Improved Cold-resistant Performance in Transgenic Grape (*Vitis vinifera* L.) Overexpressing Cold-inducible Transcription Factors *AtDREB1b*

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Abstract. Dehydration response element binding (*DREB*)*1b* is a cold-inducible transcription factor in *Arabidopsis thaliana*. *DREB1b* driven by cauliflower mosaic virus 35S promoter was genetically introduced into grape *Vitis vinifera* L. cv. Centennial Seedless through *Agrobacterium*-mediated transformation for improving its cold resistance and exploring new genetic breeding approaches to obtain cold-resistant cultivars. In this study, Southern blot analysis showed the *DREB1b* gene was integrated into the transgenic grapevines with one to two copies. Northern blot analysis showed the presence of *DREB1b* transcripts in the independent transgenic lines 3, 5, 6, and 7. Further characterization of transgenic grapevines confirmed that both electrolyte leakage conductivity and the freezing point of the transgenic plants were lower than those of wild-type plants. After the cold treatment at -4°C for 12 h, 26% of transgenic plants wilted among which 95% plants recovered once being placed under the condition of temperature 22 to 25°C . However, subjected to the same treatment, 98% of non-transgenic plants wilted and only 2% recovered. Our results lead to the conclusion that activity of *DREB1b* in the transgenic grape could significantly improve its resistance to cold stress.

Temperature is a key environmental factor that influences plant growth and development as well as geographical distributions of many plant species. Low temperature often adversely affects crop quality and productivity (Thomshow, 1999). Grapes are a widely grown fruit crop in the world. ‘Centennial Seedless’ of *Vitis vinifera* L. is one of the most widely grown grape varieties in China

because it is suitable for both table consumption and wine production (Kong, 2004). The berries of the variety are heart-shaped, medium-sized, and thin-skinned. The berry flesh is firm and has a strong Muscat flavor. The soluble solid content of the berries usually ranges from 15% to 17% (Kong, 2004). However, ‘Centennial Seedless’ is quite sensitive to low temperature and its production is often seriously reduced as a result of the early spring or late fall frosts in China (Chao et al., 2001).

A cold-resistant variety of grape may be bred by conventional cross, although it is time-consuming and has low efficiency. Now, the development of plant genetic biotechnology has offered a new avenue for the genetic improvement of grape cultivars (Perl et al., 2000). Transgenic techniques can readily transfer a foreign gene into the genome of a plant in a faster and more efficient manner.

For plant cold resistance improvement, it is known that plant cold resistance depends on multiple gene interactions. A lot of cold-responsive genes contain CCGAC, the core sequence of the DRE/CRT (dehydration-responsive element/C-repeat) *cis*-element in their promoters (Stockinger et al., 1997). A family of transcription factors, DREB1s binds to this element and activates transcription of the downstream cold genes (Chinnusamy et al., 2003). *DREB1b* is a cold-inducible transcription factor functioning as a molecular switch for cold-regulated (COR) genes such as LTI (low-temperature-induced), CAS (cold acclimation-specific), and RD (responsive to desiccation). The *Arabidopsis DREB1b* gene is located in tandem array on chromosome 4 (Gilmour et al., 1998) and the protein of *DREB1b*, including the AP2 DNA-binding domain, can bind to the DRE/CRT *cis*-element in the promoter regions of many cold response genes, consequently leading to the activation of this gene transcription. Overexpression of *DREB1b* can significantly activate COR genes, which in turn increases the degree of cold resistance in *Arabidopsis* plants (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Stockinger et al., 1997; Van Buskirk et al., 2006). Therefore, introduction of *AtDREB1b* into different plants probably can activate the relevant gene regulatory network in the host plants and consequently significantly improve the transgenic plant resistance to cold stress. In this article, the *Arabidopsis* transcription factor *DREB1b* was introduced into the genome of *Vitis vinifera* L. through *Agrobacterium tumefaciens* with the aim to improve cold resistance in grape.

