

RESEARCH PAPER

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

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## Abstract

Rapid alkalization 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 alkalization 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,

Abbreviations: BL, brassinolide; BR, brassinosteroid; CT, threshold cycle; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HRGP, hydroxyproline-rich glycoprotein; IAA, indole-3-acetic acid; MS, Murashige and Skoog; PRP, proline-rich protein; RALF, rapid alkalization factor; SD, standard deviation.

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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 LeRALF). AtRALF1, a root-specific isoform from *Arabidopsis thaliana*, causes a transient increase in the cytoplasmic Ca<sup>2+</sup> concentration, suggesting that RALF peptides mediate a Ca<sup>2+</sup>-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 AtRALFs 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 Lampert, 1994; Cosgrove, 2005). Cell-wall enzymes such as expansin and xyloglucan endotransglucosylase (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 (Smalle *et al.*, 1997; Wang *et al.*, 2002; Fu and Harberd, 2003; Parry and Estelle, 2006; Vanstraelen 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 BRI1 (Clouse *et al.*, 1996; He *et al.*, 2000). BR binding to BRI1 triggers the autophosphorylation of the kinase domain and the subsequent recruitment of the co-receptor BRI1-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* 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 report 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 pk7GWIWG2I (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 pk7GWIWG2I 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<sup>-1</sup> 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-epi-brassinolide (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 (<sub>His</sub>AtRALF1) and BL were added 1 d 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 <sub>His</sub>AtRALF1 and/or BL were then added to the medium.

### Production and purification of recombinant <sub>His</sub>AtRALF1

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-1-thiogalactopyranoside (1 mM) for 4 h to induce protein expression. Bacterial cells were harvested by centrifugation and resuspended in denaturing buffer [100 mM NaH<sub>2</sub>PO<sub>4</sub>, 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 16 000 g 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<sub>T</sub>*) was determined automatically by the instrument, and the fold change in each gene was calculated using the equation 2<sup>-ΔΔ*C<sub>T</sub>*</sup> (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<sup>-1</sup> 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

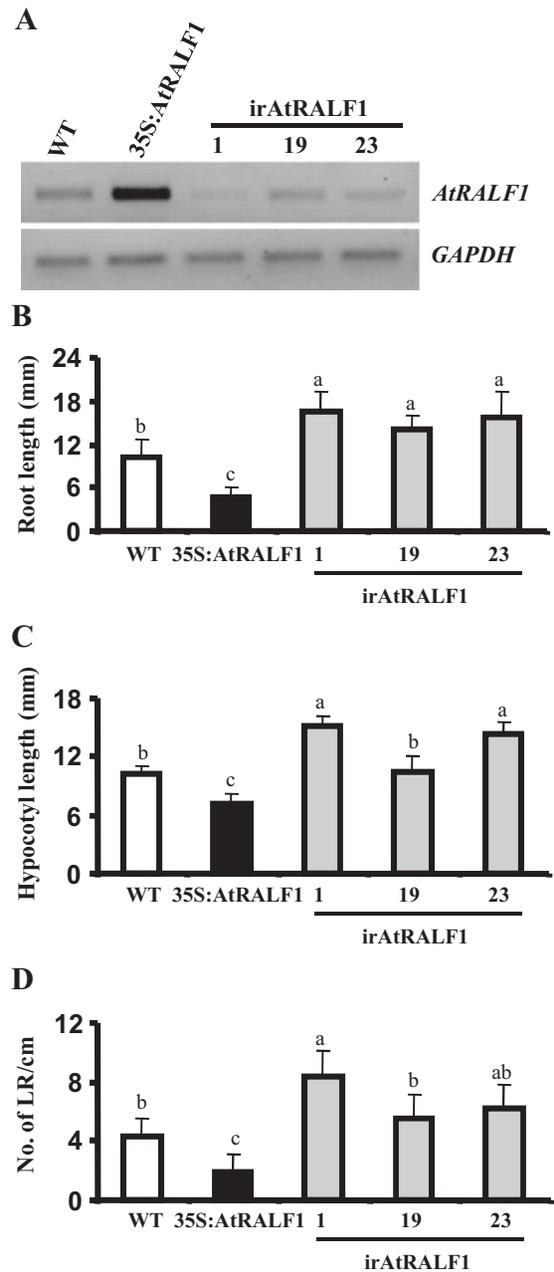
### *Reduction of AtRALF1 transcripts in irAtRALF1 plants increases root length, lateral root number, hypocotyl elongation, and cell length*

