Overexpression of Stress-Related Genes, \textit{BrERF4} and \textit{AtMYB44}, in \textit{Arabidopsis thaliana} Alters Cell Expansion but Not Cell Proliferation During Leaf Growth

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Abstract We have previously shown that overexpression of \textit{BrERF4} (\textit{Brassica rapa} ETHYLENE-RESPONSIVE FACTOR4) increases salt and drought tolerance in Arabidopsis plants, and also retarded organ growth. In the present study, we investigated in detail the leaf growth retardation phenotype at the cellular level. Results showed that \textit{BrERF4}-overexpressing Arabidopsis plants developed small leaves by reducing their cell size but not the cell number. Detailed kinematic analysis revealed that changes in cell size appeared from the very early stages of leaf development, directly affecting the size of leaf organs. RT-PCR analysis showed that expression of expansin genes was reduced in the overexpressors, whereas expression of the cell cycle gene, \textit{CYCB1;1}, was not altered at all. In addition, overexpression of \textit{AtMYB44}, another stress-related transcription factor gene, reduced leaf growth, which also resulted from reduction in cell size but not in cell number. These results suggest that overexpression of those transcription factors negatively affects cell expansion during leaf growth without altering cell number. We discuss about the advantages that the \textit{BrERF4}- or \textit{AtMYB44}-induced cell expansion retardation confers on plants under natural environmental adversity.

Keywords: \textit{AtMYB44}; \textit{BrERF4}; cell number; cell size; environmental stress; leaf size; organ growth

Introduction

Plant growth and development is influenced not only by internal developmental signals but also by external environmental factors (Mizukami and Fischer, 2000). Developmental signals determine the intrinsic size of organs (Mizukami, 2001). In organ growth two distinct but integrated processes are involved, i.e., cell proliferation and cell expansion. Significant progress has been made in identifying and characterizing regulatory factors that affect organ growth, which act either on cell proliferation or cell expansion (Breuninger and Lenhard, 2010).

Environmental stress, such as cold, drought, and salinity, adversely affect plant growth (Xiong et al. 2002). Changes in morphological and developmental patterns are among the responses when plants are subjected to environmental stress conditions. Several studies have been conducted to determine the cellular and molecular mechanisms underlying stress-induced morphogenetic growth response (Jing et al. 2009; Pasternak et al. 2005; Skirycz et al. 2011). Mild osmotic stress can result in the cell cycle arrest, reduction of cell proliferation, and activation of ethylene signaling (Skirycz et al. 2011). Ethylene inhibited cyclin-dependent kinase A activity, which is a main component in the cell cycle progression, and further exposure to stress led to mitotic exit and cell differentiation. Oxidative stress caused by copper induced changes in plant architecture, comprising both stimulatory and inhibitory effects: the former included germination, root/leaf formation, and lateral root formation, and the latter root elongation and leaf expansion (Pasternak et al. 2005). The \textit{small organ 1} mutant with a lesion in the tryptophan synthase \textbeta-subunit displayed tryptophan deficiency.
and small size of aerial organs, which was due to reduction in cell expansion (Jing et al. 2009).

Environmental stress signaling studies in Arabidopsis have reported a small plant size phenotype produced by overexpressing the stress-induced transcription factors, including MYBs such as AtMYB44, and ERFs (ETHYLENE-RESPONSIVE FACTORS), such as AtERF1 and DREB1 (DEHYDRATION RESPONSIVE ELEMENT BINDING1) (Jung et al. 2008; Lorenzo et al. 2003; Kasuga et al. 1999). Expression of AtMYB44 gene is induced by various phytohormones, such as abscisic acid (ABA), jasmonic acid (JA), salicylic acid, and ethylene as well as by abiotic stresses, specifically, dehydration, low temperature, and salinity (Jung et al. 2010). Overexpression of AtMYB44 confers abiotic stress tolerance specifically against drought and salt stresses, and the transgenic plants were dwarfed (Jung et al. 2008). Overexpression of other ERF genes, such as rice OsERF1 and tomato PtERF4, also resulted in small plants (Hu et al. 2008; Wu et al. 2002). It has been reported that an ERF protein, whose expression is triggered by a pathogen, drought, ethylene, ABA, or JA, activates a plant environmental stress response by binding to the GCC-box elements (Ohme-Takagi and Shinshi, 1995).

