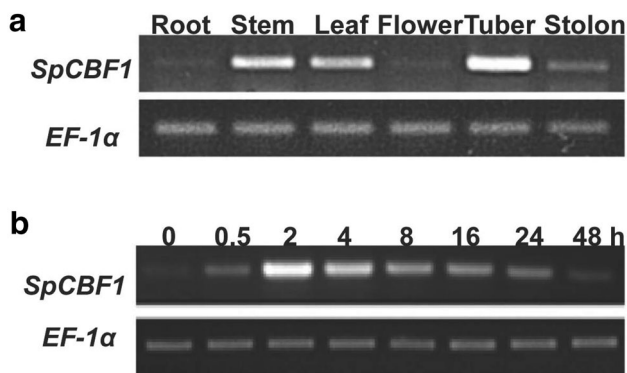
Under low temperature, some physiological and biochemical indexes of plants, such as RWC, MDA, ROS, soluble protein, proline and soluble sugar, will be changed to the corresponding adversity. In this study, the six indexes were checked in A2, A3, A6 and WT under 0 °C condition. The result showed that the contents of RWC, SOD, soluble proteins, proline and soluble sugars in OE lines were higher, in contrast with the WT (Fig. 5a, c–f) except MDA which was lower (Fig. 5b). These results showed that overexpression of CBF1 could make the physiological indexes in favor of adaptation to cold stress, which enhanced the cold resistance of plants.

### Analysis of cold-tolerance abilities of potato transgenic lines

Plants that grow at chilling temperature often exhibit symptoms of damage on the phenotype, such as water-soaking, wilting, and necrotic lesions. In order to get closer insights, three potato lines (A3, WT and antisense line)



**Fig. 1** Sequence alignment, tertiary structure model and phylogenetic analysis of SpCBF1. **a** Amino acid sequences alignment. Sequences used for alignment are derived from *Solanum tuberosum* (StCBF1, ACB45096.1), *Solanum commersonii* (ScCBF1, ACJ26751.1), *Solanum habrochaites* (ShCBF1, ACB45087.1), *Solanum lycopersicum* (SICBF1, AAS77820.1) *Lycopersicon hirsutum* (LhCBF1, BAE17131.1), *Capsicum annuum* (CaCBF1, AAR88363.1), *Populus trichocarpa* (PtCBF1, ABO48363.1), *Arabidopsis thaliana* (AtCBF3, ABV27138.1), *Arabidopsis thaliana* (AtCBF1, AAV80413.1), *Arabidopsis thaliana* (AtCBF2, AAV80415.1). Boxes indicate conserved regions of CBFs. **b** Tertiary structure model of SpCBF1. **c** Phylogenetic analysis. Sequences were aligned by CLUSTALX and analyzed using the MEGA 4.1. Construction was carried out by the parsimony method with 1000 bootstrap replicates and a consensus tree was generated. Database accession numbers are as follows: *Solanum pinnatisectum* (SpCBF1), *Solanum tuberosum* (StCBF1, ACB45096.1), *Solanum lycopersicum* (SICBF1, AAS77820.1; SICBF2, AAS77821.1), *Solanum commersonii* (ScCBF1, ACB45093.1; ScCBF2, ACB45094.1; ScCBF3, ACB45092.1), *Solanum habrochaites* (ShCBF1, ACB45087.1; ShCBF2, ACB45088.1; ShCBF3, ACB45078.1), *Capsicum annuum* (CaCBF1, AAR88363.1), *Populus trichocarpa* (PtCBF1, ABO48363.1), *Manihot esculenta* (MeCBF1, AFB83707.1), *Morus alba* var. *multicaulis* (MamCBF1, AFQ59977.1), *Arabidopsis thaliana* (AtCBF1, AAV80413.1; AtCBF2, AAV80415.1; AtCBF3, ABV27149.1), *Nicotiana tabacum* (NtCBF2, ACE73694.1; NtCBF3, ACE73695.1), *Betula pendula* (BpeCBF1, ADZ23479.1), *Betula platyphylla* (BplCBF1, ADZ23479.1), *Malus domestica* (MdCBF1, AAZ20446.1), *Camellia sinensis* (CsCBF1, AFN93974.1), *Gossypium hirsutum* (GhCBF1, ABD65473.1), *Bruguiera gymnorhiza* (BgCBF1, AFU81298.1), *Oryza sativa Japonica Group* (OsCBF, NP\_001056909.1), *Lolium perenne* (LpCBF, BAF36838.1), *Avena sativa* (AsCBF, CAJ21276.1), *Aegilops biuncialis* (AbCBF2, CBX87016.1)