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 His-AtRALF1 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



**Fig. 1.** *AtRALF1* gene silencing in transgenic lines (irAtRALF1). (A) Transcript levels of the *AtRALF1* gene in transgenic plants. Transcript levels were examined by RT-PCR using RNA samples extracted from 10-d-old roots of plants grown on half-strength MS agar plates. *GAPDH* was used as an internal control. WT, wild type. (B) Root length of *AtRALF1*-overexpressing (35S:AtRALF1, black columns) and *AtRALF1*-silenced (irAtRALF1, grey columns) transgenic lines. (C) Hypocotyl length of etiolated transgenic plants. (D) Number of emerged lateral roots per cm in transgenic plants. Root length and number of emerged lateral roots were measured and counted in 5- and 10-d-old seedlings respectively ( $n > 30$ ). Hypocotyl length was measured in 5-d-old etiolated plants. Error bars indicate standard deviation (SD). Columns with the same letter are not significantly different ( $P < 0.01$ ). All experiments were repeated at least three times (independent biological replicates).

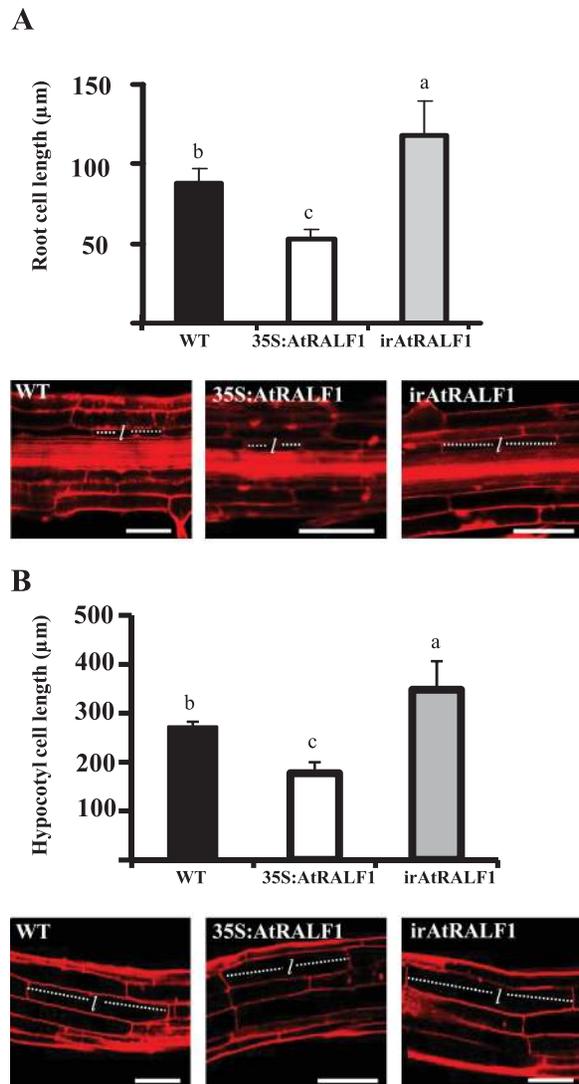
35S:AtRALF1 plants and compared these with cells from wild-type plants (Fig. 2A, B). Cells from the endodermis of the differentiation zone of the roots of 35S:AtRALF1 plants were 44% smaller than wild-type root cells in the same root

