Molecular cloning, promoter analysis, and expression profile of *VvERF3b* gene in *Vitis vinifera*

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Abstract

Ethylene response factors (ERFs) are involved in many plant development events and stress defenses. In this study, an ERF gene, *VvERF3b*, was cloned from the leaves of *Vitis vinifera*. *VvERF3b* belongs to ERF group VIIIa. Expression of the gene was induced by abscisic acid, ethephon, and salicylic acid, but not by NaCl. Promoter sequence analysis of the *VvERF3b* gene revealed that there are several potential *cis*-acting elements that may be potentially recognized and bound by the transcription factors related to hormones and stress responses. Deletion analysis showed that the 5'-flanking sequence of -1047 to -585 from the transcriptional start site is essential to the high expression of the *VvERF3b* gene, whereas the sequence fragment of -1324 to -1047 revealed suppression effect. The result indicated that the region appears to contain *cis*-acting elements that can be bound by the proteins in a transcription complex to induce the inhibition of gene expression.

Additional key words: abscisic acid, *cis*-acting elements, ethephon, ethylene response factor, salicylic acid.

Introduction

AP2/ERF is a large family of transcription factors with one or two conserved AP2/ERF domains. The family is divided into four subfamilies, namely, AP2, CBF/DREB, ERF, and RAV, based on their sequence similarities and numbers of AP2/ERF domains (Sakuma *et al.* 2002). ERF genes, which encode a number of plant-specific transcription factors, play very important roles in plant growth and developmental processes, such as hormone signal transduction, metabolism regulation, and stress responses. The ERF protein was first isolated from tobacco as GCC-binding protein (Ohme-Takagi and Shinshi 1995). Today, many ERF genes are cloned from various plants, such as *Arabidopsis* (*AtERF1-5*; Fujimoto *et al.* 2000), tomato (*LeERF1-4*; Tournier *et al.* 2003), rice (*OsERF1*; Hu *et al.* 2008), and soybean (*GmERF3*; Zhang *et al.* 2009). These ERF proteins contain a single highly conserved DNA binding domain known as AP2/ERF consisting of 58 or 59 amino acids (Ohme-Takagi and Shinshi 1995). These are composed of a three-stranded anti-parallel β-sheet and an α-helix packed approximately parallel to the β-sheet (Allen *et al.* 1998). A flanking region at the N-terminus of the conserved ERF domain is also required for stable binding to the GCC box (Hao *et al.* 1998).

The ERF gene family in *Arabidopsis* is divided into 12 groups (Nakano *et al.* 2006). Some genes including *AtERF1*, *AtERF2*, and *AtERF5* that belong to group IX function as transcription activator, whereas some ERF genes including *AtERF3* and *AtERF4* that belong to group VIIIa function as transcription repressors (Fujimoto *et al.* 2000). The predicted proteins of group VIIIa has a highly conserved sequence motif of L/FDLNL/F(x)P, ERF-associated amphiphilic repression (EAR) motif, which is essential for repression function (Ohta *et al.* 2001), and an acidic domain located near the C-terminal of the protein.

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Abbreviations: ABA - abscisic acid; EAR - ERF-associated amphiphilic repression; ERF - ethylene response factor; ET - ethephon; MS - Murashige and Skoog; MTD-PCR - modified touchdown polymerase chain reaction; ORF - open reading frame; RT-PCR - reverse transcription - polymerase chain reaction; SA - salicylic acid.

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Many experiments indicate that the expression of many ERF genes can be induced by pathogen infection, *e.g.*, *AtERFs*, *TiERF1*, and *GmERF3* (Berrocal-Lobo *et al.* 2002, Onate-Sanchez and Singh 2002, Liang *et al.* 2008, Zhang *et al.* 2009), whereas *JERF1*, *AtERF4*, *Pti4*, and *GhERF4* can be induced by phytohormones (Wu *et al.* 2002, Zhang *et al.* 2004, Yang *et al.* 2005, Jin and Liu 2008). Further, ERF proteins that act as transcription factors can modulate not only the expression of GCC box-containing genes but also DRE/CRT, CE1, JERE, and CT-rich elements (Chakravarthy *et al.* 2003, Wu *et al.* 2008, Agarwal *et al.* 2010) in order to regulate many non-GCC box defense-regulated gene expression. These are ERF subfamily transcription factors involved in cross-talk among plant defense signaling pathways.

Although identified in many plant species, the ERF gene has not been reported in grapevine. In this study, we clone the ERF gene *VvERF3b* from *V. vinifera* and present an expression analysis of the gene as induced by some plant hormones such as ABA, ET, and SA. The sequence deletions of the promoter region are investigated to gain insight into the expression profile of *VvERF3b*.

