**Research Paper**

**Arabidopsis thaliana** RALF1 opposes brassinosteroid effects on root cell elongation and lateral root formation

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**Abstract**

Rapid alkalinization factor (RALF) is a peptide signal that plays a basic role in cell biology and most likely regulates cell expansion. In this study, transgenic *Arabidopsis thaliana* lines with high and low levels of *AtRALF1* transcripts were used to investigate this peptide’s mechanism of action. Overexpression of the root-specific isoform *AtRALF1* resulted in reduced cell size. Conversely, *AtRALF1* silencing increased root length by increasing the size of root cells. *AtRALF1*-silenced plants also showed an increase in the number of lateral roots, whereas *AtRALF1* overexpression produced the opposite effect. In addition, four *AtRALF1*-inducible genes were identified: two genes encoding proline-rich proteins (*AtPRP1* and *AtPRP3*), one encoding a hydroxyproline-rich glycoprotein (*AtHRPG2*), and one encoding a xyloglucan endotransglucosylase (*TCH4*). These genes were expressed in roots and involved in cell-wall rearrangement, and their induction was concentration dependent. Furthermore, *AtRALF1*-overexpressing plants were less sensitive to exogenous brassinolide (BL); upon BL treatment, the plants showed no increase in root length and a compromised increase in hypocotyl elongation. In addition, the treatment had no effect on the number of emerged lateral roots. *AtRALF1* also induces two brassinosteroid (BR)-downregulated genes involved in the BR biosynthetic pathway: the cytochrome P450 monooxygenases *CONSTITUTIVE PHOTOMORPHISM AND DWARFISM* (CPD) and *DWARF4* (DWF4). Simultaneous treatment with both *AtRALF1* and BL caused a reduction in *AtRALF1*-inducible gene expression levels, suggesting that these signals may compete for components shared by both pathways. Taken together, these results indicate an opposing effect of *AtRALF1* and BL, and suggest that RALF’s mechanism of action could be to interfere with the BR signalling pathway.

**Key words:** Root development, brassinolide, peptide hormone.

**Introduction**

Several physiological processes related to growth, development, defence, and reproduction are coordinated by intercellular communication. Peptide signals are involved in developmental processes and environmental responses in plants through the regulation of intercellular signalling (Ryan et al., 2002; Boller, 2005; Matsubayashi and Sakagami, 2006; Moura and Silva-Filho, 2006).

Rapid alkalinization factor (RALF) is a peptide signal found throughout the plant kingdom that can exhibit either ubiquitous or tissue-specific expression patterns (Moura and Silva-Filho, 2006).
The precursor of the RALF peptide is a preproprotein, indicating that this peptide is secreted (Pearce et al., 2001); when Escobar et al. (2003) fused the tobacco RALF cDNA with that of green fluorescent protein, the resulting chimaeric protein was detected in both the endoplasmic reticulum and the apoplast. The RALF precursor is processed by convertases that target the dibasic site upstream of the active peptide, which is located at the C terminus of the precursor (Matos et al., 2008; Srivastava et al., 2009). A structure–activity study using the tomato RALF peptide showed that the motif ‘YISY’, which is located at positions 5–8 at the N terminus of the active peptide, is essential for activity (Pearce et al., 2010).

Knowledge of the RALF signal transduction pathway is far from complete, and RALF-inducible genes are currently unknown. The rapid increase in the external pH of cell suspension cultures and the activation of a mitogen-activated protein kinase are among the earliest events in the pathway (Pearce et al., 2001). Although the RALF receptor has not yet been identified, Scheer et al. (2005) identified two cell-surface SIRALF-binding proteins when studying the peptide in Solanum lycopersicum (SIRALF, previously LcRALF). AtRALF1, a root-specific isoform from Arabidopsis thaliana, causes a transient increase in the cytoplasmic Ca2+ concentration, suggesting that RALF peptides mediate a Ca2+-dependent signal transduction pathway (Haruta et al., 2008).

A synthetic tomato RALF peptide was shown to inhibit root growth in tomato and Arabidopsis seedlings (Pearce et al., 2001). SacRALF1, a peptide isolated from the leaves of the grass sugarcane, inhibits the formation of elongated cells in cell-suspension cultures, and SacRALF1 gene expression is observed in the elongating base of the leaves but not in the mature, non-elongating leaf tips (Mingossi et al., 2010). When present in pollen germination medium, the SIRALF peptide inhibits the elongation of normal pollen tubes (Covey et al., 2010), and the AtRALF1 isoform also inhibits hypocotyl elongation in dark-grown Arabidopsis seedlings (Mingossi et al., 2010). In the most recent version of the Arabidopsis genome, 37 AtRALF1s were identified (Lamesch et al., 2011). The overexpression of two of these isoforms from Arabidopsis, AtRALF1 and AtRALF23, results in shorter and bushier Arabidopsis plants that displayed a characteristic semi-dwarf phenotype (Matos et al., 2008; Srivastava et al., 2009). When the single-copy RALF gene of the Nicotiana attenuata genome is silenced, the roots of the transgenic tobacco plants grow longer than those of the wild type (Wu et al., 2007). Thus, all the evidence gathered thus far indicates that RALF peptides have a basic role in cell biology, and they most likely regulate cell expansion (Pearce et al., 2001; Matos et al., 2008; Srivastava et al., 2009; Covey et al., 2010; Mingossi et al., 2010).