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

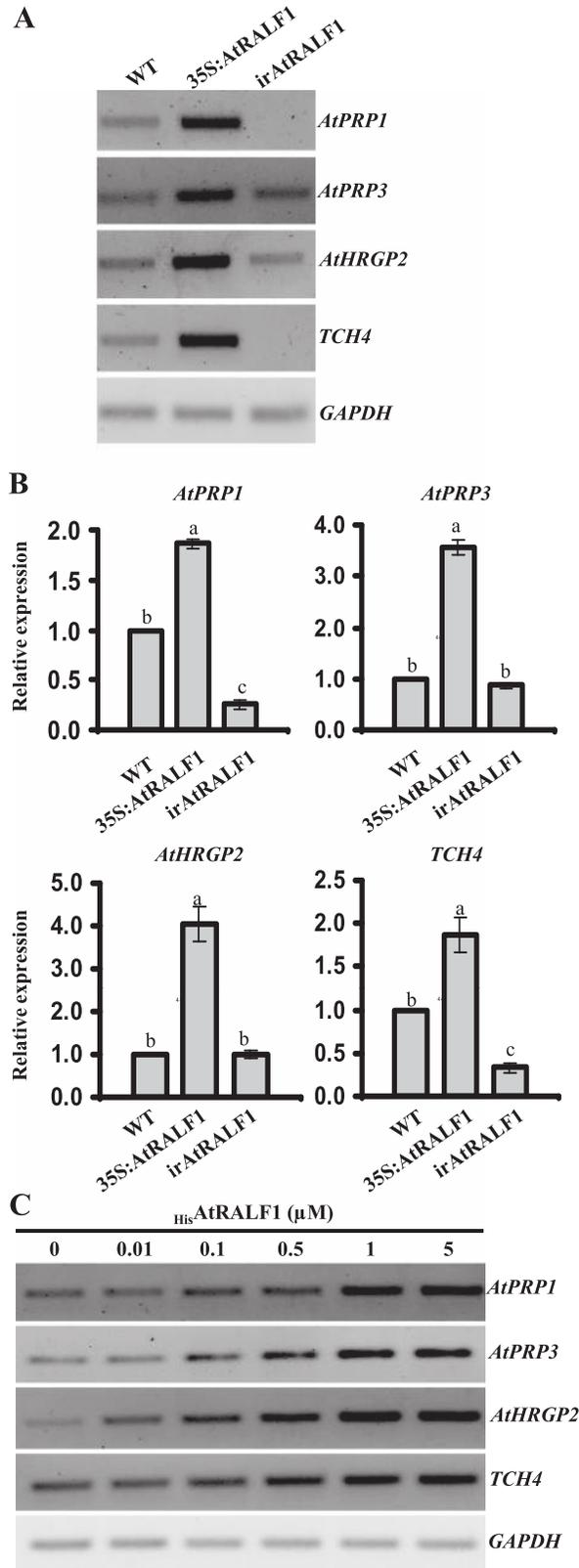
silencing also produced larger leaves and rosettes in *irAtRALF1* plants (Supplementary Fig. S2 available at *JXB* online). Whilst we did not investigate these effects, the large rosettes and leaves could be due to the unintentional silencing of another RALF isoform present in the *Arabidopsis* genome. We evaluated the gene expression of seven RALF isoforms, including those closest in sequence to *AtRALF1* (*AtRALF22*, -23, and -33), 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*, At3g62680), 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



**Fig. 2.** Root and hypocotyl cell length of *AtRALF1*-overexpressing (35S:AtRALF1) and *AtRALF1*-silenced (*irAtRALF1*) transgenic lines. (A) Root cell length of 35S:AtRALF1 plants (black columns) and *irAtRALF1* (grey columns) plants. Panels below the graph are confocal images of the root differentiation zone. Cells from root endodermis of 10-d-old seedlings were measured ( $n=30$  cells per root). WT, wild type. (B) Hypocotyl cell length of 35S:AtRALF1 (black columns) and *irAtRALF1* (grey columns) plants. Panels below the graph are confocal images of the base of hypocotyls. Epidermal cells from hypocotyls of 10-d-old seedlings were measured ( $n=6$  cells per hypocotyl). Error bars indicate SD. Columns followed by the same letter are not significantly different ( $P<0.01$ ). Bars, 100 µm. The length of representative cells is indicated by 'l'. (This figure is available in colour at *JXB* online.)



**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  $_{His}AtRALF1$ -treated 10-d-old wild-type plants. Total RNA was extracted from roots of plants after 30 min of treatment with different concentrations

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  $_{His}AtRALF1$  exhibited the induction of all four genes in a  $_{His}AtRALF1$ -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  $0.1 \mu M$   $_{His}AtRALF1$ . 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 popular 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 *cl3egl3* 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 primordia, 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  $_{His}AtRALF1$  could cause premature wall hardening, which would prevent full elongation and result in smaller cells and plants.