Materials and methods

Vitis vinifera L. plants were grown in a greenhouse (temperature 23 - 28 °C, mean irradiance 120 μmol m^{-2} s^{-1}, 16-h photoperiod). Abscisic acid (ABA; 200 μM), NaCl (200 mM), salicylic acid (SA; 2 mM), and ethephon (ET; 1 mM) were directly dissolved in water and sprayed on the fourth or fifth leaf (counted from the tip).

Seeds of wild-type *Arabidopsis thaliana* L. ecotype Columbia were sterilized by soaking in 70 % ethanol for 30 s, washing in autoclaved distilled water then soaking in 10 % sodium hypochlorite for 10 min, and then washing with sterile distilled water. The sterilized seeds were germinated on plates containing Murashige and Skoog (MS) solid medium for 10 d, and then transferred to pots filled soil mix and *Vermiculite* (1:1). The *Arabidopsis* plants were grown in growth chamber under 12-h photoperiod, irradiance 50 - 74 μmol m^{-2} s^{-1}, temperature of 23 °C and relative humidity of 50 % until use.

Total RNA was extracted from grapevine leaves following the method of Zhang *et al.* (2003). It was displayed using agarose gel electrophoresis. Of the total RNA, 1 - 5 μg was treated with DNase I (*TaKaRa*, Dalian, China) and then reverse-transcribed with the *M-MLV* RTase cDNA synthesis kit (*TaKaRa*) according to the manufacturer's instructions. The gene-specific primers VvERF3b-11 and VvERF3b-12 for reverse transcriptase (RT)-PCR were designed according to the open reading frame (ORF) sequence of *VaERF3b* (AY395745) cloned by the author and a sequence similar to *VaERF3b* in the *Genosope* database (http://compbio.dfci.harvard.edu/) using *Primer Premier 5.0* software. The sequences of the primers are listed in Table 1.

For the analysis of gene expression under different treatments, semiquantitive PCR was performed using the gene-specific primer pair designed according to the sequence of the *VvERF3b* and the sequences of the gene-specific primer pair for the *Actin* gene used as internal control. The primer sequences are listed in Table 1.

PCR reactions were performed in a final volume of 20 mm^3 with 1.0 unit of *Taq* polymerase (*TaKaRa*), 2 mm^3 10× buffer provided by the supplier, 2 mm^3 10 mM dNTPs, 0.4 mm^3 each of the primers (0.1 μM each), and 0.1 μg DNA template. The PCR conditions were as follows: 94 °C for 5 min, 35 cycles of 94 °C for 60 s, 58 °C for 60 s, and 72 °C for 60 s, followed by 72 °C for 7 min. The products were checked on 1 % (m/v) agarose gel.

The isolation of genomic DNA from *V. vinifera* leaves was performed following the method of Guillemaut and Maréchal-Drouard (1992). Genomic DNA was treated with 1 unit of RNase A (*TaKaRa*) at 37 °C for 1 h. The resulting DNA was used as PCR template. The gene-specific primers VvERF3b-pF and VvERF3b-pR were designed according to the 5'-flanking sequence of *VvERF3b* in the *Genosope* database (see Table 1). For the cloning of the promoter sequence of *VvERF3b*, modified touchdown PCR (MTD-PCR) was employed and performed following the method of Zhang *et al.* (2003).

The cloned promoter of the *VvERF3b* gene was used as template for the production of all *VvERF3b*-promoter deletions. PCR amplified the *VvERF3b*-promoter deletion fragments using six forward primers for constructs VvERF3b-P1, VvERF3b-P2, VvERF3b-P3, VvERF3b-P4, VvERF3b-P5, and VvERF3b-P6 (Fig. 3), respectively, and a common reverse primer (Table 1). The amplified products were digested with *BamH I* and *Hind III*, and ligated into *BamH I*- *Hind III*- cleaved vector pGCC (Yanagisawa *et al.* 2003) (Genebank EF090415) using T4 DNA ligase (*TaKaRa*).

Arabidopsis protoplast isolation and plasmid transfection were done as described by Fujimoto *et al.* (2000) and Yoo *et al.* (2007). Luminescence was measured with a LB960 illuminometer (*Berthold Technologies*, Oak Ridge, NT, USA) by using a *Promega* (Madison, WI, USA) kit according to the manufacturer's protocol.

Table 1. The primer sequences for the PCR reactions in this study. *a* - the *Hind III* site is underlined, b - the *BamH I* site is underlined.

