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Low-temperature-induced transcription factors in grapevine enhance cold tolerance in transgenic *Arabidopsis* plants

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ABSTRACT

We report the characterization of low-temperature-induced transcription factors in grapevine (*Vitis vinifera*). Four transcription factors were identified in low-temperature-treated grapevine. The expression of *V. vinifera* C-repeat-binding factors, *VvCBF2*, *VvCBF4*, and *VvCBFL*, and *V. vinifera* B-box-type zinc finger protein, *VvZFPL*, was immediately induced and upregulated in leaves by the low-temperature treatment. Similar induction of the gene expression was observed in low-temperature-treated stems and flowers, although *VvZFPL* was constitutively expressed in flowers. Tendrils expressed all the four genes constitutively. In berry skin, *VvCBF2* and *VvCBFL* were induced by the low-temperature treatment before the onset of véraison, while only *VvCBF2* was induced under the low-temperature condition after the onset of véraison. The overexpression of VvCBF2 and VvZFPL in *Arabidopsis* plants led to longer hypocotyls than the control plants. The rosette leaves of these plants were smaller and had lower chlorophyll contents than those of the control plants, resulting in a pale green color. Finally, the VvCBF2- and VvZFPL-overexpressing plants revealed growth retardation. These results suggest that VvCBF2 and VvZFPL may affect photomorphogenesis and growth in grapevine. Meanwhile, no morphological changes were detected in the VvCBF4- and VvCBFL-overexpressing plants. The cold tolerance test demonstrated that all of the overexpressing plants remained viable and noticeably healthy compared with the control plants even after exposure to severe cold treatment, suggesting that VvCBF2, VvCBF4, VvCBFL, or VvZFPL may enhance cold tolerance in grapevine.

Introduction

Low temperature is an important environmental factor that affects the growth and development of grapevine in the next growing season. During the dormant season in winter, grapevine cultivated in cool climate areas is frequently exposed to temperatures below 0 °C. The temperature required to kill half of dormant buds is −26 °C for *Vitis labrusca* and −23 °C for *Vitis vinifera* (Proebsting et al., 1980). Michigan State University reported freezing damage to grapevines during development in Southwest Michigan (Longstroth, 1998, 2002). Late spring frost on June 3, 1998 damaged leaves on the outside of the canopy and killed most of the leaves and flower clusters.

Molecular approaches have been proposed to induce or enhance tolerance to abiotic stress in target plants (Vinocur and Altman, 2005). First, the stress-associated genes should be identified and characterized to understand the stress response mechanisms in target plants. Thereafter, the stress-associated genes should be introduced to model plants, including *Arabidopsis thaliana* and *Nicotiana tabacum*, and the transformants could be employed to elucidate the function of the stress-associated genes under stress conditions. Finally, crop breeding to confer stress tolerance could be achieved by molecular breeding techniques. Using this approach, heat-shock-related genes were characterized in grapevine, which conferred thermotolerance to *Arabidopsis* plants (Kobayashi et al., 2010).

Although grapevines are capable of surviving under freezing conditions, the signaling pathway involved in the tolerance to low temperature is poorly understood. The expression of many genes is changed in plants in response to low temperature. To clarify how grapevine recognizes low temperature and acquires cold tolerance, it will be necessary to identify the components of the signaling pathway. In the model plant *A. thaliana*, the transcription network regulated by C-repeat-binding factors (CBFs) plays a critical role in cold acclimation (Medina et al., 2010). In *Arabidopsis*, CBFs activate the transcription of cold-responsive genes, resulting in the acquisition of tolerance to low temperature (Liu et al., 1998). In grapevine, *VvCBF1*, *VvCBF2*, *VvCBF3*, and *VvCBF4* have been identified (Xiao et al., 2006, 2008). The transcription of all the *VvCBFs* was enhanced upon exposure to 4 °C, although the transcription patterns were different among them. However, functional confir-

Abbreviations: CBF, C-repeat-binding factor; ZFP, zinc finger protein.

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mation of the role of VvCBFs in cold acclimation or cold tolerance in mutants or transgenic plants has not been demonstrated.

To understand the molecular mechanism related to cold tolerance in grapevine, in the present study, the transcription of seven low-temperature-induced transcription factors published previously (Xiao et al., 2006, 2008; Tattersall et al., 2007), including CBFs, zinc finger proteins (ZFPs), and homeobox-leucine zipper protein (HZP) was confirmed in *V. vinifera* grapevine, and four low-temperature-induced transcription factors were overexpressed in *Arabidopsis* plants. We report here the characterization of the transcription factors and their effects on cold tolerance and growth in transgenic plants.

Materials and methods

Plant materials

Grapevines of *Vitis vinifera* L. cv. Koshu were cultivated in the experimental vineyard of the Institute of Enology and Viticulture, University of Yamanashi, Yamanashi, Japan. Flowers, stems, young leaves, and tendrils were collected on June 2, 2010. The onset of véraison of Koshu berries was confirmed at day 84 after flowering. Berry skins were collected at 74, 84, and 94 days post flowering.

Low-temperature treatment

Young leaves were used to identify low-temperature-induced transcription factors in grapevine. Leaves were submitted to 4 °C for 1 h or 4 h, and immediately frozen in liquid nitrogen for RNA isolation. To determine the time-dependent gene expression on exposure to low temperature, young leaves were treated at 4 °C for 1–6 h. Detached flowers, tendrils, stems, and berries were also treated at 4 °C for 4 h. After the low-temperature treatment, berry skins were peeled off with a razor.