Cell expansion occurs through the loosening of the existing cell-wall architecture, which is accompanied by the synthesis of new wall components (Carpita and Gibeaut, 1993; McCann et al., 2001; Cosgrove, 2005; Benatti et al., 2012). The primary cell wall is a complex matrix of polysaccharides, structural proteins, and enzymes (Carpita and Gibeaut, 1993; Fry, 2004). Hydroxyproline-rich glycoproteins (HRGPs) and proline-rich proteins (PRPs) are structural proteins that may be involved in cell-wall expansion (Showalter, 1993; Kieliszewski and Lamport, 1994; Cosgrove, 2005). Cell-wall enzymes such as expansin and xyloglucan endotransglycosylase (XET) are also related to cell-wall expansion due to their promotion of wall loosening (Fry et al., 1992; Cosgrove, 1999; Eklöf and Brumer, 2012). The mechanisms involved in cell expansion are regulated by brassinosteroid (BR), auxin, ethylene, gibberellin, and cytokinin signalling (Smallle et al., 1997; Wang et al., 2002; Fu and Harberd, 2003; Parry and Estelle, 2006; Vantraelen and Benková, 2012).

BRs such as brassinolide (BL) are involved in hypocotyl elongation and root growth (Clouse et al., 1996; Clouse and Sasse, 1998; Müssig et al., 2003). BR-mediated cell expansion involves wall-modifying proteins, such as XETs, as well as cortical microtubule reorientation (Mayumi and Shibaoka, 1995; Clouse and Sasse, 1998; Wang et al., 2012). The activity of BL is mediated by a leucine-rich repeat receptor kinase called BR11 (Clouse et al., 1996; He et al., 2000). BR binding to BR11 triggers the autophosphorylation of the kinase domain and the subsequent recruitment of the co-receptor BR11-associated receptor kinase (BAK1), resulting in the activation of BR-responsive genes (Clouse, 2004; Vert et al., 2005; Chinchilla et al., 2009; Li and Jin, 2007).

Little is known about the cross-talk between RALF peptides and other plant signals. In poplar, the PtdRALF2 gene is downregulated after methyl jasmonate treatment, whereas auxin and cytokinin treatments do not affect PtdRALF2 RNA levels (Haruta and Constabel, 2003). The Arabidopsis gene AtRALF23 in Brassica napus is downregulated upon treatment with BRs, and plants that over-express the AtRALF23 gene exhibit compromised BL-induced hypocotyl elongation (Srivastava et al., 2009).

In this study, we showed that silencing of the AtRALF1 gene in Arabidopsis plants increased cell elongation and the formation of lateral roots, whereas AtRALF1 overexpression showed the opposite effects. To track the effects of the peptide at the molecular level, we identified and reported for the first time six RALF-inducible genes. Four of these genes are related to cell-wall rearrangement and two were characterized previously as BR-downregulated genes involved in the BR biosynthetic pathway. In addition, we showed that AtRALF1-overexpressing plants have a compromised response to exogenously applied BR. Our data suggest that AtRALF1 opposes the action of BR in Arabidopsis plants and that the interplay between these two signals might collaborate in the regulation of cell expansion.

Materials and methods

Plant materials and growth conditions

Arabidopsis plants that overexpress AtRALF1 have been described previously (Matos et al., 2008). Seeds for the BR-insensitive mutant bril (At4g39400, CS3723) were obtained from the Arabidopsis Biological Resource Center, Ohio State University, Columbus, USA. For all seedling experiments, Arabidopsis seeds (ecotype Col-0) were surface sterilized and cold treated (4°C) for 4 d in the dark. Seedlings were grown in soil or in half-strength medium containing Murashige and Skoog (MS) salts without vitamins and sucrose (PhytoTechnology Laboratories), KOH-adjusted pH 5.8, and containing 0.9% (w/v) of Gellan Gum Powder (PhytoTechnology Laboratories) in a growth room at 22±2°C with a light regime of 16 h light and 8 h dark.
**Plasmid construction**

The *AtRALF1* coding region was amplified from *Arabidopsis* genomic DNA using standard PCR with the primers AtRALF1F and AtRALF1R (Supplementary Table S1 available at JXB online). The interfering RNA construct used to silence the *AtRALF1* gene (irAtRALF1) was generated using Gateway technology (Invitrogen) and the vectors pENTR/D-TOPO (Invitrogen) and pk7GWIG2I (Karimi et al., 2002) following the manufacturer’s instructions. All constructs were verified by DNA sequencing.

**Plant transformation**

Plants were inoculated with the *Agrobacterium tumefaciens* strain GV3101 carrying the pk7GWIG2I vector using the floral dip method (Clough and Bent, 1998). Seeds from transformed plants were plated in half-strength medium containing MS salts without vitamins and sucrose and 100 mg l⁻¹ of kanamycin (Sigma). Transgenic homozygous *Arabidopsis* lines from the T3 generation were used in all experiments.