Materials and Methods

Plant materials and explants preparation. Grape cultivar Centennial Seedless (*Vitis vinifera* L.) was used in this study. Six-week-old nonwoody shoots of the cultivar were cut into one-node segments. The shoot segments were washed with tap water for 60 min and surface-sterilized by immersing them in 0.1% HgCl_2 for 8 min. After being rinsed three times in sterile distilled water, the segments were cultured in 40 mL Murashige and Skoog (MS) solid medium (Murashige and Skoog, 1962) containing 3% sucrose and combinations of $0.5\text{ mg}\cdot\text{L}^{-1}$ 6-benzyl amino purine and $0.2\text{ mg}\cdot\text{L}^{-1}$ indole-3-butyric acid (IBA) under a 16/8-h light/dark regime and $30\text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ illuminance at $25 \pm 2^{\circ}\text{C}$. After 20 d, young shoots from axillary buds were transplanted onto the media containing half-strength MS salts and full-strength vitamins, $30\text{ g}\cdot\text{L}^{-1}$ sucrose, $6\text{ g}\cdot\text{L}^{-1}$ agar, and $0.2\text{ mg}\cdot\text{L}^{-1}$ IBA.

Transformation and regeneration of grape explants. The *DREB1b* gene was isolated from *Arabidopsis* and placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in the pBPDREB binary vector carrying the selectable marker of neomycin phosphotransferase II (*npt II*) gene (Fig. 1). The pBPDREB vector was then

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Fig. 1. Diagram of binary vector pBPDREB with the *DREB1b* gene. LB and RB = left and right borders of T-DNA, respectively; NPTII = neomycin phosphotransferase II; CaMV 35S = cauliflower mosaic virus 35S promoter; nos3 = terminator of nitric oxide synthase 3.

introduced into *Agrobacterium tumefaciens* strain LBA4404 for transformation experiments. *Agrobacterium*-mediated grape transformation was carried out according to the protocols of Das et al. (2002) and Jin et al. (2008) with some modifications. Leaf discs 4 to 6 mm in diameter were excised from 35-d-old aseptic plantlets and infected with overnight *Agrobacterium* culture. After being blotted dry, the infected leaf discs were placed on solid MS medium plus 2.0 mg·L⁻¹ thidiazuron. Adventitious shoots (1 to 2 cm long) generated from the infected leaf discs were excised and subcultured in solid MS medium containing 10 mg·L⁻¹ kanamycin for selection.

DNA extraction and polymerase chain reaction analysis. Fresh leaves (250 mg) from each of the independent transformants were homogenized in liquid nitrogen for genomic DNA extraction with the cetyl triethyl ammonium bromide method (Thomas et al., 1993). A pair of primers, DREB1b-F (5'-GTACTCTAGTCAATTGAGACTC-3') and DREB1b-R (5'-GAAACGACTATCGAATATTAG-3'), was used for detecting the presence of *DREB1b* in polymerase chain reaction (PCR) analyses, which was conducted following the protocol described by Zhen et al. (2000).

Southern blot analysis. Genomic DNA samples (15 µg) from both the transgenic and wild-type plants were digested with

*Hind*III (Takara Company, Dalian, Liaoning Province, China), electrophoresed in a 1.5% agarose gel, and transferred to Hybond-N⁺ nylon membranes (Dingguo Company, Beijing, China). The *DREB1b* coding sequence was labeled with digoxigenin (DIG) using the DIG nucleic acid labeling kit (Roche Company, Basel, Switzerland) and then used as the hybridizing probe. Hybridization signal detection was carried out according to Roche's protocol.

RNA isolation and Northern blot analysis. Total RNA was isolated from young leaves of both transgenic and wild-type plants with Trizol agent (Dongsheng Company, Beijing, China). RNA was subject to formaldehyde-containing agarose gel electrophoresis and transferred onto Hybond-N nylon membranes. The *DREB1b* coding sequence was used as hybridizing probes after being labeled with DIG. Hybridization signal detection was carried out according to the manufacturer's protocol.