PCR reaction	Name of primer	Sequence of the primer
Cloning of VvERF3b	VvERF3b-11	5'-ATGGCGCCGAGAGACAAACC-3'
	VvERF3b-12	5'-TCAAGCGACTTCCGCTGGAGGATG-3'
Semiquantitive PCR	VvERF3bF	5'-CACAGCGACTCCGACTCAT-3'
	VvERF3bR	5'-GAGGGAACAACATTAAGAAAATC-3'
	ActinF	5'-TCCTGCTACAAACATCCT-3'
	ActinR	5'-GAATCTGGTCCATCCATT-3'
Promoter cloning	VvERF3b-pF	5'-TGGGCGACAAAATCTGGG-3'
	VvERF3b-pR	5'-ACACCAACCGCAGGGTAACG-3'
Primer deletion assay	VvERF3b P-R	5'-CCCAAGCTTTTGTTTTGTGTTTTGGTACAAGTG-3'*a*
	VvERF3b P1-F	5'-CGGGATCCTGGGCGACAAAATCTGGG-3'*b*
	VvERF3b P2-F	5'-CGGGATCCCGTTGAGAAAAGCGGGTT-3'*b*
	VvERF3b P3-F	5'-CGGGATCCCGTCAAAACCGCCGTTAG-3'*b*
	VvERF3b P4-F	5'-CGGGATCCGCGGATTTTTGGTTATCAT-3'*b*
	VvERF3b P5-F	5'-CGGGATCCACAATATATCCAAATGGC-3'*b*
	VvERF3b P6-F	5'-CGGGATCCTAGGTGAGGTGGGCATTCG-3'*b*

Results and discussion

The ORF had a sequence of 663 bp and encoded a protein of 220 amino acids with the predicated molecular mass of 23.69 kDa, with the pI of 9.22. Sequence alignment (Fig. 1) revealed that the deduced protein owns a single 58-amino acid AP2/ERF domain located close to the N-terminal with two conserved amino acid residues, alanine in position 14 and aspartic acid in position 19. The protein also has an acidic domain and an EAR motif in the C-terminal. Compared with the *VvERF3b* amino acid sequence having ERF proteins that function as repressors (Fig. 1), the ERF domain in *VvERF3b* is highly conserved, whereas the other parts of the deduced amino acid sequences were notably lower. Phylogenic analysis (Fig. 2) indicated that *VvERF3b* can be assigned to group VIIIa of the previously described ERF groups (Nakano *et al.* 2006) because the ERF proteins in the group have a conserved motif, EAR, in the C-terminus. Previous studies found that some ERF proteins such as *AtERF3*, *AtERF4*, *AtERF7*, and *LeERF3* (Fujimoto *et al.* 2000, Tournier *et al.* 2003, Song *et al.* 2005) have the EAR domain which is essential for their repression function. This is because mutation within the motif eliminated the capacity of repression. The result implied that the *VvERF3b* gene may function as a repressor in the regulation of gene expression.