Total RNA isolation

Plant materials were placed in a mortar containing liquid nitrogen and homogenized with a pestle. Approximately 100 mg of the powder was transferred to a microtube. Total RNA isolation and purification were performed as described by Kobayashi et al. (2010).

RT-PCR analysis

One μg of total RNA was reverse transcribed with random hexamer primers (Takara, Otsu, Japan). PCR conditions were as follows: after incubation at 95 °C for 3 min, PCR amplification was performed for 25 cycles at 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s, with a final extension step at 72 °C for 5 min. The nucleotide sequences of the primers for the gene expression analysis of low-temperature-induced transcription factors in grapevine were as follows: *VvCBF1* (5′-AACCCAACTGCACCATCTTC-3′ and 5′-CATCATCCCAGCTGAATCCT-3′, from *V. vinifera* CBF 1, GenBank accession no. AY390372), *VvCBF2* (5′-ATGGACTTGGACCGTGAGTC-3′ and 5′-ATCGGGAAAATTGAGGGAAG-3′, from *V. vinifera* CBF 2, GenBank accession no. AY390376), *VvCBF3* (5′-CCCTCATCCTCCTCTTCCTC-3′ and 5′-TCCCCAGCTGAAGATGACTT-3′, from *V. vinifera* CBF 3, GenBank accession no. AY390375), *VvCBF4* (5′-GAACTCCGGGAAGTGGGTAT-3′ and 5′-TCCCTCCTCTCATCTTGCAT-3′, from *V. vinifera* CBF-like protein, GenBank accession no. AY706986), *VvCBFL* (5′-AGACGCGACACCCGATATAC-3′ and 5′-TGGAAGATGACGATGATGGA-3′, from *V. vinifera* similar to CBF-like transcription factor, GenBank accession no. XM_002270601), *VvZFPL* (5′-TGGGGCTAAATGGTAGTTGC-3′ and 5′-TGGTCTCCGTCTCTCCATCT-3′, from *V. vinifera* ZFP-like protein, GenBank accession no. HQ179976), and *VvHZP* (5′-CAACCGCCAGAAAGAAGAAG-3′ and 5′-GGATTCAAACCGAGAAACCA-3′, from *V. vinifera* homeobox-leucine zipper protein, GenBank accession no. XM_002262914). Grapevine β-actin primers (5′-GTTATGCACTTCCCCATGCT-3′ and 5′-ACGGAATCTCTCAGCTCCAA-3′, from *V. vinifera* β-actin, GenBank accession no. AF369524) were used as control. RT-PCR products were separated on 2% agarose gel and visualized by ethidium bromide staining under UV illumination.

Cluster analysis

Cluster analysis of *Arabidopsis* ZFPs and VvZFPL was performed. Briefly, the amino acid sequences of *Arabidopsis* ZFPs were collected from NCBI database (http://www.ncbi.nlm.nih.gov). The sequences were subjected to the neighbor-joining (NJ) method using Molecular Evolutionary Genetics Analysis software, MEGA4 (http://www.megasoftware.net).

Transformation of low-temperature-induced transcription factors in Arabidopsis thaliana

Open reading frames of VvCBF2, VvCBF4, VvCBFL, and VvZFPL were amplified from low-temperature-treated leaves by RT-PCR. The nucleotide sequences of the primers were as follows: 5′-CATATGATGGACTTGGACCGTGAGTC-3′ containing an *Nde*I site (underlined) and 5′-GAATTCTTAAGATAGGAAATCATGAT-3′ containing an *Eco*RI site (underlined) for VvCBF2; 5′-CATATGATGAATACTACTTCTCCACC-3′ containing an *Nde*I site (underlined) and 5′-GAATTCCTAAATAGAGTAACTCCATA-3′ containing an *Eco*RI site (underlined) for VvCBF4; 5′-CATATGATGGACTTGGACCGTGAGTC-3′ containing an *Nde*I site (underlined) and 5′-GAATTCTTAAGACAGGAAATCATGAT-3′ containing an *Eco*RI site (underlined) for VvCBFL; and 5′-CATATGATGAAGGGTAGGGTTTGTGA-3′ containing an *Nde*I site (underlined) and 5′-GAATTCTCATCTAAATTCTTCCCCAG-3′ containing an *Eco*RI site (underlined) for VvZFPL. PCR products were digested with *Nde*I and *Eco*RI and ligated to the *Nde*I and *Eco*RI sites of the binary vector pRI101-AN (Takara), resulting in plant expression plasmids. The plant expression plasmids were introduced into *Agrobacterium tumefaciens* strain LBA4404. *A. thaliana* Col-0 was transformed with *Agrobacterium* by the floral dip method (Clough and Bent, 1998) and selected on 1× Murashige and Skoog (MS) medium plates containing 50 μg/mL kanamycin. T2 seeds propagated from the transgenic lines were obtained and used for phenotypic analysis and the cold tolerance test. One transgenic line transformed with pRI101-AN was used as the control plant in the present study.

Phenotypic analysis of Arabidopsis transgenic plants

To observe the general growth and development of *Arabidopsis* transgenic plants, T2 seeds were plated on MS medium containing 50 μg/mL kanamycin and incubated at 22 °C for 10 d in an incubator (11.8 Wm^{-2}/16 h/d). Kanamycin-resistant seedlings were planted in soil. To determine meristem transition from vegetative to reproductive growth, the number of rosette leaves per plant was measured before the appearance of inflorescence meristems. The size of the plants was measured at days 30, 35, and 40 after planting in soil. Different phenotypes from control plants were photographed at each growth stage.