Cold resistance test of the transgenic grape. Biological tests for cold resistance of the transgenic grapevine were conducted as follows: the methods of electrolyte leakage test by (Jaglo-Ottosen et al. 1998, 2001) were used for analysis of cold resistance of the transgenic plant using 10 leaf discs for each set with a total of three replicates. Freezing temperature is another indicator of the degree of cold resistance of a plant (Kang et al., 1998). Freezing points of leaf, stem, and petiole samples of both the transgenic and wild-type grapevines were estimated according to Wang et al. (2003). There were three replications per treatment, including leaf, stem, and petiole treatments. Duncan's multiple comparison tests were conducted. Moreover, 100 clonal grapevines from both 3-month-old transgenic Line 3 and wild-type grapevines were used for the growth recovery test after a cold stress treatment according to (Jaglo-Ottosen et al. 1998, 2001).

Results

Generation and characterization of the transgenic lines. After *Agrobacterium* culture infection, grape leaf discs turned dark brown during the first several days. The number of leaf explants cocultivated with

the *Agrobacterium* strain was 4480. Callus appeared 8 to 10 d after inoculation and 100% calli were produced and 35.6% regenerative calli were induced 28 d after cultured in the selective media. Transformants were generated from green or light green calli as shown in Figure 2. The number of explants regenerating on kanamycin selective medium was 326. The number of plants regenerated and surviving on kanamycin selective medium was 45.

To test the presence of the transgene in the putative transformants produced in the selective media, PCR analyses were conducted using genomic DNA of the 45 transformants and *DREB1b*-specific primers. Thirty-nine independent transgenic plants were produced and integration of the *DREB1b* gene was confirmed by PCR analyses and the PCR-positive rate was 87%. Among those positive transgenic plants, there were four, labeled Line 3, 5, 6, or 7, respectively, containing the *DREB1b* gene with the expected fragment size of 640 bp, as found from positive plasmid control (Fig. 3A). These four transgenic plant lines were used for further studies.

Southern blot analysis confirmed the PCR results of the transgenic plants. There was either one or two copies of the transgene present in the transformant plants in this study (Fig. 3B). Transgenic lines (3, 5, 6, or 7) showed distinguishable banding patterns indicating that these four lines were independent of each other.

Northern blot analyses were performed to examine the transcript levels of the *DREB1b* gene in the four transgenic plants. Hybridization signal bands were present in all four transgenic lines but absent in wild-type plants (Fig. 3C). Furthermore, two bands of *DREB1b* mRNA were detected in transgenic Lines 3 and 7, both of which just contained a single copy of *DREB1b* transgenic plant lines.

Cold resistance for the transgenic grape. Cell membrane damage can cause the leakage of cell components. Electrolyte leakage, a measurement of the extent of cell membrane damage, is commonly accepted as a cold-resistance indicator of plants (Jaglo-Ottosen et al., 1998). Detached leaves of transgenic Lines 3 and 7 as well as wild-type plants were subjected to various subzero temperatures.

Fig. 2. Regeneration of the transgenic grapevines. (A) Adventitious shoots generated from the infected leaf discs; (B) elongated shoots with expended leaves; (C) roots formed on the medium with kanamycin.

Fig. 3. Molecular analysis of transgenic grapevines. (A) Polymerase chain reaction analysis of transgenic plants. M = DNA marker (λ DNA/HindIII + EcoRI); Lane 1, plasmid DNA of pBPDREB; Lane 2, wild-type grapevine; Lanes 3–8, independent lines survived from selective media containing kanamycin; (B) Southern blot analysis of transgenic grapevines. Genomic DNA was extracted from transgenic Lines 3, 5, 6, and 7. The 640-bp *DREB1b* coding region from plasmid (P) and DNA from wild-type plant (WT) were used as positive and negative control, respectively; (C) Northern blot analysis of the transgenic grapevines: (1) Northern blot results; (2) growth status of the plants, in order of wild-type plants, transgenic Lines 3, 7, 5, and 6 from left to right.

