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Antagonistic relationship between AtRALF1 and brassinosteroid regulates cell expansion-related genes

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Rapid alkalization factor (RALF) is a peptide signal that plays a role in plant cell expansion. We have recently proposed that AtRALF1 negatively regulates root cell elongation and lateral root formation by opposing the effects of brassinosteroid (BR). We reported 6 AtRALF1-inducible cell wall-related genes and 2 P450 monooxygenase -encoding genes involved in the BR biosynthetic pathway. The AtRALF1-inducible genes implicated in cell wall remodeling were not downregulated by brassinolide (BL) treatment alone; their induction was only compromised following simultaneous treatment with AtRALF1 and BL. We further examined the cell wall-remodeling gene EXPANSIN A5 (*AtEXPA5*), which is upregulated by BL and has been shown to positively affect root cell elongation. Herein, we report that *AtEXPA5* expression is downregulated by AtRALF1 in a dose-dependent manner in the roots and hypocotyls of *Arabidopsis* plants. *AtEXPA5* is also downregulated in plants that overexpress AtRALF1, and it is upregulated in plants in which the AtRALF1 gene is partially silenced. The AtRALF1 peptide is also able to repress *AtEXPA5* induction following a pre-treatment with BL. A schematic diagram showing the gene regulatory network connecting the recently reported genes with the regulation of cell expansion by *AtEXPA5* is presented.

Cell-cell communication is essential in several growth and developmental processes in plants. Peptide signals regulate a variety of developmental processes and have been demonstrated to be involved in cellular signaling.^{1,2} Rapid alkalization factor (RALF) genes are present throughout the plant kingdom and can exhibit either ubiquitous or tissue-specific expression patterns.³⁻⁶ RALF proteins have an N-terminal signal sequence and a conserved C-terminal region that covers the active peptide.^{3,4} The inhibitory activities of RALF peptides on root growth, hypocotyl elongation and pollen tube growth are all related to their roles as negative regulators of cell expansion.^{3,5,7,8}

The primary cell wall is composed of polysaccharides, structural proteins, and enzymes.⁹ Among cell wall proteins, expansins induce cell wall loosening by disrupting the hydrogen bonds between cellulose microfibrils and cross-linking glycans, resulting in cell expansion.¹⁰ The concerted activities of plant hormones, such as auxin, brassinosteroid (BR) and ethylene, control expansin gene expression, and therefore, their functions in cell expansion.¹⁰

The *AtRALF1* gene is mainly expressed in roots, and the protein it encodes inhibits cell elongation when applied exogenously.^{5,7} Recent studies have shown that FERONIA, which is a ubiquitously expressed receptor kinase in *Arabidopsis* plants,

is an AtRALF1 receptor and that root growth inhibition is mediated by the inhibition of the plasma membrane H⁺-ATPase2.¹¹ Our recent study highlighted a role for AtRALF1 in cell expansion through interference with the BR signaling pathway. We have reported 6 AtRALF1-inducible genes, including 4 involved in cell wall rearrangement and 2 in BR biosynthesis.¹² Both genes involved in BR biosynthesis, CONSTITUTIVE PHOTOMORPHISM AND DWARFISM (*CPD*) and DWARF4 (*DWF4*), were downregulated by brassinolide (BL) treatment. However, the 4 AtRALF1-inducible genes related to cell wall remodeling, the proline-rich proteins *AtPRP1* and *AtPRP3*, the hydroxyproline-rich glycoprotein *AtHRPG2* and the xyloglucan endotransglucosylase *TCH4*, were not repressed by BL itself but were induced at lower levels following simultaneous treatment with AtRALF1 and BL. We selected an expansin gene, *AtEXPA5*, and evaluated its response following exposure to AtRALF1. *AtEXPA5* was induced by BL treatment, played a regulatory role in the elongation of both roots and hypocotyls, and was downregulated by ethylene and upregulated in *edt1* roots, which is an enhanced drought-tolerant mutant with a well-developed root system.¹³⁻¹⁶

Roots of *Arabidopsis* plants treated with increasing doses of the recombinant AtRALF1 peptide (_{His}AtRALF1) showed