**Hormone treatments**

Seedlings were grown on vertical plates containing half-strength medium containing MS salts without vitamins and sucrose with different concentrations of 24-epibrassinolide (BL; PhytoTechnology Laboratories) or indole-3-acetic acid (IAA; PhytoTechnology Laboratories). BL and IAA were prepared as 2 mM stock solutions; IAA was dissolved in 10 M potassium hydroxide and then diluted in water. The BL stock solution was prepared in 10% (w/v) ethanol. Further dilutions were made in water, and the final concentration of the solvents used was applied in the controls and the experimental conditions. Seedlings were photographed using a Nikon (CoolPix S202) digital camera 10 d after germination, and primary root lengths, hypocotyl elongation, rosettes, and leaves were measured using ImageJ (National Institutes of Health, USA). The number of emerged lateral roots (>1 mm) was also recorded 10 d after germination. All experiments were repeated at least three times (independent biological replicates).

Hypocotyl measurements were taken as described by Weigel and Glazebrook, (2002). After cold treatment (4 d at 4 °C), the seeds were placed in half-strength liquid medium containing MS salts without vitamins and sucrose, and incubated with gentle agitation on a rotary shaker in the growth room. Seeds were exposed to light for 1 h and were then kept in the dark (for dark experiments) or maintained in the growth room with 16 h light and 8 h dark (for light experiments). The recombinant AtRALF1 peptide (His6AtRALF1) and BL were added 1 h after germination, and measurements were taken 6 d after treatment.

For semi-quantitative reverse transcription (RT)-PCR and quantitative (q)RT-PCR analysis, 10-d-old seedlings were placed in half-strength liquid medium containing MS salts without vitamins and sucrose, and incubated with gentle agitation on a rotary shaker in the growth room. Different concentrations of His6AtRALF1 and/or BL were then added to the medium.

**Production and purification of recombinant His6AtRALF1**

The AtRALF1 coding region was amplified using standard PCR with genomic DNA as the template and specific primers for the correction of rare codons [primers A (forward) and C (reverse); Supplementary Table S1 available at JXB online]. The final amplification of the insert was performed using specific primers [primers D (forward) and C (reverse); Supplementary Table S1 available at JXB online]. The amplified fragment was fused to the C terminus of a 6×His tag using the pET28b expression vector (Novagen) and introduced into *Escherichia coli* strain BL21. Cells harbouring the plasmids were grown at 37 °C at 250 rpm until they reached an optical density at 600 nm of 0.7 and then were treated with isopropyl β-D-thiogalactopyranoside (1 mM) for 4 h to induce protein expression. Bacterial cells were harvested by centrifugation and resuspended in denaturing buffer [100 mM NaH₂PO₄, 10 mM Tris/HCl (pH 8.0), 8 M urea] and lysed using cell disruption by nitrogen decompression in a Parr bomb. The lysate was centrifuged at 16 000g for 40 min at room temperature. The supernatant was then applied to an affinity chromatography column containing Ni-NTA resin (Qiagen) to purify the peptide. The purified peptide was lyophilized, purified, and quantified by high-performance liquid chromatography using a C18 reversed-phase column (Kromasil) as described previously (Pearce et al., 2001).

**Semi-quantitative RT-PCR and qRT-PCR analysis**

Total RNA was isolated from the roots of *Arabidopsis* plants using Trizol reagent according to the manufacturer’s instructions (Invitrogen) and treated with DNase I (Invitrogen). cDNA was synthesized using 1 μg of RNA and the ImProm-II Reverse Transcription System (Promega). For semi-quantitative RT-PCR analyses, an aliquot of cDNA was used as template in the PCR, which was performed for 27 cycles, unless indicated otherwise, using gene-specific primers (Supplementary Table S1 available at JXB online). The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as a reference to show the equal loading of cDNA in the reactions. All the experiments were performed at least three times (independent biological replicates), and a representative experimental result is shown. Evaluation of RT-PCR signals via densitometry after normalization to the housekeeping gene *GAPDH* was made using ImageJ.

qRT-PCR was performed using 20-fold-diluted cDNA and the Maxima SYBR Green Rox/qPCR Master Mix (Thermo Scientific) on a StepOne™ Real-Time PCR System (Applied Biosystems). The primers used are listed in Supplementary Table S1 (available at JXB online). The *GAPDH* gene was used as an internal control. Three replicates were analysed for each biological sample along with a template-free reaction as a negative control. The threshold cycle (*C*ₚ) was determined automatically by the instrument, and the fold change in each gene was calculated using the equation 2^{−ΔΔCₚ} (Livak and Schmittgen, 2001). An arbitrary value of 1 was attributed to control treatments and wild-type plants. Graphs shown are from one representative biological replicate.

**Measurements of cell length**

Seedlings were grown vertically on plates containing half-strength MS salts without vitamins and sucrose for root measurements. For hypocotyl measurements, unless described otherwise, seedlings were kept in the dark in plates containing half-strength liquid medium containing MS salts without vitamins and sucrose. Measurements were taken 10 d after germination in epidermal cells located at the base of the hypocotyls. For propidium iodide staining of the cell wall, the growth medium was replaced with a 1 mg ml⁻¹ propidium iodide solution (Sigma), and the cells were imaged 15 min after the addition of the stain. Cells in the endodermis of the root differentiation zone (presence of root hairs), located at approximately 800 μm from the tip, were visualized and measured using confocal microscopy (Olympus FV1000). At least 30 cells per root and six cells per hypocotyl were analysed. Fifteen plants from each genetic background were used for root and hypocotyl cell measurements. The wavelengths for excitation and emission were 555 and 655 nm, respectively. Image processing was completed using Olympus Fluoview software.