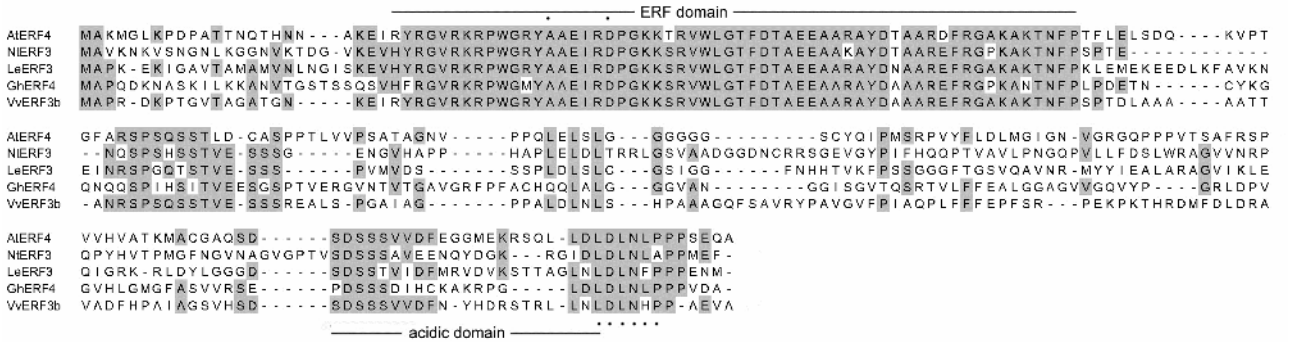

Fig. 1. Amino acid sequence alignment of the grapevine *VvERF3b* gene with some analogs from other plants. Residues matching the consensus (≥ 3 sequences) are *shaded in gray*. Comparison of the derived amino acid sequence of the grapevine *VvERF3b* with ERFs from *AtERF4*, *LeERF3*, tobacco *NtERF3*, and cotton *GhERF4*. The AP2/ERF domain and the C-terminal acidic domain are represented by lines. The alanine and aspartic acid residues at positions 14 and 19 in the AP2/ERF domain are marked by dots above the sequence, whereas the EAR motif is represented by a dot under the sequence. Dashes show gaps in the amino acid sequences, which are introduced to optimize alignment. Alignments were made using *BioEdit* software. The GenBank accession numbers of the ERF proteins are listed as follows: *AtERF4* (NP_188139.1), *NtERF3* (Q40477), *GhERF4* (AAX07461.1), *LeERF3* (AAO34705.1).

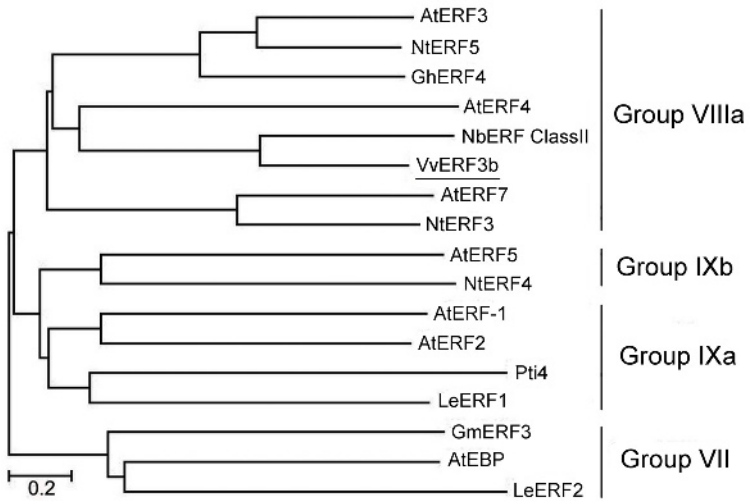

Fig. 2. Phylogenic relationships of the grapevine *VvERF3b* gene with some analogs from other plants. Phylogenic comparison of the VvERF3b protein and some AP2-ERF-related protein sequences. The tree was created by the Neighbor-Joining program of *MEGA 3.1* software after the ERF protein sequences were aligned using *Clustal X*. The GenBank accession numbers of the ERF proteins are listed as follows: *AtERF-1* (NP_567530), *AtEBP* (NP_188299.1), *AtERF2* (NP_199533.1), *AtERF3* (NP_175479.1), *AtERF5* (BAA97157.1), *NtERF5* (Q9SXS8.1), *AtERF7* (NP_188666.1), *NbERF classII* (BAD99476.1), *AtERF4* (NP_188139.1), *NtERF3* (Q40477), *LeERF2* (AAR87866.1), *NtERF4* (Q40478.1), *GmERF3* (ACD47129.1), *GhERF4* (AAX07461.1), *Pti 4* (AAC50047.1), *LeERF1*(AAO34703.1).

To better understand the expression regulatory region of the *VvERF3b* gene, a 1466 bp sequence fragment upstream *VvERF3b* ORF was amplified from grapevine genomic DNA. A promoter *cis*-acting motif search of the *VvERF3b* promoter sequence was performed using the *PLACE* database (Higo *et al.* 1999) (http://www.dna.affrc.go.jp/htdocs/PLACE/). The putative transcription start site was predicted using *TSSP-TCM* online software (Shahmuradov *et al.* 2005) (http://mendel.cs.rhul.ac.uk/mendel.php?topic=fgen). Analysis of the sequence of the *VvERF3b* promoter region showed that there are many putative *cis*-acting elements associated with hormone and stress responses, such as ABRE, DPBF, ERE, GCC, S-box, TCA-element, and W-box. The result showed that the gene may be induced or regulated by many different elicitors.

A series of 5'-deletions of the *VvERF3b* promoter sequence was constructed (Fig. 3) using the pGCC expression vector with a luciferase (*LUC*) reporter gene (Yanagisawa *et al.* 2003). The effects of the deleted sequences on the transcription of the vector in plant cells were monitored by detecting the levels of luciferase synthesis. We see the levels of luciferase synthesis of *VvERF3b-p6* as control (standardized to 100), and the relative values of *VvERF3b-p1*, *VvERF3b-p2*, *VvERF3b-p3*, *VvERF3b-p4*, and *VvERF3b-p5* are 207.7, 703.6, 1776.6, 386.1, and 373.6. From these results, we can see that the basal expression of the reporter gene in cells carrying the P4, P5, and P6 promoter deletions were significantly lower than that observed for cells carrying the P3 promoter region, suggesting that -1047 to -585 region is essential for the high expression of the gene. Removal of the -1324 to -1185 and -1324 to -1047 regions caused an increased activity of luciferase in cells, which indicates that the region between -1324 and -1047 may contain at least two putative negative elements causing such decreases.