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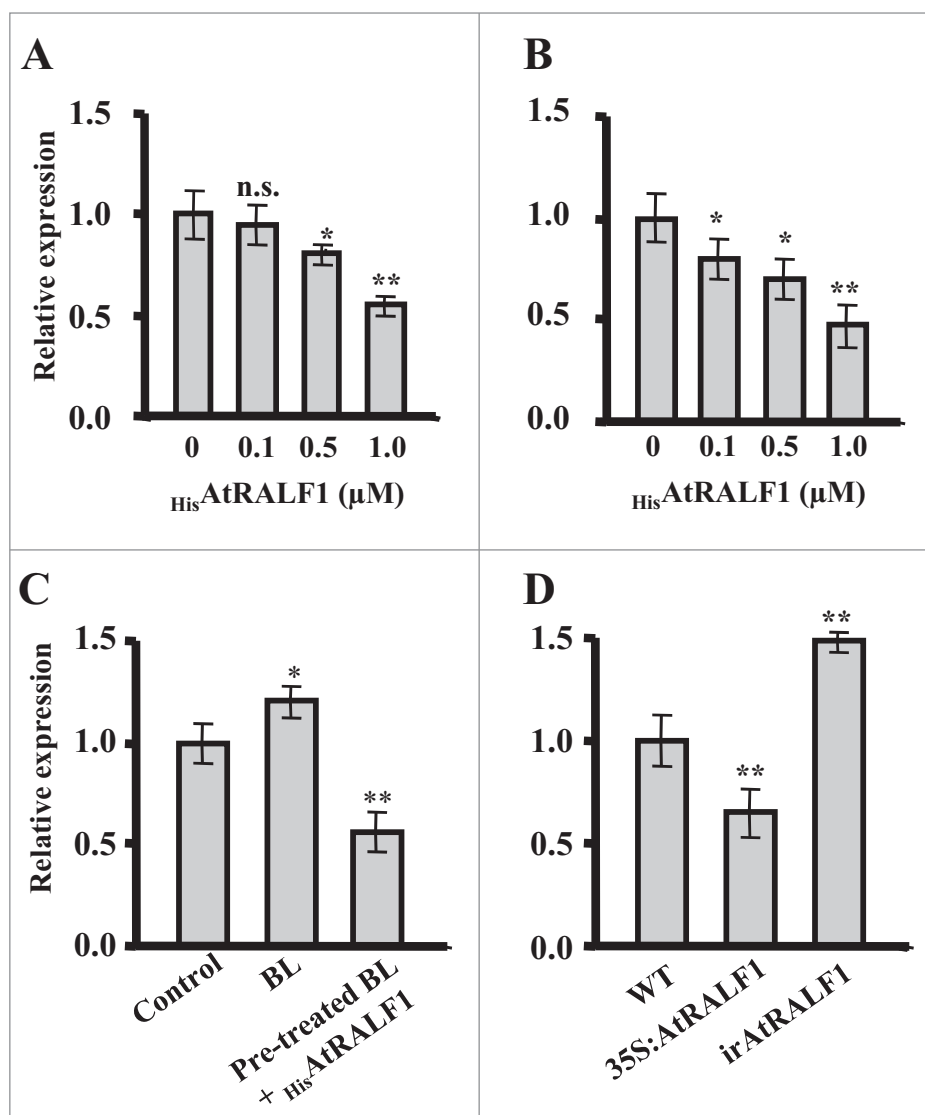


Figure 1. Quantitative RT-PCR gene expression analyses of EXPANSIN A5 (*AtEXPA5*, At3 g29030). (A) HisAtRALF1 -treated 10-d-old wild-type plants. Total RNA was extracted from roots after 30 min of treatment with 0, 0.1, 0.5 and 1 μM of the peptide. (B) Dark-grown 4-d-old wild-type plants treated with HisAtRALF1 . Total RNA was extracted from hypocotyls after 30 min of treatment with 0, 0.1, 0.5 and 1 μM of the peptide. (C) Arabidopsis plants (10-d-old) treated with 100 nM of brassinolide (BL) for 6 h or pre-treated with 100 nM of BL for 5.5 h and treated with 1 μM of HisAtRALF1 for 30 min. Control plants were treated with H_2O . Total RNA was extracted from the roots after each treatment. (D) *AtRALF1*-overexpressing (35S:*AtRALF1*), *AtRALF1*-silencing (*irAtRALF1*) and wild-type (WT) plants. Total RNA was extracted from roots of 10-d-old plants. Error bars indicate SD (* = p value < 0.05, ** = p value < 0.01, t -test). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, At1 g13440) expression was used as a control. All experiments were repeated at least 3 times (independent biological replicates).