**Statistical analyses**

For statistical analyses, the Infostat Statistics Base software package (version 2012e; Córdoba, Argentina) was used. Means were compared using the Tukey HSD (honestly significant difference) test in conjunction with analysis of variance (Steel et al., 1996).

**Results and discussion**

**Reduction of AtRALF1 transcripts in irAtRALF1 plants increases root length, lateral root number, hypocotyl elongation, and cell length**

An interfering RNA construct (irAtRALF1) was used to silence the *AtRALF1* gene and to investigate the effects of
the lack of AtRALF1 peptide in Arabidopsis plants. Thirty lines were obtained, and three lines with different levels of AtRALF1 transcripts were selected for the evaluation of root growth, hypocotyl elongation, and lateral root formation. The AtRALF1 transcript levels in the roots of selected irAtRALF1 lines 1, 19, and 23 were lower, similar, and slightly lower, respectively, when compared with wild type (Fig. 1A). Ratios of the AtRALF1 band intensities in the transgenic plants versus the control wild-type plants are shown in Supplementary Table S2 available at JXB online. A plant line that overexpresses AtRALF1 (35S:AtRALF1) was obtained previously (Matos et al., 2008) and is shown for comparison. irAtRALF1 plants showed an average 42.8% increase in root length (Fig. 1B), whereas, in 35S:AtRALF1 plants, root length was observed to decrease by a similar percentage. The increase in root length observed in irAtRALF1 plants was proportional to the level of AtRALF1 transcripts detected by RT-PCR; the lower the transcript level, the longer the roots. Similar results were reported by Wu et al. (2007) in N. attenuata plants bearing a construct to silence the only N. attenuata RALF isoform. Hypocotyl elongation in dark-grown Arabidopsis plants is due to an increase in cell length and not to cell division (Gendreau et al., 1997). Hypocotyl elongation was compromised in the 35S:AtRALF1 plants, and irAtRALF1 lines 1 and 23 showed longer hypocotyls compared with controls (Fig. 1C). Again, the increase in hypocotyl length in irAtRALF1 plants, approximately 33% in irAtRALF1 lines 1 and 23, was proportional to the decrease observed in 35S:AtRALF1 plants, which was approximately 30%. The irAtRALF1-19 line showed normal hypocotyl elongation in spite of the reduced levels of AtRALF1 transcripts. Because the irAtRALF1-19 line had the least reduction in transcript levels, we believe that the reduction in this line is not sufficient to cause a visible effect on the hypocotyls. The irAtRALF1-1 line showed the lowest levels of AtRALF1 transcripts and was used for further experiments. The wild-type phenotype was recovered in irAtRALF1 plants upon exogenous treatment with the HSLAtRALF1 peptide (Supplementary Fig. S1 available at JXB online).

In Arabidopsis, the lateral roots are derived from pericycle founder cells located opposite xylem poles (Blakely et al., 1982; Malamy and Benfey, 1997; Péret et al., 2009). Auxin, cytokinin, BRs, and ethylene act to control lateral root formation and emergence (Casimiro et al., 2001; Bao et al., 2004; Laplaze et al., 2007; Negi et al., 2008). Cytokinin and ethylene act as auxin antagonists (Laplaze et al., 2007; Lewis et al., 2011), whereas auxin and BRs act synergistically (Bao et al., 2004). Interestingly, the number of emerged lateral roots in 35S:AtRALF1 plants was lower than in wild-type plants (average reduction of 60%), whereas the opposite occurred in irAtRALF1 (average increase of 60%) (Fig. 1D). These data indicated the possible interference of the peptide with root architecture maintenance. Recently, overexpression of the AtRALF8 gene in Arabidopsis also caused a reduction in the number of lateral roots (Atkinson et al., 2013).

To prove that the increase in root length and hypocotyl elongation was due to an increase in cell length, we measured the lengths of root and hypocotyl cells in irAtRALF1 and 35S:AtRALF1 plants and compared these with cells from wild-type plants (Fig. 2A, B). Cells from the endodermis of the differentiation zone of the roots of 35S:AtRALF1 plants were 44% smaller than wild-type root cells in the same root
zone, and root cells from irAtRALF1 plants were 30% larger than wild-type cells also from the differentiation zone. The endodermis layer was chosen for measurements due to the high levels of endogenous AtRALF1 gene expression predicted by *in silico* data (eFP-browser, Birnbaum et al., 2003; Winter et al., 2007). Hypocotyl measurements performed on epidermal cells along the axis of elongation showed that 35S:AtRALF1 cells were 34% shorter than in the wild type, and irAtRALF1 cells were 27.6% longer than the hypocotyl cells from wild-type plants. Although *AtRALF1* is expressed predominantly in roots (Haruta et al., 2008), its partial silencing also produced larger leaves and rosettes in irAtRALF1 plants (Supplementary Fig. S2 available at *JXB* online). Whilst we did not investigate these effects, the large rosettes and leaves could be due to the unintentional silencing of another RALF isoform present in the *Arabidopsis* genome.