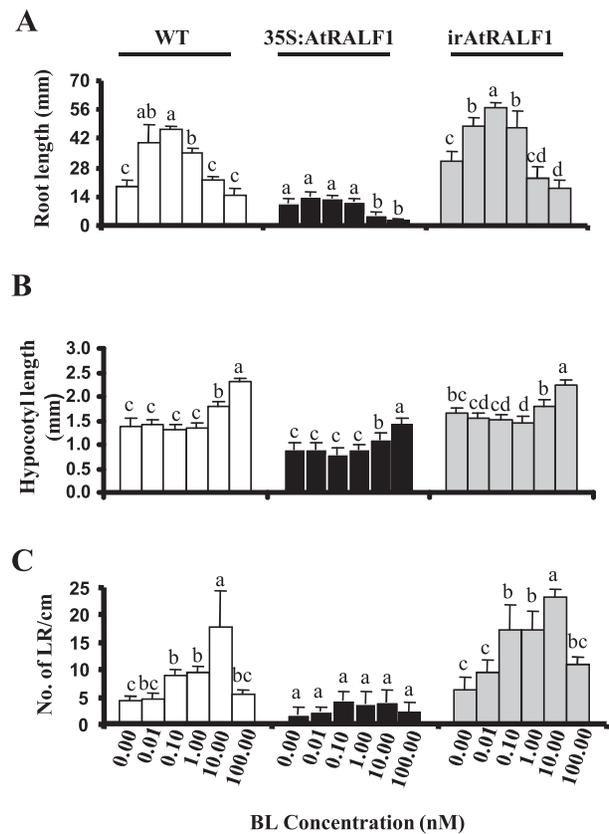
of the peptide. *GAPDH* expression was used as a control. *AtPRP1* and *AtPRP3*, proline-rich proteins 1 and 3 (*AtPRP1*, At1g54970 and *AtPRP3*, At3g62680); *AtHRGP2*, hydroxyproline-rich glycoprotein (At5g19800); *TCH4*, XET gene *TOUCH4* (At5g57560); *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase (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



**Fig. 4.** AtRALF1-overexpressing (35S:AtRALF1, black columns), AtRALF1-silencing (irAtRALF1, grey columns) transgenic lines and wild-type (WT) plants (white columns) treated with different concentrations of BL. (A) Root length. (B) Hypocotyl length of light-grown plants. (C) Number of emerged lateral roots per cm. Root length, hypocotyl length, and number of emerged lateral roots were measured in 10-d-old seedlings ( $n > 30$ ) grown in the presence of different concentrations of BL. Error bars indicate SD. Statistical analysis was made for each genetic background separately. Columns within each genotype that are followed by the same letter are not significantly different ( $P < 0.01$ ). All experiments were repeated at least three times.

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.

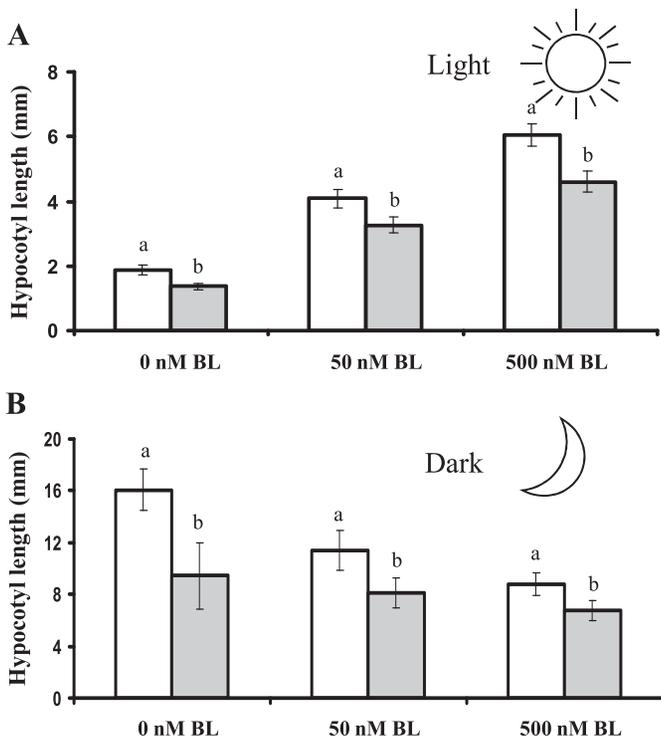
BL has a different and opposite effect on hypocotyl elongation when it is applied under dark rather than light conditions. Specifically, BL promotes elongation when seedlings are exposed to light and inhibits elongation when seedlings are grown in the dark (Mandava, 1988; Turk *et al.*, 2003). To analyse 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 HisAtRALF1 and high concentrations of BL (50 and 500 nM) under both light and dark conditions. HisAtRALF1 decreased hypocotyl elongation in plants grown in both light and dark