**Fig. 2** Expression analysis of *SpCBF1* gene in different tissues under normal conditions (**a**) and in response to low temperature in time course (0, 0.5, 2, 4, 8, 16, 24, 48 h) (**b**). The *EF-1α* gene was used as the internal control

were placed under natural conditions in winter for cold-tolerance assay. Phenotypic observation showed that water-soaking, wilting, and necrotic lesions occur on both of OE and the control plants after 2 weeks of growth at low temperature (1–10 °C), but the degrees of injuries varied among the different lines. There were less necrotic lesions on the leaves of A3 line and more serious water-soaking,

wilting and necrotic lesions on the leaves of antisense-*CBF1* line and WT (Fig. 6a).

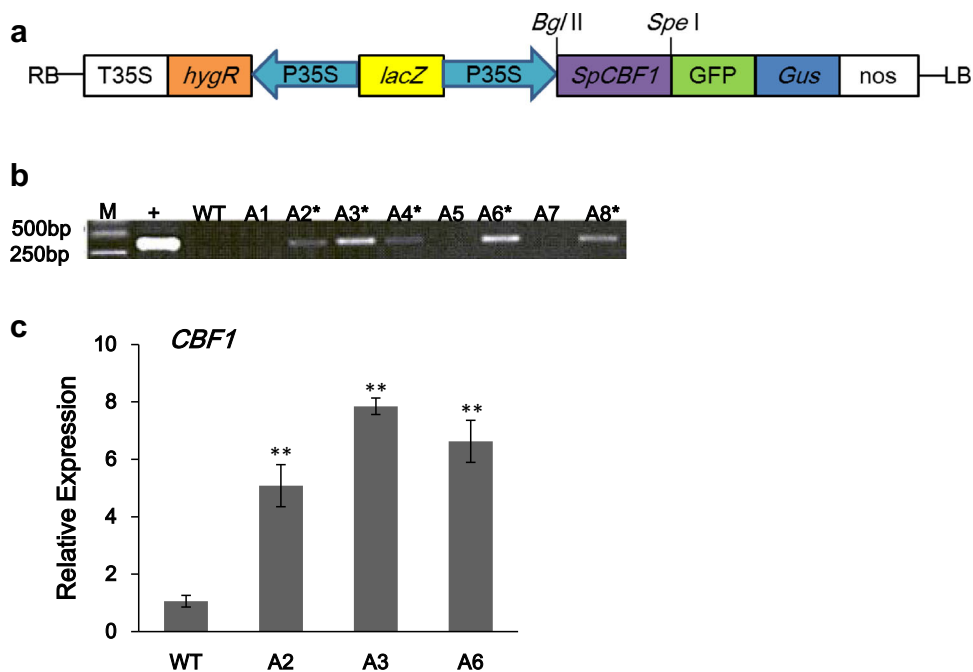
Necrotic lesion has been used in earlier studies as reliable parameter to evaluate the degree of chilling injury (Janowiak and Markowski 1987; Janowiak et al. 2002). Damage analysis showed that after growth at low temperature for one month, the percentage of necrotic lesions on leaves was evidently lower in A3 line (6.25%) and higher (11.67 and 15.08%) in WT and antisense-*CBF1* line (Fig. 6b). The results demonstrated that over-expression of *SpCBF1* enhanced potato plant tolerance to cold stress, which were consistent with the results of physiological and biochemical indexes analyzed above.

## Discussion

*CBF1* gene, an important member of *CBF* family, has been isolated from different plants which can be mentioned as *Arabidopsis thaliana* (Stockinger et al. 1997), *Brassica campestris* (Gao et al. 2002), *Hordeum vulgare* (Xue 2003), *Solanum lycopersicum* (Hsieh et al. 2002) etc. In potato crop, *CBF* genes are only reported in *Solanum commersonii* and *Solanum tuberosum*, and the expression of *CBF1* and *CBF4* is induced by low temperature (Pen-ycooke et al. 2008). In this study, we cloned a *CBF1* gene from *S. pinnatisectum*, a potato wild relative with strong cold resistance. The *SpCBF1* shared 95% homology with *S. commersonii* *CBF1* (Fig. 1a) and its encoding protein was highly similar to *CBF1* from *Solanum* plants (Fig. 1c) and had an AP2 domain, demonstrating that *SpCBF1* belonged to *CBF* gene family.