Previously, we have demonstrated that the overexpression of a Chinese cabbage ERF gene (BrERF4, Brassica rapa ETHYLENE-RESPONSIVE FACTOR4) in Arabidopsis resulted in increased salt and drought tolerance as well as small sizes of the plant aerial parts and delayed flowering (Seo et al. 2010). In this study, we investigated in detail the cellular basis for the growth retardation of the BrERF4 overexpressors and found that it was entirely due to reduction in cell expansion, but not due to cell proliferation at all. We also found that overexpression of AtMYB44, another stress-related transcription factor, exerted the same effect with respect to organ growth. We discuss the cellular behavior in relevance to stress responses.

**Materials and Methods**

**Plant Materials**

The Arabidopsis BrERF4 overexpressor lines at the third generation, BrERF4-8 and BrERF4-4, were described in the previous study (Seo et al. 2010). The AtMYB44 overexpressor T-18 line was a kind gift from Dr. Yang Do Choi (Seoul National University, Korea; Jung et al. 2008). Columbia-0 wild-type plants were used as a control. Seeds were soaked in 0.1% agar solution and then stratified at 4°C for 7 d. The culture soil consisted of 7:1 Sunshine mix 5 (Sun Gro Horticulture, Canada) and vermiculite (GFC, Korea). Growth condition was 14 h light/10 h darkness, 22°C and 50% humidity.

**Leaf Area Measurement**

Digital images of detached leaves were obtained using a scanner. Leaf length and width were measured in the first and second leaves. Leaf area was measured using the image analysis program Scionimage (Scion Corp., Maryland, USA). For kinematic analysis, 4-9-d-old leaves were mounted on glass slides for microscopic imaging and analysis (Kwon et al. 2009).

**Measurement of Cell Number and Cell Area**

Leaf samples were fixed in a mixture of ethanol and acetic acid (6:1) for 4 h, washed 3 times with 100% ethanol and then washed with 70% ethanol. The tissues were cleared in a chloral hydrate solution (8 g chloral hydrate, 1 mL glycerol, and 2 mL distilled water) and mounted on glass slides. Microscopic images were obtained through a DIC microscope (Zeiss Axiosplan, Carl Zeiss, Region Bayern, Germany). Adaxial subepidermal palisade cells aligned along the longitudinal axis just beside the midvein or along the horizontal axis in the maximum width region located in a 0.25 mm² area were counted. To determine the cell area, 20 cells grouped half-way from the midvein to the organ margin at the widest point were analyzed using Scionimage software (Kwon et al. 2009).

**RNA Isolation and RT-PCR Analysis**

Total RNA was isolated from the first two leaves of 20-d-old wild-type and BrERF4-overexpressing plants, and treated with DNase I (Takara, Japan) and subjected to reverse transcriptase-polymerase chain reaction (RT-PCR). The primers used for RT-PCR are listed in Table 1.

**Table 1. Primer sequences used for RT-PCR**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>BrERF4</td>
<td>5'-TGAACATCTACGAGGGTGTCG-3</td>
<td>5'-CTCCTCAGATTAAGTTTTTTC-3</td>
</tr>
<tr>
<td>A1EXP5</td>
<td>5'-ATGGCCTTATTGGAACACCGCC-3</td>
<td>5'-GACACGAGATCTCAATGAGG-3</td>
</tr>
<tr>
<td>A1EXP6</td>
<td>5'-GACACTTCTCTATCTACAC-3</td>
<td>5'-CAATGACGTTCTCAAG-3</td>
</tr>
<tr>
<td>A1EXP10</td>
<td>5'-AGTACTGCTGGATGATC-3</td>
<td>5'-GAGGATTTACCAACCGGTC-3</td>
</tr>
<tr>
<td>CYCB1;1</td>
<td>5'-AGAAGAGAACAGCAGACCAAACCAGC-3</td>
<td>5'-ATATCCCAAGGCCTGAGG-3</td>
</tr>
<tr>
<td>ACTIN</td>
<td>5'-ATGAAATGTTAGGGTCGTGG-3</td>
<td>5'-TCCGAGTTTGAAGAGGCTAC-3</td>
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</tbody>
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