Leaf cell membrane damage was then estimated by measuring the electrolyte leakage. Our results showed that the electric conductivities of transgenic plants were significantly lower than those of the wild-type plants (Fig. 4). The conductivity of wild-type plants was remarkably increased at -4°C , whereas such a significant increase in the conductivity of the transgenic plants was found at -6°C . No significant differences in the conductivity were found between Line 3 and Line 7. These results indicated that the transgenic grapevines overexpressing the *DREB1b* gene were more resistant to cold stress.

Table 1 showed that the freezing points of transgenic Lines 3 and 7 were 1.51 and 2.02°C lower than that of wild-type, respectively. In all transgenic lines and wild-type plants, the organs listed in an order of as-

cended freezing points were root $\rightarrow$ stem $\rightarrow$ leaves.

To evaluate the cold resistance of the transgenic plants, 100 of 30-d-old clonal plants from both transgenic Line 3 and wild-type plants were transplanted into pots and grown at room temperature for 2 months and then subjected to -4°C for 12 h. After the cold stress, water stains and wilting were observed on the leaves of the tested plants. There were 26% of transgenic plants wilted, whereas wilted wild-type plants were 98%. These cold-stressed plants were then placed at 22 to 25°C for 2 weeks for recovery. Ninety-five percent of the transgenic plants and only 2% of the wild-type seedlings recovered (Fig. 5), whereas 98% of the wild-type seedlings and only 5% of the transgenic plants died.

Discussion

Genetic transformation of grapes started in the late 1990s by means of somatic embryonic regeneration, mostly using pollen as an explant. *Agrobacterium tumefaciens* mediated *nptII* gene transformations in *Vitis rupestris* Scheele species (Mullins et al., 1990) and in grape cultivar of 'Sultana' (*Vitis vinifera* L.) (Franks et al., 1998). The coat protein gene of grapevine fanleaf virus was also introduced into rootstock varieties 'SO4' (*Vitis vinifera* $\times$ *Vitis berlandieri*) and '41B' (*Vitis berlandieri* $\times$ *Vitis riparia*) (Mauro et al., 1995) through *Agrobacterium*-mediated gene transformation. In this experiment, we successfully introduced the *DREB1b* gene into grape cultivar Centennial Seedless through *Agrobacterium tumefaciens* using

Fig. 4. Cold tolerance of transgenic and wild-type leaves. The tested plants were treated at the indicated temperatures and degree of the cellular damage was estimated by measuring electrolyte leakage. Error bars indicated as sds.

Table 1. Freezing points of the transgenic Lines 3 and 7 and wild-type (WT).

Material	Leaf tissue	Stem tissue	Roots
WT	-4.25 ± 0.28 b	-7.04 ± 0.37 b	-9.51 ± 0.33 b
Transgenic Line 3	-6.18 ± 0.30 a	-8.55 ± 0.31 a	-11.35 ± 0.46 a
Transgenic Line 7	-6.29 ± 0.35 a	-8.90 ± 0.43 a	-11.63 ± 0.32 a

Note: Different letters indicate statistically different variations at $P = 0.05$.

Fig. 5. Recovery of the plants at 22 to 25 °C after being treated at -4 °C for 12 h.

grape leaf discs as an explant. *DREB1b* gene integration and expression in the transgenic plants were confirmed using PCR, Southern blot, and Northern blot analyses.

Foreign gene integrated into a plant genome with a single or low copy number is often desired for many different reasons. One is that it is much easier to perform the genetic segregation analysis in the offspring and it is also less likely to get the foreign gene

cosuppressed or silenced. Franks et al. (1998) reported that the expression of a foreign gene with four copies is much stronger than that of a foreign gene with five copies in grape, because methylation in the transgenic plants was detected by Southern hybridization analyses using isoschizomer pairs of restriction endonucleases to digest DNA extracted from two transgenic plants. In this experiment, one to two copies of the foreign gene insertion

were verified in transgenic grape lines by Southern blot analysis and their enhanced transcript levels were also found.