We evaluated the gene expression of seven RALF isoforms, including those closest in sequence to *AtRALF1* (*AtRALF22*, *AtRALF23*, and *AtRALF33*), and did not observe any significant reduction in their mRNA levels (Supplementary Fig. S3 available at *JXB* online). The results showed that the reduced root growth and hypocotyl elongation observed in 35S:AtRALF1 plants were a consequence of reduced cell size and that partial silencing of the *AtRALF1* gene led to an increase in cell size. Our data support a role for AtRALF1 in cell expansion through the inhibition of cell elongation.

**AtRALF1 induces the expression of genes involved in cell-wall rearrangement**

To understand the mechanism by which the AtRALF1 peptide inhibits cell elongation, we took advantage of an experiment comparing gene expression in 35S:AtRALF1 and wild-type seedlings whose results are deposited in the NCBI Gene Expression Omnibus (Edgar et al., 2002) and are accessible using GEO Series accession number GSE641 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE641). We selected candidate genes to be validated using the following criteria: gene expression is altered in at least one sample; the gene is expressed in roots; and the gene is involved in cell elongation, growth, or expansion. We validated four genes, two that encode PRPs (*AtPRP1*, At1g54970, and *AtPRP3*, At5g62680), one that encodes a hydroxyproline-rich glycoprotein (here named *AtHRGP2*, At5g19800), and the XET gene *TOUCH4* (*TCH4*, At5g57560). All of these genes are upregulated in 35S:AtRALF1 plants, as predicted by the microarray data and confirmed by RT-PCR and qRT-PCR (Fig. 3A, B, see Supplementary Table S2, available at *JXB* online for ratios of the band intensities). Of the four AtRALF1-upregulated genes, *AtPRP1* and *TCH4* were also downregulated in irAtRALF1 plants. *AtPRP3* and *AtHRGP2* transcript levels in wild-type and irAtRALF1 plants were not significantly different. At least two reasons could be proposed to explain the normal expression levels of the *AtPRP3* and *AtHRGP2* genes in the irAtRALF1 plants. The first is that the residual level of *AtRALF1* expression in our knockdown line could be sufficient to support detectable levels of both *AtPRP3* and *AtHRGP2* expression. The second reason is related to the 37 RALF isoforms in the *Arabidopsis* genome and the possible functional redundancy of these genes. The lack of specificity for the *Arabidopsis* RALF isoforms can be inferred from the similarity of the independently obtained semi-dwarf phenotypes caused by the overexpression of AtRALF1, AtRALF23, or AtRALF8 (Matos et al., 2008; Srivastava et al., 2009; Atkinson et al., 2013). In our irAtRALF1 line, we showed that the closest AtRALF isoforms were not affected by the AtRALF1 RNA interference construct (Supplementary Fig. S3 available at *JXB* online); in this case, any other AtRALF
isoform that is expressed in roots could be responsible for maintaining the normal $AtPRP3$ and $AtHRGP2$ gene expression levels. Wild-type plants treated with exogenous H$_2$OAtRALF1 exhibited the induction of all four genes in a H$_2$OAtRALF1-concentration dependent manner (Fig. 3C, see Supplementary Table S2, available at JXB online, for ratios of the band intensities). Gene induction was rapid and could be detected by RT-PCR in as little as 30 min using 0.1 μM H$_2$OAtRALF1. After 3 h of exposure to the peptide, all genes, except $TCH4$, returned to basal levels (Supplementary Fig. S4 available at JXB online). Both the fast response and the fact that expression returned to control levels after only 3 h suggested a rapid turnover of the peptide or a mechanism of desensitization. The concentration dependence also suggested that the induction over time may be dependent on the availability of the signal. Desensitization has been shown for the peptide systemin (Yalamanchili and Stratmann, 2002), and the rapid degradation of RALF peptides in poplar cell culture medium has also been suggested (Haruta and Constabel, 2003).

Cell expansion is the result of the deposition of new cell-wall material and the rearrangement of the existing cell walls. PRPs are structural cell-wall proteins that have been linked to the wound response and to plant development (Tierney et al., 1988; Hong et al., 1990; Carpita and Gibeaut, 1993; Showalter, 1993). The $AtPRP1$ and $AtPRP3$ genes are expressed only in roots and are most likely involved in epidermal cell differentiation (Fowler et al., 1999; Bernhardt and Tierney, 2000; Bruex et al., 2012). Although its function is still unknown, the $AtHRGP2$ gene was induced 2 h after treatment with cytokinin (Lee et al., 2007), and its transcript was detected at high levels in the cl3eg3 mutant, which produces excessive root-hair cells. It was also found as a low-level transcript in cpctry, a non-hair-cell mutant line (Bruex et al., 2012). The $TCH4$ gene encodes a XET involved in cell-wall rearrangement and is expressed in young expanding leaves, lateral root primordium, and elongating hypocotyls, among other tissues (Xu et al., 1995). The $TCH4$ gene is regulated by several environmental stimuli such as cold, heat, and touch, as well as by hormones such as auxin and BRs. Auxin-stimulated induction occurs after 30 min, similar to AtRALF1 induction, whereas BR induction occurs later and is detectable only 2 h after treatment (Xu et al., 1995 and Supplementary Fig. S5 available at JXB online). The identity of the AtRALF1-induced genes suggested that the effect of RALF in plant tissues may be a product of cell-wall rearrangement resulting in wall stiffening. High levels of the peptide in 35S:AtRALF1 plants and in plants treated with exogenous H$_2$OAtRALF1 could cause premature wall hardening, which would prevent full elongation and result in smaller cells and plants.

