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RESEARCH PAPER

Strigolactones interact with ethylene and auxin in regulating root-hair elongation in *Arabidopsis*

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Abstract

Strigolactones (SLs) or derivatives thereof have been identified as phytohormones, and shown to act as long-distance shoot-branching inhibitors. In *Arabidopsis* roots, SLs have been suggested to have a positive effect on root-hair (RH) elongation, mediated via the MAX2 F-box. Two other phytohormones, auxin and ethylene, have been shown to have positive effects on RH elongation. Hence, in the present work, *Arabidopsis* RH elongation was used as a bioassay to determine epistatic relations between SLs, auxin, and ethylene. Analysis of the effect of hormonal treatments on RH elongation in the wild type and hormone-signalling mutants suggested that SLs and ethylene regulate RH elongation via a common regulatory pathway, in which ethylene is epistatic to SLs, whereas the effect of SLs on RH elongation requires ethylene synthesis. SL signalling was not needed for the auxin response, whereas auxin signalling was not necessary, but enhanced RH response to SLs, suggesting that the SL and auxin hormonal pathways converge for regulation of RH elongation. The ethylene pathway requirement for the RH response to SLs suggests that ethylene forms a cross-talk junction between the SL and auxin pathways.

Key words: Auxin, ethylene, root-hair elongation, signalling, strigolactone, synthesis.

Introduction

Strigolactones (SLs) or derivatives thereof have been recently identified as phytohormones, and have been shown to act as long-distance branching inhibitors and to play a role in suppressing the growth of pre-formed axillary buds (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). SL production has been demonstrated in both monocotyledons and eudicotyledons (reviewed by Xie *et al.*, 2010); they are synthesized mainly in the roots and in some parts of the stem (Foo *et al.*, 2001; reviewed by Dun *et al.*, 2009) and then move towards the shoot apex (Foo *et al.*, 2001; Brewer *et al.* 2009; Ferguson and Beveridge, 2009).

SL pathways have so far been identified in several plant species (e.g. Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008; Drummond *et al.*, 2009; Liang *et al.*, 2010; Vogel *et al.*, 2010; reviewed by Dun *et al.*, 2009; reviewed by Leyser, 2009). Several enzymes involved in SL biosynthesis have been identified (Umehara *et al.*, 2008; Liang *et al.*, 2010; Vogel *et al.*, 2010; reviewed by Dun *et al.*, 2009; reviewed by Leyser, 2009). In addition, although SL receptors have not yet been identified, MAX2, an F-box protein, has been suggested to be a component of SL signalling and function in the degradation of as yet unknown protein targets, mediated by ubiquitin (Stirnberg *et al.*, 2007; Umehara *et al.*, 2008).

More recently, based on analyses of mutants flawed in SL biosynthesis or signalling and the treatment of seedlings with GR24 (a bioactive, synthetic SL; Johnson *et al.*, 1981), SLs have been suggested to repress lateral root initiation (Kapulnik *et al.*, 2011; Ruyter-Spira *et al.*, 2010) and to have a positive effect on root-hair (RH) elongation in

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ARF, auxin-response factor; AVG, aminoethoxyvinylglycine; IAA, indole-3-acetic acid; RH, root-hair; SL, strigolactone; WT, wild type.

Arabidopsis; these effects are suggested to be mediated via the MAX2 F-box (Kapulnik *et al.*, 2011). In addition, SLs have been suggested to interfere with the inhibitory effect of exogenously applied auxin on tomato root elongation via an increase in root-cell length, and to lead to alterations in RH elongation (Koltai *et al.*, 2010).

Several pieces of evidence suggest intimate cross-talk between auxin and SLs. SLs were suggested to move up into the buds to repress their outgrowth as auxin-promoted secondary messengers (Brewer *et al.*, 2009; Ferguson and Beveridge, 2009; reviewed by Dun *et al.*, 2009). Alternatively, it was suggested that SLs inhibit polar auxin transport from the buds by reducing the capacity for such transport from the apical meristem, resulting in restrained bud outgrowth (e.g. Bennett *et al.*, 2006; Mouchel and Leyser, 2007; Ongaro and Leyser, 2008; Leyser, 2009). However, it was shown that SLs can inhibit branching in auxin-depleted decapitated pea plants and in auxin-signalling mutants (Brewer *et al.*, 2009). In addition, auxin and SLs were shown to change each other's levels and distribution in a dynamic feedback loop (Hayward *et al.*, 2009). Therefore, the cross-talk between SLs and auxin is still under debate; additional evidence is needed to determine the epistatic relations between the two.