conditions (Fig. 5). In light conditions, HisAtRALF1 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 HisAtRALF1 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*

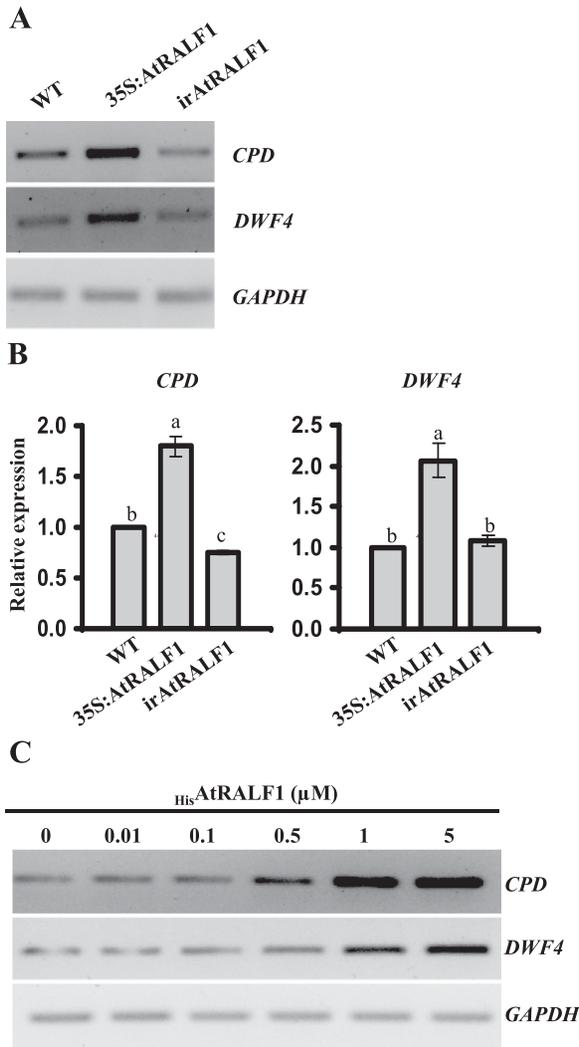
To investigate further the effect of AtRALF1 on BL responses, we evaluated two BL-downregulated genes that encode cytochrome P450 monooxygenases, *CONSTITUTIVE PHOTOMORPHISM AND DWARFISM (CPD)* and *DWARF4 (DWF4)*, both of which are involved in the biosynthesis of BRs (Mathur *et al.*, 1998; Goda *et 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 HisAtRALF1, 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.



**Fig. 5.** AtRALF1 effect on hypocotyl elongation in the presence or absence of BL at high concentrations. (A) Hypocotyl length of light-grown 5-d-old seedlings HisAtRALF1 treated (1 μM, grey columns) or untreated (control, white columns). (B) Hypocotyl elongation of dark-grown 5-d-old seedlings HisAtRALF1 treated (1 μM, grey columns) or untreated (control, white columns). Error bars indicate SD. Within each concentration of BL, columns followed by the same letter are not significantly different ( $P < 0.01$ ). All experiments were repeated at least three times.

