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BRIEF COMMUNICATION

Identification of a C2H2-type zinc finger gene family from *Eucalyptus grandis* and its response to various abiotic stresses

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Abstract

C2H2-type zinc finger proteins belong to a major family of transcription factors that play a crucial role in plant stress responses. In this study, seven C2H2-type zinc finger genes (*EgrZFP1-7*) in *Eucalyptus grandis* were cloned using the RACE PCR method. EgrZFP1-7 proteins included a QALGGH motif, two zinc finger domains, and an EAR motif. They belong to a Q-type C2H2 zinc finger protein family and are classified into the subset C1. *EgrZFP4* and *EgrZFP6* had a higher transcription in roots than in leaves and stems, whereas the expression of the other genes did not differ in various plant tissues. The expression of *EgrZFP* genes in seedlings was induced by low temperatures. Time course experiments at temperatures lower than 4 °C revealed oscillating expression of *EgrZFP1-6* during the cold treatment. However, *EgrZFP7* showed a phasic expression pattern at the same conditions. The expression of *EgrZFP1-6* was found to be enhanced by 200 mM NaCl, whereas the expression of *EgrZFP7* was inhibited.

Additional key words: gene expression, low temperature, NaCl, RACE PCR, transcription factors.

Plants often encounter various environmental stresses during their growth and development, and evolved a number of mechanisms at the molecular level to adapt to these conditions, *e.g.*, *via* transcription regulation (Zhu 2002, Nakashima *et al.* 2009). Previous studies have shown that certain transcription factors (TFs) play an important roles in abiotic stress responses, such as DREB (Mizoi *et al.* 2012), MYB (Qin *et al.* 2012), NAC (Zheng *et al.* 2009), bZIP (Gao *et al.* 2011), WRKY (Niu *et al.* 2012) and zinc-finger proteins (ZFPs; Takatsuji 1999). TFs containing zinc-finger (ZF) motifs are one of the largest groups in plants; zinc finger as the key feature of ZFPs is a small peptide domain with a special secondary structure stabilized by a zinc ion bound to the cystein (Cys) and histidine (His) residues (Miller *et al.* 1985). Based on the numbers and positions of the Cys and His residues, ZF transcription factors are divided into different types, such as C2H2, C2HC, C2C2, C2HCC2C2, and C2C2C2C2 (Berg and Shi 1996). C2H2-type zinc finger transcription factors are ubiquitous in the biological world. They represent a large portion of the transcription factors in plants. Over 2000 potential transcription factors have been identified in *Arabidopsis thaliana*, of which 9 % belong to the C2H2-ZFP family (Gourcilleau *et al.* 2011). The domain of C2H2-type zinc finger has approximately 30 amino acid residues which can be represented as CX2-4CX3FX5LX2HX3-5H (Pabo *et al.* 2001). According to the number, type, and arrangement of ZFs, the C2H2-type ZFPs can be divided into three classes: A, B, and C. In plants, most C2H2-type zinc fingers belong to the class C with a highly conserved QALGGH motif containing a single ZF or several dispersed ZFs, also called Q-type ZFs. Based on the number of amino acid (AA) residues separating the two

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Abbreviations: ABA - abscisic acid; DMSO - dimethylsulphoxide; EAR - ERF-associated amphiphilic repression; SD - short day; TF - transcription factor; ZF - zinc-finger; ZFP - zinc-finger protein;

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invariant His of ZFs: three, four, or five residues, the class C of C2H2-type ZFs can be further grouped into subset C1, C2 and C3, respectively (Englbrecht *et al.* 2004). So far, several of the C2H2-type ZFPs identified were found to be involved in various biological processes. The *A. thaliana* ZF, AtZFP1, also known as AZF1, which had one canonical C2H2-type zinc finger motif, is known to impact shoot development (Chrispeels *et al.* 2000). AZF1, AZF3, and STZ, containing two canonical C2H2-type zinc finger motifs, are known to be induced by salt and cold stresses, whereas AZF2 can be induced by a salt stress and ABA (Kodaira *et al.* 2011). Overexpression of *OsCOIN*, a putative cold inducible zinc finger protein, increased the tolerance to chilling, salt, and drought in rice (Liu *et al.* 2007). Furthermore, most of the C2H2-type zinc finger transcription factors with two canonical C2H2-type zinc finger motifs were shown to respond to abiotic stresses in various other plant species, such as *A. thaliana*, *Populus trichocarpa*, and *Solanum tuberosum* (Gourcilleau *et al.* 2011, Kodaira *et al.* 2011, Tian *et al.* 2010).