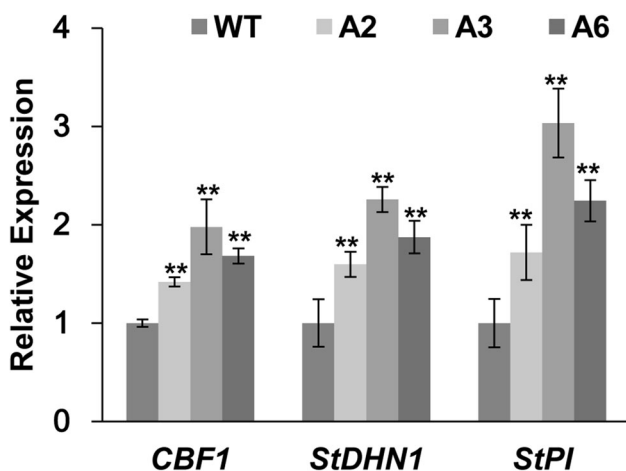
In *Arabidopsis*, tomato and some potatoes, *CBF1* is a low temperature inducible gene (Novillo et al. 2007; Pen-ycooke et al. 2008). In this study, we examined the expression of *SpCBF1* under 0 °C and the result showed that it was also induced by chilling temperature, whose value reached the highest level after 2 h (Fig. 2b), suggesting that *SpCBF1* is a cold-sensitive gene like that in *Arabidopsis* and tomato.

It has been reported that *CBF1* is involved in the process of drought resistance and cold resistance in plants (Stockinger et al. 1997; Xu et al. 2014). Over-expression of *AtCBF1* could obviously improve cold tolerance of plants such as potato, strawberry, tomato and cucumber (Pino 2006; Jin et al. 2009; Singh et al. 2011; Gupta et al. 2012). In order to study the function of *SpCBF1*, we constructed *SpCBF1* overexpressing transgenic lines. Then, we detected the cold resistance index and performance of the transgenic lines in molecular, physiological and phenotypic aspects (Figs. 4, 5, 6). Cold resistance analysis showed that cold injury in the transgenic line overexpressing *SpCBF1* was only 1/2 of WT and 1/3 of *StCBF1* antisense line. The



**Fig. 3** The construction and PCR identifications of *SpCBF1* transgenic potato lines. **a** schematic representations of pCAMBIA1304-*SpCBF1* vector with site of restriction enzymes. **b** PCR-based identifications of over-expression transgenic lines. The DNA template was extracted from bacteria (lane +), non-transformed (lane WT) and transformed (lane A1-8) lines. \* means the positive line. M, marker DL2000. **c** Quantitative RT-PCR analyses of *CBF1* transcripts in

over-expression transgenic lines. Total RNAs were extracted from leaves of transgenic lines (A2, 3 and 6) and non-transgenic lines (WT). EF-1 $\alpha$  expression was used as the loading control. Error bars indicate SE. The asterisk indicates a significant difference (\* $P < 0.05$ ; \*\* $P < 0.01$ ) between transgenic lines and WT at 25 °C. Student's *t* test (two-tailed) was conducted