The gaseous plant hormone, ethylene, has been suggested to be involved in diverse developmental processes, including RH elongation (Pitts *et al.*, 1998), and has been suggested to partially function in some of these processes via cross-talk with auxin (reviewed by Stepanova and Alonso, 2009). For example, recording of hormonal effects on lateral root formation and primary root elongation suggested that at low levels, ethylene promotes auxin synthesis in the root apex (Stepanova *et al.*, 2007; Swarup *et al.*, 2007; Ivanchenko *et al.*, 2008), and auxin transport (Negi *et al.*, 2008, 2010). However, nothing is yet known on the possible cross-talk between SLs and ethylene.

Since all three phytohormones (SLs, auxin, and ethylene) have a positive effect on RH elongation (Pitts *et al.*, 1998; Kapulnik *et al.*, 2011), this process was used as a bioassay to determine the epistatic relations between SLs, auxin, and ethylene. Results suggested that ethylene is epistatic to SLs, implying that SLs and ethylene regulate RH elongation in *Arabidopsis* via a common regulatory pathway, whereas SL and auxin hormonal pathways may converge via the ethylene pathway for regulation of RH elongation.

Materials and methods

Arabidopsis *strains and growth conditions*

Seeds of *Arabidopsis* wild type (WT; Columbia; Col-0), and *max2-1*, *etr1-1*, *ein2-1*, *tir1-1*, *arf7arf19*, and *aux1-7ein2-1* mutants (Col background), obtained from the ABRC stock centre (http://abrc.osu.edu/), were surface sterilized and germinated on half-strength Murashige and Skoog (MS) plates supplemented with 1.5% (w/v) sucrose. Plates were incubated vertically in the dark at 4 °C for 2 d to synchronize germination. Three days after germination, seedlings were gently transferred using forceps to half-strength MS plates containing various concentrations of hormones or inhibitors (as detailed below) and controls. The location of the root tip of the transferred seedling was marked on the plate. The plate was left unsealed to prevent accumulation of gases (e.g. ethylene). Plates were positioned in an upright 45° position, and incubated at 22 °C. Light intensity at plate level was 50–60 μE m^{-2} s^{-1} provided by white fluorescent tubes with a photoperiod of 16/8 h (light/dark) for 6 d.

GR24 (Johnson *et al.*, 1981) treatments were conducted at concentrations ranging from 0 to 3×10^{-6} M. GR24 was initially dissolved in acetone (5 mg of GR24 in 3 ml of acetone) to give a 4.5 mM solution, and this solution was then further diluted with double-distilled sterile water. The ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) was diluted in double-distilled sterile water to give a stock solution of 1 mM, and was used at final concentrations ranging from 0 to 4×10^{-11} M; the ethylene-synthesis inhibitor 2-aminoethoxyvinylglycine (AVG) was diluted in double-distilled sterile water to give a stock solution of 1 mM, and was used at a final concentration of 2×10^{-7} M; indole-3-acetic acid (IAA) was diluted in double-distilled sterile water to give a stock solution of 1 mM, and was used at final concentrations ranging from 0 to 5×10^{-8} M (Massuci and Schiefelbein, 1994).

Hence, in addition to untreated seedling roots, experimental controls included seedling roots treated with acetone at the concentrations used in the respective GR24 treatment. In each of the experiments, untreated roots were compared with the respective acetone control. Where no difference was observed between the various controls, only untreated roots are shown. Where differences were recorded between untreated and acetone controls, the comparison is presented between treated roots and the acetone-treated controls.

Determination of RH length

For examination of RH length, roots were grown on treatment and control plates as described above. Following 6 d of growth, roots were examined on the plates using a stereomicroscope (Leica MZFLIII; Leica Microsystems GmbH, Wetzlar, Germany). Pictures were taken of root segments that had grown on the plates for 48 h under the examined conditions, and were in the mature part of the root. Pictures were taken with a Nikon DS-Fi1 camera of 10 separate roots per treatment. Measurements of RH length were performed on 10 pictures per treatment; 15–20 different RHs were measured per picture using IMAGEJ (http://rsbweb.nih.gov/ij/) (n=150–200). Means of replicates were subjected to statistical analysis by multiple-range test ($P\leqslant0.05$) using the JMP statistical package.