In this study, we identified and characterized seven C2H2-type zinc finger genes *EgrZFP1-7* from *Eucalyptus grandis* and analyzed their expression patterns under abiotic stress conditions.

The seedlings of *E. grandis* plants used in this study were obtained from the nursery of the Zhejiang A&F University, Hangzhou, Zhejiang, China. They were maintained in a growth chamber (a 16-h photoperiod, irradiance of 150 μmol m^{-2} s^{-1}, day/night temperatures of 28/22 °C, and relative humidity of 70 %) and were planted in 3-dm^3 plastic pots. Roots, stems and leaves were harvested from the 3-month-old seedlings and were immediately frozen in liquid nitrogen for the RNA extraction and subsequent analyses.

A *BLAST* search (*blastp* with default parameters) against the *nr* protein database of *NCBI* was performed, using the AA sequence of the *PtaZFP2* gene (Martin *et al.* 2009) as query sequence. We used five *Populus trichocarpa* proteins, two *Ricinus communis* proteins, three *Vitis vinifera* proteins, two *Petunia hybrida* proteins, five *Glycine max* proteins, and six *A. thaliana* proteins from the acquired sequences in the *BLAST* search to design a pair of degenerated primers based on their conserved regions, and the primers EgrZFP-DP forward: TGCAAGACKTGCAAHCGCSARTTYYCN and EgrZFP-DP reverse: CCTGTGYYTCCKATG TGDCCDCCVAR were used to amplify the homologs with cDNA templates from RNA of *E. grandis*. The PCR products from cDNA amplification were cloned into the *pGEM-T easy* vector (*Promega*, Madison, USA), and then transformed into *Escherichia coli* cells (DH5α) for sequencing. The obtained sequences were submitted to the *NCBI* database for the *BLAST* analysis.

The full-length cDNAs of the C2H2 zinc finger genes of *E. grandis* were obtained with the *SMARTer*™ RACE cDNA amplification kit (*ClonTech*, Mountain View, USA) according to the manufacturers' instructions using the specific primers (Supplement data). The primers were designed using the obtained sequences and mRNA from the *E. grandis* leaves. The obtained 5' and 3' products were sequenced as the products cloned using the degenerated primers. DNA sequencing was performed by *Shanghai BGI* (Shanghai, China).

The functional domains of the proteins were predicted by *SMART* (http://smart.embl-heidelberg.de). Fourteen *A. thaliana* proteins belonging to the subset C1 subclasses C1-2iA, C1-2iB, C1-2iC, C1-2iD (Englbrecht *et al.* 2004) and six ZFPs from *Populus* (Gourcilleau *et al.* 2011) were downloaded from the *NCBI* (http://www.ncbi.nlm.nih.gov/) and from the plant transcription factor database (http://plntfdb.bio.uni-potsdam.de/v3.0/). Multiple sequence alignment was performed using the *ClustalX* program (http://www.clustal.org/clustal2/) and *GeneDoc* software (http://www.nrbsc.org/gfx/genedoc/). An unrooted neighbor-joining tree with 1000 bootstrap replications was constructed using the *MEGA v. 4.0* software (http://www.megasoftware.net/).

The seedlings of *E. grandis* were placed in the dark for 24 h before the cold treatment. For cold-stress induction, the *E. grandis* seedlings were maintained in separate growth chambers at 0, 2, 4, 6, and 8 °C in the dark. For each treatment, leaves of 3 plantlets were harvested 2 h after the treatment. Furthermore, the *E. grandis* seedlings were maintained at 4 °C in the dark and leaves of 3 plantlets were harvested after 0, 2, 4, 8, 24, and 48 h.