![Fig. 3. AtRALF1-inducible genes. (A, B) Semi-quantitative RT-PCR (A) and qRT-PCR gene expression analyses (B) performed in roots of 10-d-old AtRALF1-overexpressing (35S:AtRALF1), AtRALF1-silenced (irAtRALF1) transgenic lines and wild-type (WT) plants. Error bars indicate SD. Columns followed by the same letter are not significantly different ($p$<0.01). (C) Semi-quantitative RT-PCR gene expression analyses performed in roots of H$_2$OAtRALF1-treated 10-d-old wild-type plants. Total RNA was extracted from roots of plants after 30 min of treatment with different concentrations of the peptide. GAPDH expression was used as a control. $AtPRP1$ and $AtPRP3$, proline-rich proteins 1 and 3 ($AtPRP1$, At1g54970 and $AtPRP3$, At3g62680); $AtHRGP2$, hydroxyproline-rich glycoprotein (At5g19800); $TCH4$, XET gene TOUCH4 (At5g57560); GAPDH, glyceraldehyde-3-phosphate dehydrogenase (At1g13440). All experiments were repeated at least three times (independent biological replicates).]
AtRALF1 opposes BL effects

Mingossi et al. (2010) showed that SacRALF peptides isolated from sugarcane are probably involved in cell expansion because their coding genes are expressed in expanding tissues. However, it is unclear why a peptide whose actions lead to a halt in cell elongation is located in regions of cell expansion. We hypothesize that RALF counteracts other signals that promote cell elongation. Among the signals that have been associated with cell elongation, BR is a good candidate because BL has been shown to downregulate AtRALF23 gene expression, and plants transformed with the AtRALF23 gene under the control of the 35S promoter exhibited impaired BL-induced hypocotyl elongation (Srivastava et al., 2009). As opposed to AtRALF23, AtRALF1 gene expression is not affected by BL (Srivastava et al., 2009).

Primary root elongation, hypocotyl elongation, and lateral root formation are three well-known effects of exogenously applied BR in Arabidopsis plants (Müssig et al., 2003; Bao et al., 2004; Clouse, 2011). BL induces root growth at 0.01, 0.1, and 1 nM concentrations (Sasse, 1994; Müssig et al., 2003). Transgenic plants overexpressing AtRALF1 (35S:AtRALF1) were less sensitive to root growth-stimulating doses of BL, whereas plants with low levels of AtRALF1 (irAtRALF1) were sensitive to the same concentrations (Fig. 4A). When compared with untreated plants, irAtRALF1 and wild-type plants showed 150 and 200% average increases in root growth, respectively, when treated with 0.1 nM BL (Fig. 4A). When both 35S:AtRALF1 and irAtRALF1 transgenic plants were exposed to higher concentrations of BL (10 and 100 nM), the characteristic inhibitory effect of BL on root growth was observed (Fig. 4A, and Roddick et al., 1993; Müssig et al., 2003). BRs act as inhibitors if a threshold level is exceeded, and this threshold level is dependent on the genotype (Müssig et al., 2003); the 35S:AtRALF1 genotype was more sensitive to inhibitory concentrations of BL. Roots from 35S:AtRALF1 plants treated with 10 nM BL were shorter than those from untreated plants (0 BL), whereas wild-type and irAtRALF1 plants exposed to the same concentration did not differ from the untreated plants, indicating that the threshold was lower in the 35S:AtRALF1 plants than in wild-type plants (Fig. 4A).

BL also induces hypocotyl elongation in light conditions (Mandava, 1988; Sasse, 1990; Clouse and Sasse, 1998). The BL-induced hypocotyl elongation response was 5 and 10% smaller in 35S:AtRALF1 plants exposed to 10 and 100 nM BL, respectively, than in wild-type plants. However, the concentrations that caused elongation were the same within each genotype (Fig. 4B). Plants overexpressing AtRALF1 already have shorter hypocotyls (64.3% of wild type), and when both 35S:AtRALF1 and wild-type plants were treated with BL, 35S:AtRALF1 plants had hypocotyls that were 56.5% smaller than those of wild-type plants (Fig. 4B). A similar response was reported for plants that overexpress AtRALF23 (Srivastava et al., 2009). Hypocotyls of light-grown irAtRALF1 plants are larger than hypocotyls of wild-type plants. In addition, when irAtRALF1 plants are exposed to BL, they elongate further but do not surpass the size of the hypocotyls of BL-treated wild-type plants, suggesting the lack of additive effects (Fig. 4B). One reason for the limited hypocotyl elongation response in irAtRALF1 plants could be that the cells had reached the limit of elongation in our experimental conditions, and even with an additional stimulus, they would not have been able to overcome light-mediated inhibition. A similar response has been described for auxin-overproducing plants (Romano et al., 1995). Although the AtRALF1 gene is mainly expressed in roots, we noticed that 35S:AtRALF1 plants, due to ectopic expression of the AtRALF1 gene, also exhibited a compromised sensitivity to BL when rosette width and leaf length were evaluated (Supplementary Fig. S6 available at JXB online).