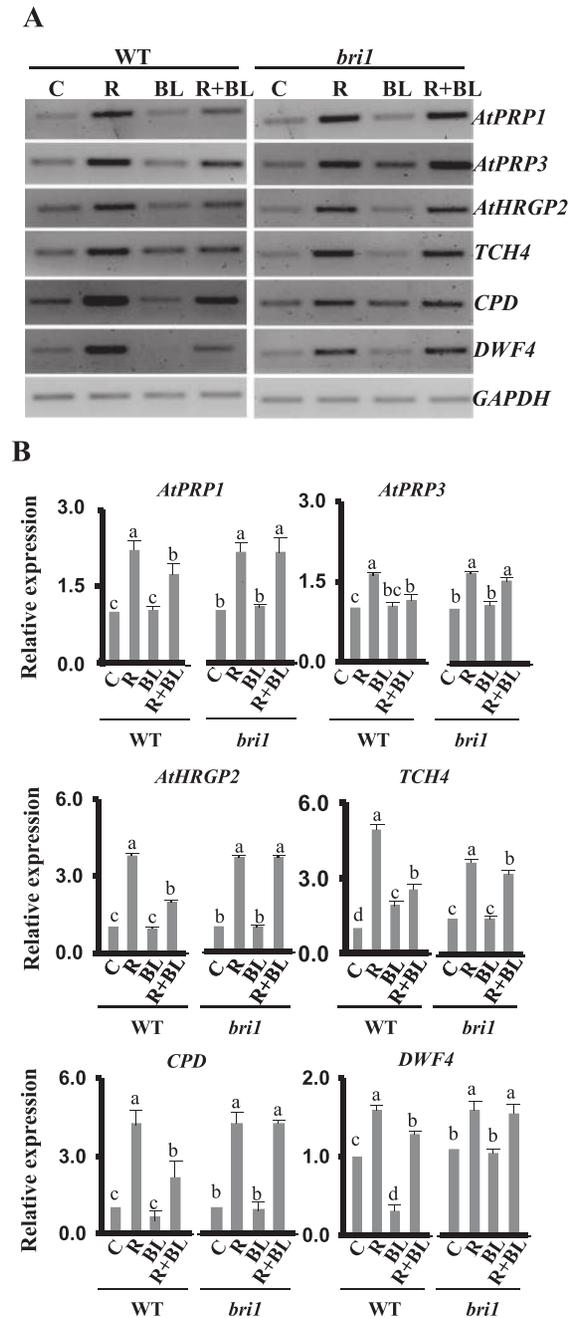
#### *BL decreases the mRNA levels of genes upregulated by AtRALF1*

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



**Fig. 6.** AtRALF1 effect on BL-downregulated genes *CPD* (At5g05690) and *DWF4* (At3g50660). (A, B) Semi-quantitative RT-PCR gene expression analyses performed in roots of 10-d-old AtRALF1-overexpressing (35S::AtRALF1) and AtRALF1-silenced (irAtRALF1) transgenic lines and wild-type plants (A) and quantitative qRT-PCR gene expression analyses (B). 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  $_{\text{His}}$ AtRALF1-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* (At1g13440) expression was used as a control. All experiments were repeated at least three times (independent biological replicates).

4 and 5). 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



**Fig. 7.** Gene expression analyses of AtRALF1-inducible genes upon simultaneous treatment with  $_{\text{His}}$ AtRALF1 (R, 1  $\mu\text{M}$  for 30 min) and BL (1  $\mu\text{M}$  for 30 min). (A) Semi-quantitative RT-PCR performed using total RNA extracted from roots of untreated (C, control plants) or treated (R, BL, or R+BL) 10-d-old wild-type (WT) or BL-insensitive mutant *bri1* plants. (B) qRT-PCR performed using total RNA extracted from roots of untreated (C, control plants) or treated (R, BL, or R+BL) 10-d-old wild-type (WT) or BL-insensitive mutant *bri1* plants. *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*, xyloglucan endotransglucosylase *TOUCH4* (At5g57560); *CPD*, constitutive photomorphism and dwarfism (At5g05690). *DWF4*, DWARF4 (At3g50660); *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase (At1g13440). *DWF4* and *AtPRP1* were amplified using 26 and 29 PCR cycles, respectively. Error bars indicate SD. Statistical analysis was made for each genetic background separately. Columns within each genotype that are followed by the same letter are not significantly different ( $P < 0.01$ ). The experiment was performed at least three times (independent biological replicates).

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 (Caño-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 *irAtRALF1*-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 xyloglucan endotransglucosylase *TOUCH4* (At5g57560) gene expression after treatment with 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  $\mu$ M for 30 min) and brassinolide (BL, 1  $\mu$ M 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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