Salinity treatments were done according to the method of Kayal (2006). In brief, 10 leaf discs with a diameter of 1.5 cm from fully expanded leaves were incubated in 200 mM NaCl or in distilled water as a control for 2 h. The salinity treatments were carried on in a growth chamber under conditions mentioned above and were replicated 3 times.

Total RNA was extracted from all the treated materials using the cetyltrimethyl ammonium bromide (CTAB) method (Jaya *et al.* 2010). The quality and concentration of the extracted RNA was checked by agarose gel electrophoresis and measured by a *NanoDrop 1000* spectrophotometer (*Thermo Scientific*, Waltham, MA, USA). cDNA was synthesized from these RNA preparations by priming with *oligo (dT) 18* using *M-MLV* reverse transcriptase (*Promega*).

The target *E. grandis* gene was amplified using cDNA from leaves, stems, and roots. The *Takara* (Shuzo, Japan) *Taq* polymerase with specific primers (Supplement data) was used for PCR. The RT-PCR was conducted in a heated-lid thermal cycler (*Bio-Rad*, Hercules, USA). *Egr18SrRNA* was used as an internal standard to adjust the relative quantity of cDNA in each sample (Kayal *et al.* 2006). The experiment was replicated 3 times and the results were analyzed with *SPSS v. 13.0*.