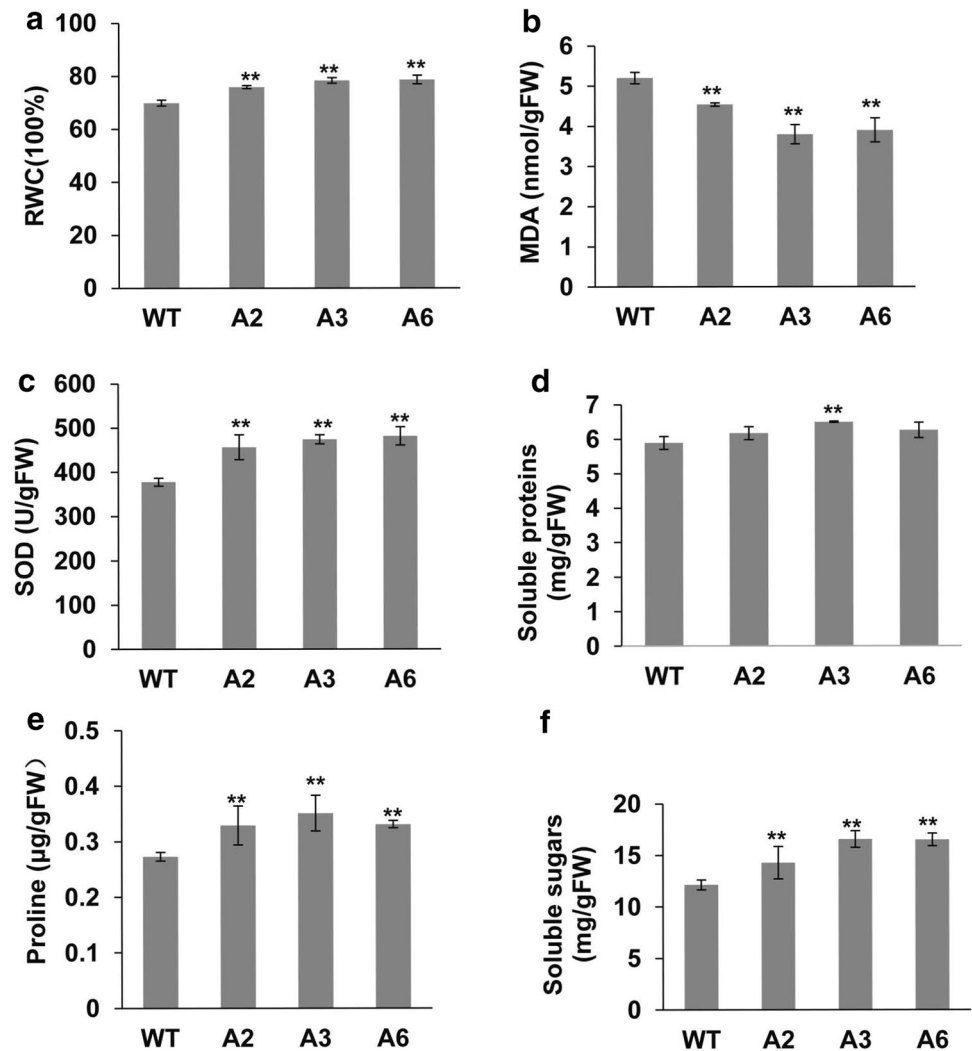
**Fig. 4** Quantitative RT-PCR analyses of *CBF1*, *StDHN10* and *StPI* transcripts in transgenic lines and WT under 0 °C after 24 h treatment. Total RNAs were extracted from leaves of transgenic lines (A2, 3 and 6) and WT. EF-1 $\alpha$  expression was used as the loading control. Error bars indicate SE. The asterisk indicates a significant difference (\* $P < 0.05$ ; \*\* $P < 0.01$ ) between the transgenic lines and WT. Student's *t* test (two-tailed) was conducted

*COR* genes are the targets of transcription factors CBFs (Jaglo et al. 2001), so we detected the transcriptional levels of two *COR* genes *StDHN10* and *StPI*, and the expression of *StDHN10* and *StPI* was induced by constitutively

expressing *SpCBF1* (Fig. 4). In *Arabidopsis*, ectopic constitutive over-expression of *CBF* genes can induce the expression of *COR* genes in whole plant cold-resistance in the absence of chilling stress (Van Buskirk and Thomashow 2006). Similar result is also observed in tomato where a set of *COR* genes are responsive to *CBF* over-expression (Zhang et al. 2004). Low temperature stress may cause a series of physiological changes in plants. Physiological analysis revealed that in transgenic lines RWC, ROS, the contents of soluble protein, proline and soluble sugar were increased and MDA was declined, compared with WT. RWC is an important index that reflects the growth status of plant under cold stress. MDA is a kind of product of membrane-lipid peroxidation whose content reflects the level of membrane injury under chilling stress. When facing chilling stress, the plant can increase the level of MDA and reduce ROS (reactive oxygen species) accumulation and subsequent oxidative damage to increase its tolerance. Soluble proteins, proline and soluble sugars, as osmolytes in plants, can prevent cell from cold-induced water loss which can cause many dysfunctions. Our results suggest that *SpCBF1*, by inducing the expression of *COR* genes and modulating the stability of the cell membrane, positively regulates the cold tolerance of potato.