Alternative splicing is an important post-transcriptional regulatory mechanism. Transgenic plant had both the endogenous mature mRNA and unspliced transcripts by Northern blot analysis (Liu et al., 2003). Alternative splicing is often regulated by specific factors expressed in response to developmental or environmental condition. In pumpkin, two highly homologous cDNAs for hydroxypyruvate reductase (HPR) were obtained and analyses showed that the amounts of the two HPR mRNAs was changed in response to light (Mano et al., 2000). In the same way, two bands of *DREB1b* mRNA, one with the expected full size of the transcript, another with a smaller size, were detected in transgenic Lines 3 and 7, both of which just contained a single copy of *DREB1b* transgenic plant lines. However, it is unclear why transgenic Lines 3 and 7 exhibited two different sizes of *DREB1b* transcripts. Further studies are needed to find out whether two different *DREB1b* transcripts resulted from posttranscriptional processing.

Cold stress often leads to a decrease in crop yield. Gene engineering techniques provide an entirely new method to develop a cold-resistant variety in plants. The cold resistance trait of a plant is genetically controlled by multigene interactions and the genes are regulated by transcription factors. *DREB1b* is a transcription factor for COR genes in plants, and overexpression of *DREB1b* can increase a plant's resistance to cold stress because *DREB1b*-regulated COR genes are overexpressed. Zhen et al. (2000) found that overexpression of the *DREB1b* gene increases cold resistance significantly in grape but slightly in tobacco. Electrolyte leakage analyses of transgenic *Arabidopsis thaliana* overexpressing the *CBF1/DREB1b* gene showed that overexpression of the transgenic plant increases cold resistance of the transgenic plants by 3.3 °C (Jaglo-Ottosen et al., 1998).

DREB1b-like genes exist in both monocotyledon and dicotyledon plants such as sour cherry and strawberry (Owens et al., 2002), rice (Dubouzet et al., 2003), grape (Xiao et al., 2006), *Brassica napus*, and some other plants (Jaglo-Ottosen et al., 2001). Overexpression of the *DREB* cDNA results in a severe dwarfed phenotype in *Arabidopsis* and tomato (Gilmour et al., 2004; Liu et al., 1998; Zhang et al., 2004). Gilmour found that transgenic plants that overexpress *DREB1b* are smaller in size and grow more slowly and exhibit a more prostrate growth habit compared with wild-type plants (Gilmour et al., 2004). Examination of cross-sections of the leaves by confocal microscopy indicated that the structure of the leaves from transgenic plants also differs from that of the wild-type plants. Whereas wild-type *Arabidopsis* leaves have a single layer of palisade mesophyll cells, transgenic ones have a double layer of palisade cells, which are slightly longer and thinner than those of wild-type

plants. Zhang et al. (2004) found that transgenic tomato was stunted in growth and delayed in flowering because of the overexpression of the *DREB* gene. In addition, they produce fewer fruit per plant than wild-type tomato plants. Pino et al. (2007) transformed the *Solanum tuberosum* 'Umatilla' with three *Arabidopsis* *DREB* genes driven by either a constitutive CaMV 35S or a stress-inducible *Arabidopsis* rd29A promoter. *DREB1b* and *DREB1c* overexpression increases freezing tolerance, whereas *DREB1a* overexpression does not. Transgenic plants overexpressing *DREB1b* and *DREB1c* driven by the rd29A promoter reach the same level of freezing tolerance as the 35S versions within a few hours of exposure to low but nonfreezing temperatures and grow similarly to wild-type plants. In our experiments, the plants of transgenic grape (Line 3) overexpressing the *DREB1b* gene exhibited similar phenotypes to wild-type plants in young stage. *DREB1b* overexpression induced COR gene expression and COR gene expression could bring about the full array of biochemical and physiological changes in grapevines. The freezing tolerance was arrayed by an electrolyte leakage test. The electrolyte leakage test could analyze the biochemical and physiological changes. Our experimental results demonstrated that *Arabidopsis* *DREB1b* gene also functioned in grape for improving plants' cold resistance.

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