For quantitative real-time PCR of *EgrZFPs*, the

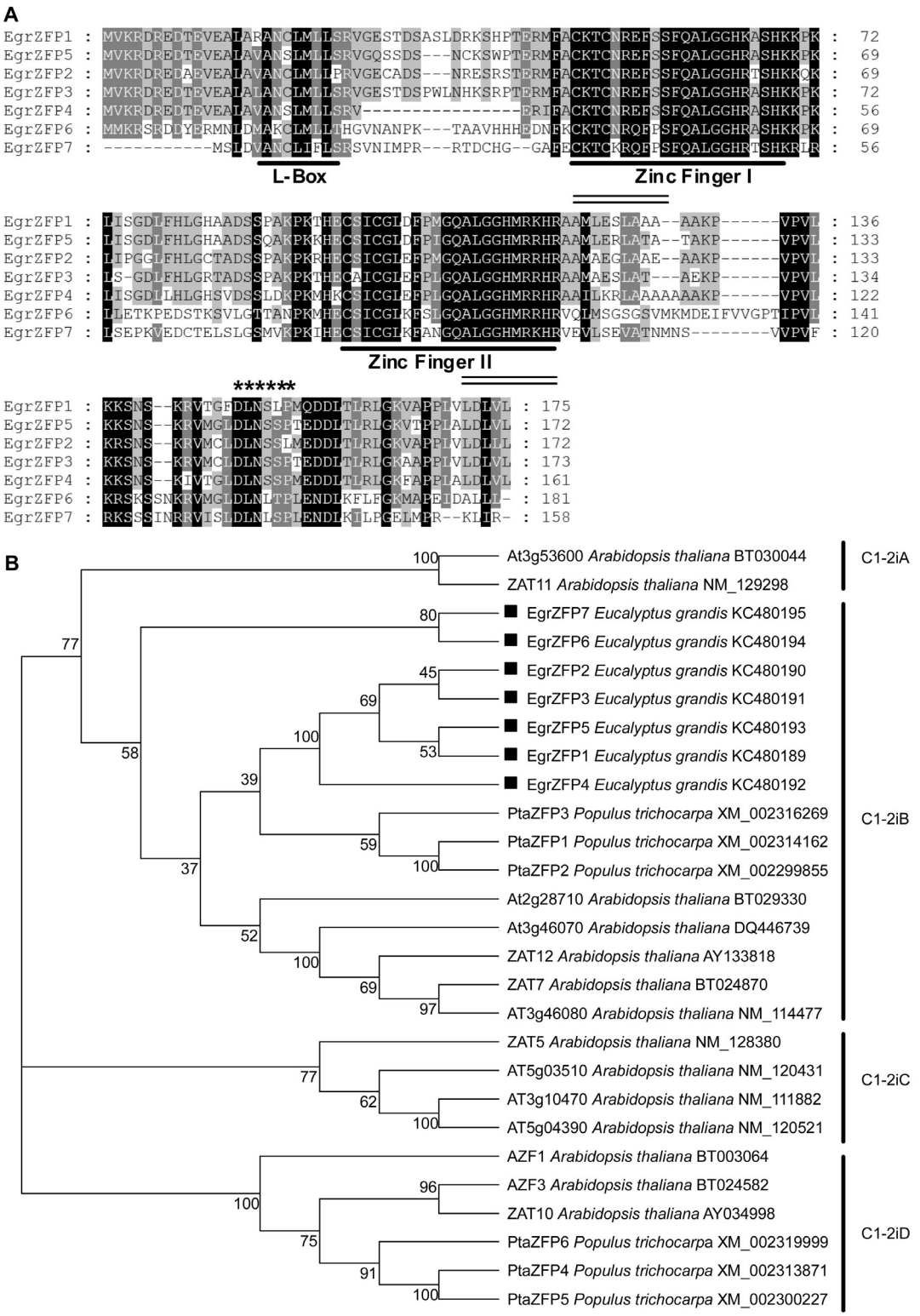

Fig. 1. C2H2-type zinc finger protein sequences analysis. *A* - Comparison of the deduced amino acid sequence of the seven *EgrZFPs* genes. Residues conserved among all seven sequences are highlighted *in black*. The two zinc finger motifs and L-Box are *underlined*. The sites used to design the degenerate primers are marked by a *double straight line*. The main AA residues of the EAR motif are marked by the *asterisks*. *B* - Phylogenetic analysis of *Eucalyptus grandis C2H2*-type zinc finger genes and other plant *C2H2*-type zinc finger homologs. The EgrZFPs were marked by a *black box*.

Table 1. Relative gene expression under different abiotic stresses (0, 2, 4, 6, and 8 °C for 2 h, 4 °C for 0, 2, 4, 8, 24, and 48 h, or 200 mM NaCl for 2 h) analyzed by qRT-PCR. Means ± SE, $n = 3$. The mean within every group of a row indicated by different lowercase letters are significantly different at $P < 0.05$ (the LSD test).

Stress	*EgrZFP1*	*EgrZFP2*	*EgrZFP3*	*EgrZFP4*	*EgrZFP5*	*EgrZFP6*	*EgrZFP7*
28 °C	0.066±0.002^{a}	0.036±0.002	0.104±0.004^{a}	0.150±0.019^{a}	0.045±0.003^{a}	0.147±0.013^{a}	0.220±0.021^{a}
0 °C	0.146±0.002^{b}	0.142±0.007^{b}	0.187±0.009^{b}	0.314±0.013^{b}	0.126±0.016^{a}	0.297±0.004^{b}	0.397±0.029^{b}
2 °C	1.720±0.042^{e}	1.120±0.039^{e}	1.720±0.064^{e}	0.469±0.037^{c}	1.760±0.125^{c}	0.408±0.007^{c}	0.541±0.057^{b}
4 °C	1.080±0.042^{d}	1.420±0.069^{f}	1.280±0.054^{d}	1.380±0.034^{e}	1.110±0.060^{b}	1.380±0.053^{f}	1.490±0.187^{d}
6 °C	0.195±0.019^{b}	0.261±0.023^{c}	0.245±0.021^{b}	0.536±0.031^{d}	0.125±0.010^{a}	0.493±0.031^{d}	0.720±0.082^{c}
8 °C	0.195±0.018^{b}	0.424±0.013^{d}	0.474±0.006^{c}	0.633±0.035^{d}	0.036±0.013^{a}	0.618±0.015^{e}	0.926±0.057^{c}
0 h	0.014±0.000^{a}	0.010±0.000^{a}	0.014±0.001^{a}	0.024±0.001^{a}	0.008±0.000^{a}	0.030±0.002^{a}	0.099±0.019^{a}
2 h	0.190±0.005^{d}	0.274±0.006^{d}	0.138±0.003^{e}	0.239±0.008^{c}	0.162±0.002^{e}	0.264±0.006^{c}	0.324±0.060^{b}
4 h	0.069±0.003^{b}	0.209±0.009^{c}	0.058±0.002^{b}	0.090±0.007^{ab}	0.043±0.002^{c}	0.079±0.002^{a}	0.140±0.043^{a}
8 h	0.136±0.007^{c}	0.294±0.018^{e}	0.119±0.006^{d}	1.200±0.055^{e}	0.105±0.004^{d}	0.910±0.040^{d}	1.326±0.120^{c}
24 h	0.049±0.001^{b}	0.108±0.007^{b}	0.068±0.002^{c}	0.134±0.007^{bc}	0.027±0.001^{b}	0.185±0.009^{b}	0.298±0.052^{b}
48 h	1.110±0.066^{e}	1.110±0.057^{f}	1.110±0.070^{f}	1.040±0.068^{d}	1.040±0.051^{f}	1.040±0.050^{e}	0.152±0.019^{a}
NaCl	1.000±0.024^{b}	1.000±0.024^{b}	1.000±0.025^{b}	1.000±0.120^{b}	1.000±0.021^{b}	1.000±0.040^{b}	0.735±0.047^{a}
Control	0.062±0.003^{a}	0.113±0.003^{a}	0.102±0.003^{a}	0.299±0.012^{a}	0.044±0.002^{a}	0.381±0.018^{a}	2.370±0.214^{b}