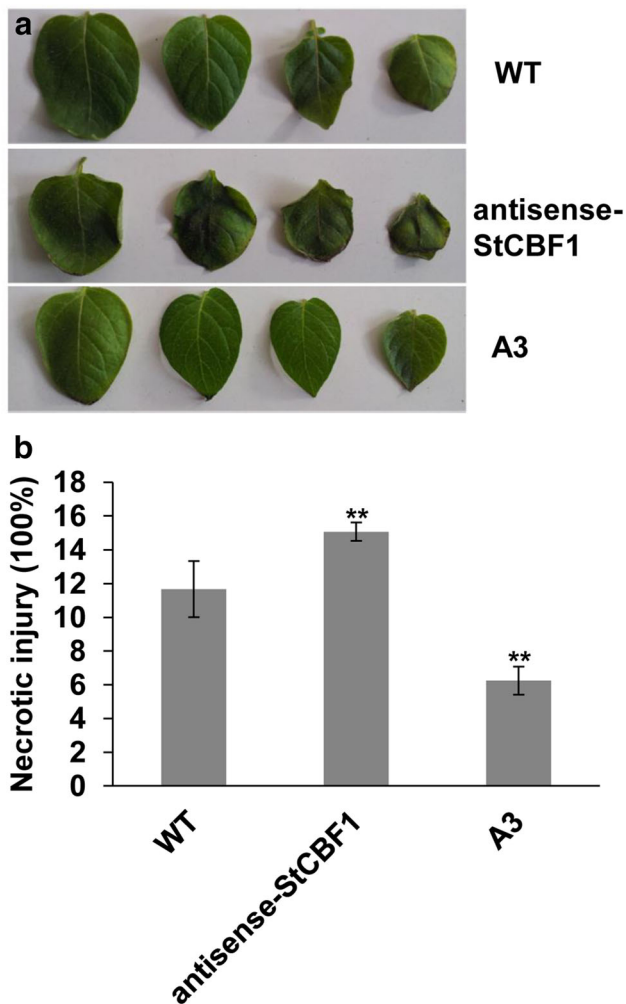
**Fig. 5** Physiological and biochemical index of transgenic lines (A2, 3 and 6) and WT under 0 °C for 48 h. Determination of RWC (a), MDA (b), SOD (c), soluble proteins (d), Proline (e) and soluble sugars (f). Error bars indicate SE. The asterisk indicates a significant difference (\* $P < 0.05$ ; \*\* $P < 0.01$ ) between transgenic lines and WT. Student's  $t$  test (two-tailed) was conducted



Different *Solanum* species evolve different cold-resistant mechanisms. *Solanum tuberosum* cv. Désirée could not acclimatize well when exposed at 4 °C for 3 weeks while another two species *Solanum phureja* CHS and *Solanum tuberosum* PS3 are more cold-resistant (Oufir et al. 2008). Analysis of transcriptome data and contents of soluble carbohydrates, polyols and free polyamines in these three *Solanum* plants showed their different responses to the cold stress were caused by many affected genes involved in carbohydrate and polyamine metabolism (Oufir et al. 2008). This result implied that there is a differential response to cold stress among different species due to the transcript expression. In our work, overexpressing *SpCBF1* in cultivar Désirée enhanced its cold tolerance (Fig. 6), indicating that the different cold tolerance of *Solanum tuberosum* and *Solanum pinnatisectum* may due to the different expression of *CBF1* gene or the different sequence between these two potatoes, which needs to be further studied.

*CBF1* not only conferred tolerance to low temperatures (Lee et al. 2004; Wei et al. 2006; Jin et al. 2009; Hsieh et al. 2002; Singh et al. 2011; Gupta et al. 2012), but also confers tolerance to other stresses. Overexpressing *ScCBF1* in *Solanum tuberosum* and *Solanum commersonii* plants enhanced their drought tolerance (Pino et al. 2013). *AtCBF1* overexpression in potato plants enhanced salt tolerance (Kwon et al. 2013) and confers tolerance to high light conditions at 22 °C by enhancing the ability to cope with an excess of radiant energy (Storani et al. 2015). In our work, we only detected the chilling tolerance of overexpressing *SpCBF1* in cultivar Désirée, further tolerance assays for other stresses like drought, salinity and high-light condition should be studied for its further function analysis.

Our work provides significant insights in the function of the gene *SpCBF1* in regulating cold tolerance in *Solanum* and *SpCBF1* may be utilized for modifying potato cultivar cold tolerance. Our data obtained show only that this



**Fig. 6** Leaves phenotypes of transgenic line A3, WT and antisense-*StCBF1* control (a) and percent of necrotic injury (b) under natural light and temperature in winter. Error bars indicate SE. The asterisk indicates a significant difference (\*\* $P < 0.01$ ) among A3, WT and antisense-*StCBF1* control. Student's *t* test (two-tailed) was conducted

protein is involved in chilling tolerance but not in freezing tolerance. Therefore, further studies in freezing tolerance are needed to elucidate its cold-resistant mechanism.

**Acknowledgements** This study was supported financially by grants from the National Natural Science Foundation of China (11171155), the National Pear Industry Technology System (NO. CARS-29) and A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions: Modern horticultural science (PAPD).

**Author contributions** Z.W., S.K., Y.X. and Y.Q. designed research; S.K., T.R., M.X., C.J. and M.C. performed research; S.K., Z.W., M.X., C.J. and Y.Q. analyzed data; and Z.W., S.K. and Y.Q. wrote the paper.

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