To better understand the expressions of the *VvERF3b* gene under various treatment conditions, grapevine leaves were exposed to ET, SA, ABA, and NaCl for 1, 3, 6 and 12 h, and expressions of the *VvERF3b* in the leaves were monitored (Fig. 4). The basic expression levels of *VvERF3b* were relatively high in grapevine leaves. However, in the ET-, ABA-, and SA-treated groups, the expressions of *VvERF3b* were strongly inhibited within 6 h of treatments and maintained low level for up to 12 h. In the NaCl-treated group, the expression of *VvERF3b* was not affected by the high concentration of salt.

Many previous studies demonstrated that the ET and SA signaling pathways play central roles in biotic and abiotic stress responses, and members of the ERF family have been shown to be involved in the ethylene, jasmonic acid, or SA signaling pathways in various plant species (Ohme-Takagi and Shinshi 1995, Van der Fits and Memelink 2001, Cheong *et al.* 2002, Lorenzo *et al.* 2003, Yang *et al.* 2005, Pirrello *et al.* 2006, Liang *et al.* 2008, Pre *et al.* 2008, Wu *et al.* 2008, Champion *et al.* 2009, Lenochová *et al.* 2009). ABA is a phytohormone that usually responds to abiotic stress. Previous experiments have confirmed that it is involved in the regulation of expression in many genes (Pandey *et al.* 2005). This study demonstrated that phytohormones like ABA, ET, and SA can suppress the expression of *VvERF3b* in grapevines, but a high salt concentration cannot.

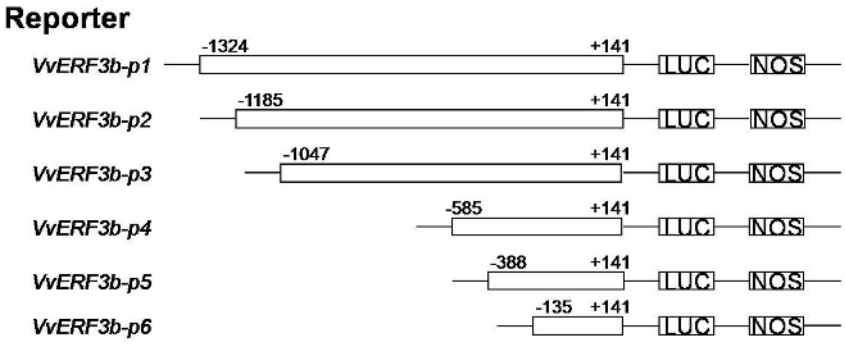

Fig. 3. Level of LUC gene expression regulated by different 5'-deletions of the *VvERF3b* promoter. Schematic of the reporter plasmid constructs used in the transient assay. The promoter of *VvERF3b-p1*, *VvERF3b-p2*, *VvERF3b-p3*, *VvERF3b-p4*, *VvERF3b-p5*, or *VvERF3b-p6* is fused upstream of the LUC gene in the pGCC vector.

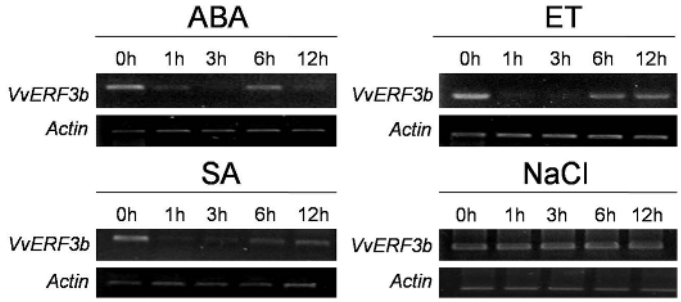

Fig. 4. Time-course of accumulation of *VvERF3b* transcripts detected upon ABA, ET, SA and NaCl treatments. Each lane was loaded with the total RNA extracted from detached grapevine leaves treated with 200 µM ABA, 2 mM SA, 1 mM ET and 200 mM NaCl for the designated times.

Therefore, *VvERF3b* may be involved in the ABA, ET, and SA signal pathways (Fig. 4).

In conclusion, *VvERF3b* might function as a suppressor to regulate other related genes. *VvERF3b* was down-regulated by phytohormones, and two putative negative elements in the promoter of the *VvERF3b* gene may be related to the suppression. Further investigations on this topic are currently underway.

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