specific primer pairs used for detecting gene expression were the same as those used for the semi-quantitative RT-PCR analysis. Real time RT-PCR was performed using the fluorescent dye *SYBR-Green* (*Takara*) and the CFX96 real-time PCR system (*Bio-Rad*). Three biological replicates were taken and triplicate quantitative assays for each replicate were performed. *Egr18SrRNA* was amplified as an internal control. The relative abundance of transcripts was calculated according to the *Bio-Rad CFX 96* manager (*v. 1.5.534*).

Like in other plants, we thought dozens-to-hundreds of *C2H2-ZFP* might be present in the *E. grandis* genome. Sixteen predicted two-fingered Q-type C2H2-ZF proteins were identified from the poplar *Phytozome* database. Most of them were induced by abiotic stresses. A cold treatment induces *PtaZFP2-6*, and mRNA levels of *PtaZFP1-7* are high when plants are treated with a high NaCl concentration (Gourcilleau *et al.* 2011). In this study, we got seven *EgrZFPs* clones from *E. grandis*. Two conserved C2H2-zinc finger domains and an L-Box were found in all of the seven putative C2H2-ZFPs, and every C2H2-zinc finger domain of EgrZFPs had an invariant QALGGH motif. This implied that these putative proteins were Q-type ZFPs (Englbrecht *et al.* 2004). Sequence alignment analysis showed that all of the C2H2-ZFPs belonged to the subset C1. In addition, barring *EgrZFP2*, the other six genes had an ERF-associated amphiphilic repression (EAR) motif defined by the consensus sequence pattern of DLNxxP (Kagale and Rozwadowski 2011) (Fig. 1). This motif has been previously described to function as a transcription repressor inhibiting many genes in plants under abiotic stress conditions (Ciftci-Yilmaz *et al.* 2007).

From the phylogenetic tree constructed by EgrZFPs, PtaZFPs, and AtZFPs, which belong to the subset C1, subclasses C1-2iA, C1-2iB, C1-2iC, and C1-2iD, we can see that EgrZFP1, EgrZFP2, EgrZFP3, EgrZFP4, and EgrZFP5 are grouped more closely with PtaZFP3, whereas EgrZFP6 and EgrZFP7 cluster in one clade with the classification standard (Englbrecht *et al.* 2004). All of the seven EgrZFPs were found to be clustered in the same clade, C1-2iB, with ZAT7 and ZAT12 from *A. thaliana* (Fig. 1).