proportional decreases in *AtEXPA5* gene expression (Fig. 1A). The downregulation of *AtEXPA5* expression was noted at concentrations as low as 0.5 μM , clearly opposing the effects of BL (Fig. 1A).^{13,14} *AtEXPA5* expression levels in the hypocotyls of Arabidopsis plants treated with *AtRALF1* were also downregulated in these tissues at an even lower *AtRALF1* concentration of 0.1 μM (Fig. 1B). Plants treated with BL for 6 h showed

expected increases in *AtEXPA5* expression; however, when they were pre-treated with BL for 5.5 h and then exposed to *AtRALF1* for 30 min, the mRNA levels of *AtEXPA5* were reduced to approximately 50% of the control levels (Fig. 1C).

To confirm the effects observed in the exogenously treated plants, we modulated the endogenous levels of *AtRALF1* in plants expressing the *AtRALF1* gene under the control of a strong 35S promoter (35S:*AtRALF1*) and plants with a partially silenced *AtRALF1* gene (*irAtRALF1*). *AtEXPA5* was downregulated in the 35S:*AtRALF1* plants to approximately 60% of the *AtEXPA5* mRNA levels found in the control plants (Fig. 1D). That is in agreement with our previous report that 35S:*AtRALF1* plants show a compromised response when treated with exogenous BL.¹² *AtEXPA5* also showed low levels of expression in the *detiolated2* (*det2*) mutant, which is defective in BR biosynthesis, and in the *brassinosteroid-insensitive1* (*bri1*), which is insensitive to BR.¹⁴ In *irAtRALF1*, *AtEXPA5* was upregulated by a factor of 1.5 (Fig. 1D). BL increased the level of *AtEXPA5* when applied exogenously,¹⁴ suggesting an antagonistic effect between *AtRALF1* and BR in the regulation of genes involved in cell expansion. Moreover, *AtEXPA5* controlled hypocotyl elongation through the transcription factor BZR1, which is involved in BR signaling.¹⁴ Curiously, ethylene also reduced *AtEXPA5* expression levels, regulating hypocotyl elongation.¹⁵ Ethylene and BR interact in plant growth and development, and BR promotes ethylene biosynthesis.¹⁷ *AtRALF1* and ethylene may act together by sharing components of the pathway or function in parallel, achieving the same effects.

A molecular model illustrating the interactions between *AtRALF1* and BR in the regulation of cell expansion-related genes shows 5 cell wall rearrangement genes and 2 involved in the BR biosynthetic pathway (Fig. 2). According to the proposed model, *AtRALF1* induces the cell wall remodeling genes *AtPRP1*, *AtPRP3*, and *AtHRGP2*. These genes, among others, are then responsible for cell wall hardening and the blocking of further elongation. The *TCH4* gene undergoes more complex regulation because it is controlled by both *AtRALF1* and BL. Enzymes such as TCH4 play roles in

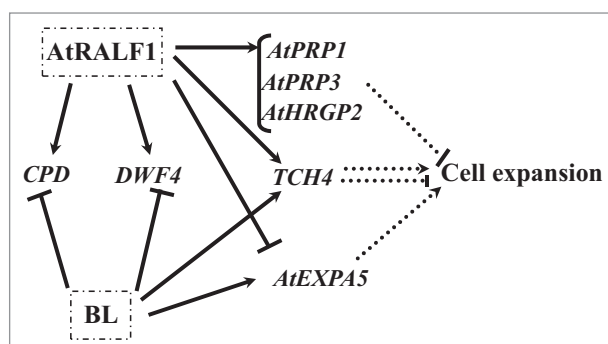


Figure 2. Schematic diagram of the AtRALF1 and BL gene regulatory network for cell expansion. Arrowed solid lines represent induction, and solid lines with bars at the end represent repression. Dashed lines represent the final effects of the actions of the genes in the cell expansion process. Dashed lines ending with arrowheads represent activation, and dashed lines ending with bars represent inhibition.

the entire elongation process and not only in the final hardening stage, which may characterize one of the hypotheses explaining such complex regulation. Finally, the *AtEXPAS5* gene may play a role in elongation only, and therefore, it is repressed by AtRALF1

to initiate cell wall stiffening. Other genes, such as the BL-down-regulated genes *CPD* and *DWF4*, are induced by AtRALF1 and may work in concert with BL during the elongation process.^{12,18,19}

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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