BL increases the lateral root number in Arabidopsis plants (Bao et al., 2004). Plants overexpressing AtRALF1 already have a reduced number of emerged lateral roots, and this number remained unchanged even upon treatment with high concentrations (e.g. 10 nM) of BL (Fig. 4C). AtRALF1-silenced plants, which already have a large number of emerged lateral roots, exhibited further increases once treated with BL.
(Fig. 4C). Bao et al. (2004) showed that BL and auxin act synergistically to promote increased lateral root numbers. However, auxin appears to be a central player in the regulation of lateral root formation (Blakely et al., 1982; Casimiro et al., 2001; Péret et al., 2009). To investigate if the peptide effect also opposes auxins in the formation of lateral roots, the transgenic plants were treated with IAA. Both overexpression (35S:AtRALF1) and suppression (irAtRALF1) of the peptide had no effect on IAA treatment, as both genotypes showed increased lateral root formation upon IAA treatment (Supplementary Fig. S7 available at JXB online). Our data regarding the effects of BL and IAA on lateral root formation showed that RALF opposes only BL and not IAA, suggesting that AtRALF1 acts downstream of or in parallel with IAA.

BL has a different and opposite effect on hypocotyl elongation when it is applied under dark rather than light conditions. Specifically, BL promotes elongation when seedlings are exposed to light and inhibits elongation when seedlings are grown in the dark (Mandava, 1988; Turk et al., 2003). To analyse whether the opposing effects of AtRALF1 and BL would be maintained even under conditions in which BL has an inhibitory effect, we simultaneously treated seedlings with hAtRALF1 and high concentrations of BL (50 and 500 nM) under both light and dark conditions. hAtRALF1 decreased hypocotyl elongation in plants grown in both light and dark conditions (Fig. 5). In light conditions, hAtRALF1 slightly inhibited the full effect of BL-induced hypocotyl elongation, even at high BL concentrations such as 500 nM (Fig. 5A). In etiolated plants, in which BL inhibits hypocotyl elongation (Turk et al., 2003), treatment with the hAtRALF1 peptide and BL led to an even greater decrease in elongation, demonstrating the characteristics of an additive effect (Fig. 5B).

BL is known as a growth-promoting hormone, and a role for RALF in the regulation of cell expansion has also been proposed. Our data showed opposite roles for AtRALF1 and BL during primary root elongation, hypocotyl elongation, and lateral root formation. An additive effect of BL and AtRALF1 was only observed on the inhibition of hypocotyl elongation when high concentrations of BL were applied to dark-grown seedlings. Overall, these results are consistent with independent, opposite effects of BR and AtRALF1 on cell elongation.

AtRALF1 induces genes of the BL biosynthetic pathway

To investigate further the effect of AtRALF1 on BL responses, we evaluated two BL-downregulated genes that encode cytochrome P450 monoxygenases, CONSTITUTIVE PHOTOMORPHISM AND DWARFISM (CPD) and DWARF4 (DWF4), both of which are involved in the biosynthesis of BRs (Mathur et al., 1998; Goda et al., 2002). Roots from plants that overexpressed AtRALF1 showed high levels of CPD and DWF4 mRNA (Fig. 6A, B; see Supplementary Table S2 (available at JXB online) for ratios of the band intensities). In irAtRALF1 plants, the mRNA levels of the CPD gene were lower than in the wild type, whereas transcript levels for DWF4 were similar to those of the wild type (Fig. 6A, B). When Arabidopsis plants were exposed to increasing concentrations of exogenously applied hAtRALF1, the CPD and DWF4 genes also exhibited increased expression in roots, as demonstrated by RT-PCR (Fig. 6C; see Supplementary Table S2 (available at JXB online) for ratios of the band intensities). The gene expression analyses of two BL-downregulated genes again showed opposite roles for AtRALF1 and BL. The AtRALF1-mediated induction of these genes could be one way that plants have evolved to counteract excess RALF, perhaps by seeking a physiological equilibrium between RALF and BL. Coordination of the cellular progression mediated by BR in roots demands a refined balance of this hormone (González-García et al., 2011; Hacham et al., 2011). The compromised sensitivity to BL of the plants overexpressing AtRALF1 and the AtRALF1 induction of two genes involved in BR biosynthesis also suggest that AtRALF1 may be used as a signal to counteract BR-regulated cell growth.