Under normal conditions, the genes *EgrZFP1-7* were expressed in the leaves, stems and roots. *EgrZFP4* showed the highest expression in the roots, whereas *EgrZFP6* expression was higher in the roots and stems than in the leaves. However, apart from *EgrZFP4* and *EgrZFP6,* the expression patterns for the other genes were not significantly different in different tissues (Fig. 2).

qRT-PCR analysis revealed that *Egr*ZFPs were induced by low temperatures (Table 1). The expressions of *EgrZFP1*, *EgrZFP3*, and *EgrZFP5* were the highest at a temperature of 2 °C. The expressions were 25.8, 16.3, and 38.8 fold higher for *EgrZFP1*, *EgrZFP3* and *EgrZFP5*, respectively, as compared to the control. However, the expressions of *EgrZFP2*, *EgrZFP4*, *EgrZFP6*, and *EgrZFP7* were the highest at 4 °C, being 39.8, 9.20, 9.35, and 6.79 fold higher, respectively, as compared to the control. All the genes showed significant differences in their expression between 4 and 2 °C indicating that the expression of *EgrZFPs* was sensitive to temperature changes.

When the *E. grandis* seedlings were treated under 4 °C for 2, 4, 8, 24, and 48 h continuously, the expression patterns of different *EgrZFPs* were diverse (Table 1). The transcription of *EgrZFP1-6* rapidly increased 2 h post the cold treatment. At the 4 and 24 h cold treatments, the

gene expression of *EgrZFP1, EgrZFP2, EgrZFP4, EgrZFP5*, and *EgrZFP6* decreased compared to 2 and 8 h; the expression of *EgrZPF3* decreased only at 24 h. However, *EgrZFP7* showed the characteristics of phasic expression, reaching the highest level at 8 h and decreasing after that. We did not observe a specific pattern for the *EgrZFP1-6* expression in our time course experiments. Perhaps, there are other factors driving the expression of these genes in abiotic stress environments. The fact that ZAT12 is not only involved in cold tolerance but is also induced by darkness indicates that perhaps circadian rhythm plays a role in stress responses mediated by the zinc finger family proteins (Lee *et al.* 2005). The phasic expression of *EgrZPF7* was found to be similar to that described for *AZF2* and *ZFP245* which have been reported to be induced by a cold treatment (4 °C) that is the highest at 6 h (Sakamoto *et al.* 2004, Huang *et al.* 2005).

EgrZFP1-6 were also found to be induced by NaCl, however, the expression of *EgrZFP7* was suppressed in response to the salinity (Table 1), implying that *EgrZPF7* responded to the cold and salinity in a manner different from *EgrZPF1-6*; therefore, *EgrZPF7* may have a different mechanism to combat various abiotic stresses.

In summary, we identified seven C2H2-type zinc finger genes from *E. grandis* in the present study. The proteins they encoded were found to share high homologies with other C2H2-type zinc finger proteins. These proteins also contained QALGGH motifs and EAR domains. The expression patterns of the *EgrZFP1-7* genes in the cold and high salinity conditions showed that they were involved in cold and salt resistance. Our study paved way for further elucidation of the role these proteins might play in the tolerance mechanisms *via* various pathways, and can help in understanding the stress tolerance mechanisms in *E. grandis.*

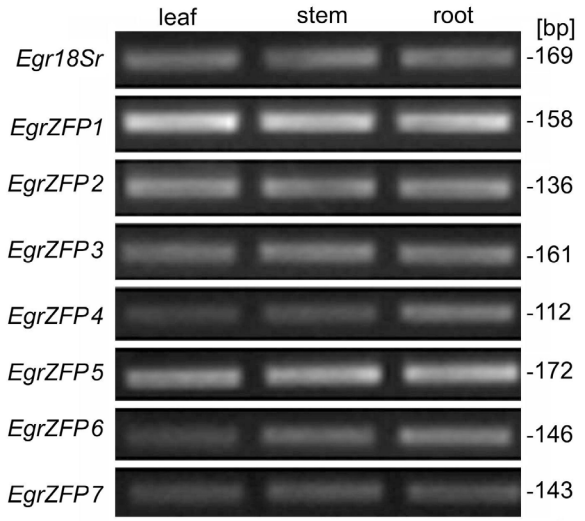

Fig. 2. Semiquantitative RT-PCR analysis of *EgrZFP1-7* in different tissues of *Eucalyptus grandis*.

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