BL decreases the mRNA levels of genes upregulated by AtRALF1

Our study of plants simultaneously exposed to AtRALF1 and BL also suggested opposite effects of the two signals on cell elongation, and transgenic 35S:AtRALF1 plants showed a compromised response when treated with exogenous BL (Figs
AtRALF1 opposes BL effects

To gain further insight into the molecular mechanism behind this inhibitory action of AtRALF1 on BL, we investigated how the expression of the AtRALF1-inducible genes, AtPRP1, AtPRP3, AtHRGP2, TCH4, CPD, and DWF4, would be affected upon simultaneous treatment with AtRALF1 and BL. BL treatment by itself had no effect on AtPRP1, AtPRP3, and AtHRGP2 gene expression and, as expected, induced TCH4 at later time points and downregulated DWF4 (Fig. 7 and Supplementary Fig. S5, available at JXB online; see Supplementary Table S2, available at JXB online, for ratios of the band intensities) (Xu et al., 1995; Mathur et al., 1998; Goda et al., 2002). The downregulation of CPD was not statistically significant at 30 min but was...
clear at the 3 h time point (Supplementary Fig. S8 available at JXB online). DWF4 was also downregulated by BL treatment at the 3 h time point (Supplementary Fig. S8 available at JXB online). When plants were simultaneously treated with HisAtRALF1 and BL, the AtPRP1, AtPRP3, and AtHRGP2 genes were induced but to a lesser degree compared with the induction by HisAtRALF1 treatment alone (Fig. 7A, B). The TCH4 gene was induced faster by HisAtRALF1 than by BL treatment, and the simultaneous addition of HisAtRALF1 and BL resulted in an intermediate level of induction (Fig. 7A, B). Xu et al. (1995) reported that TCH4 mRNA levels were weakly induced 30 min after BL treatment. AtPRP1, AtPRP3, AtHRGP2, CPD, and DWF4 mRNAs all returned to control levels by 3 h after HisAtRALF1 treatment (Supplementary Fig. S8 available at JXB online). The TCH4 gene remained induced until the end of the experiment (3 h).

TCH4 gene induction has been studied in a BR-insensitive mutant (bril-2), and it has been reported that the signalling pathways that lead to the activation of this gene are independent or at least convergent at a point downstream of the BR receptor (Iliev et al., 2002). This seems to be the case for the AtRALF1-mediated induction of the TCH4 gene because its response is not compromised in the bril mutant. AtRALF1 and BL may share a subset of transcription factors responsible for TCH4 induction. The sharing of different regulatory elements was reported in a promoter deletion study of the TCH4 gene (Iliev et al., 2002). The sharing of transcription factors could also explain the lower level of induction of AtRALF1-inducible genes upon simultaneous treatment with RALF and BL. The recruitment of shared transcription factors by BL could limit a full AtRALF1 response.

When evaluated after simultaneous treatment with the peptide and BL, the BR biosynthetic genes CPD and DWF4 also showed reduced mRNA expression levels when compared with the HisAtRALF1 treatment alone (Fig. 7A, B). To confirm that BR sensing is required to minimize the induction of the RALF-inducible genes, we evaluated the combined responses in the bril mutant (Clouse et al., 1996). Although bril mutant plants have proportionally shorter roots, treatment with the AtRALF1 peptide showed an even higher inhibition of root growth (Supplementary Fig. S9 available at JXB online). bril plants exposed to the same treatments performed in the wild type showed that bril only responded to HisAtRALF1, and no reduction in gene expression was observed upon simultaneous BL/HisAtRALF1 treatment (Fig. 7A, B). The TCH4 gene was the only one to show a minor decrease in bril plants treated with both HisAtRALF1 and BL. TCH4 responds to a complex set of signals, including auxin, BRs, environmental stimuli, and development. The inhibition of a full AtRALF1 response when bril plants are treated with HisAtRALF1 and BL could mean that BL is perhaps inhibiting AtRALF1 through one of the other BL receptors that have specific functions in cell growth (Cañó-Delgado et al., 2004).

Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1. Exogenous HisAtRALF1 effect on root length and hypocotyl elongation in irAtRALF1 plants.
Supplementary Fig. S2. AtRALF1-overexpressing (35S:AtRALF1) and AtRALF1-silenced (irAtRALF1) transgenic lines.
Supplementary Fig. S3. Semi-quantitative RT-PCR gene expression analyses performed in roots of 10-d-old AtRALF1-silenced (irAtRALF1) and wild-type (WT) plants.
Supplementary Fig. S4. Semi-quantitative RT-PCR gene expression analyses performed in roots of untreated (WT) and HisAtRALF1-treated 10-d-old wild-type plants.
Supplementary Fig. S5. Time course analysis of the xylem- and auxin-induced root growth, TOUCH4 (At5g57560) gene expression after root elongation for auxin, HisAtRALF1 and brassinolide.
Supplementary Fig. S6. AtRALF1-overexpressing (35S:AtRALF1, black columns) and AtRALF1-silenced (irAtRALF1, grey columns) transgenic lines treated with different concentrations of brassinolide (BL).
Supplementary Fig. S7. Number of emerged lateral roots in AtRALF1-overexpressing (35S:AtRALF1, black columns) and AtRALF1-silenced (irAtRALF1, grey columns) transgenic lines treated with brassinolide (BL) or indole-3-acetic acid (IAA).
Supplementary Fig. S8. Gene expression analyses of AtRALF1-inducible genes upon simultaneous treatment with HisAtRALF1 (R, 1 μM for 30 min) and brassinolide (BL, 1 μM for 30 min).
Supplementary Fig. S9. HisAtRALF1 effect on root length in bril mutants.
Supplementary Table S1. Primers used for cloning, quantitative and semi-quantitative RT-PCR analyses.
Supplementary Table S2. Evaluation of RT-PCR signals via densitometry.

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