RNA extraction

After 6 d of 3×10^{-6} M GR24 treatment or control acetone treatment as described above, WT *Arabidopsis* seedling roots were snap frozen in liquid nitrogen and RNA extraction was performed from 30 seedlings (for each biological replicate). Total RNA was extracted using TRI reagent (MRC, Cincinnati, OH, USA) and digested with Turbo DNase enzyme (Ambion, Austin, TX, USA) as per the manufacturer's instructions.

cDNA synthesis

For cDNA synthesis, 2.5 μg of total RNA and 0.1 μM random hexamer primers were heated for 5 min at 65 °C and snap-chilled on ice. The following components were added to the reaction mixture: 0.2 mM dNTP mixture (Fermentas, Glen Burnie, MD, USA), M-MuLV-reverse transcriptase (RT) buffer (1×final concentration), 40 U of RNase M-MuLV inhibitor (Fermentas), 200 U of M-MuLV-RT enzyme (Fermentas) and DEPC-treated water to a reaction volume of 21 μl. The reaction was

incubated at 42 °C for 60 min following an incubation of 70 °C for 10 min.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed on RNA extracted from roots/seedlings or leaves, as described above. The qPCR was performed using components supplied in the KAPA SYBR FAST qPCR kits (Kapa Biosystems, Woburn, MA, USA) and *Arabidopsis thaliana acs2* (ACC synthase 2; gene ID: 837082) gene-specific primers (forward 5′-GCTGGTTTATTTGCGTG-GAT-3′; reverse 5′-AGGAAGAGCCAGGAGACACA-3′). The reaction mixture consisted of the following components: 2×Master Mix with integrated antibody-mediated hot start, SYBR® Green I fluorescent dye, $MgCl_2$, dNTPs, stabilizers, 2 µl of the template, and PCR-grade water to a final volume of 10 µl. The qPCR analysis was carried out on a Rotor gene 6000 instrument (Corbett-Qiagen, Valencia, CA, USA) according to the following programme: 3 min at 95 °C, followed by 49 cycles of 95 °C for 3 s, 60 °C for 20 s, and 72 °C for 1 s. The threshold cycle (Ct) was calculated by the Rotor gene 6000 instrument software. The level of expression of the target genes was calculated relative to that of the reference mRNA; *Arabidopsis* 15S ribosomal RNA (GenBank accession no. AT1G04270.1) served as the reference gene for the amount of RNA, and was amplified using specific primers (forward) 5′-CAAAGGAGTTGATCTCGATGCTCTT-3′ and (reverse) 5′-GCCTCCCTTTTC GCTTTCC-3′. Values of the steady-state level of gene transcripts were determined as the ratio between two conditions (i.e. 3×10^{-6} M GR24 treatment versus control) using the 2ΔΔCt method (Arocho *et al.*, 2006). Values ⩽1 represent an increase or decrease, respectively, in the steady-state level of a gene transcript for the examined conditions (i.e. that of the nominator versus that of the denominator). Means+SD were calculated for three biological replicates of each examined treatment. Means of replicates were subjected to statistical analysis by multiple-range test ($P\leqslant0.05$), using the JMP statistical package.

Results

SL-signalling mutant is sensitive to ethylene precursor

To determine whether SL signalling is necessary for the ethylene response, the mutant *max2-1*, flawed in SL signalling (Stirnberg *et al.*, 2007; Umehara *et al.*, 2008), was exposed to the ethylene precursor ACC and RH elongation was measured. ACC has been shown to increase RH length in the WT (Pitts *et al.*, 1998). The response of the *max2-1* mutant to ACC was similar to that of the WT at all examined ACC concentrations (Figs 1, 2). Since *max2-1* is flawed in SL signalling, its WT-like sensitivity to ACC could suggest that SL signalling is not necessary for the ethylene response (Figs 1, 2).

Ethylene mutants show reduced sensitivity to SL treatment

Next, the possible requirement of ethylene signalling for the SL response was examined. Exposure of WT plants to the synthetic SL GR24 resulted in increased RH elongation; this response to GR24 was absent in *max2-1* mutants (Kapulnik *et al.*, 2011; Fig. 3). Exposure of the ethylene signalling-deficient mutants *ein2-1* and *etr1-1* (reviewed by Stepanova and Alonso, 2009) to GR24 resulted in a reduced response to GR24 in terms of RH elongation, in comparison with the WT (Figs 1, 3): *ein2-1* responded to GR24 significantly, but to a lesser extent than the WT, whereas *etr1-1* did not show any significant response to GR24 (Figs 1, 3). RH elongation in the mutants *ein2-1* and *etr1-1* was not responsive to ACC (not shown). These results suggest that ethylene signalling is involved in the SL response.

SLs affect RH elongation through ethylene synthesis

Since ethylene signalling was seen to be involved in the SL response, the possibility of the SL effect on RH elongation requiring ethylene synthesis was examined. WT roots were exposed to GR24 and the inhibitor of ethylene production, AVG. GR24 treatment increased RH elongation (Kapulnik *et al.*, 2011), whereas AVG treatment inhibited it (Fig. 4). Addition of both GR24 and AVG abolished the effect of GR24 on RH elongation (Figs 1, 4), suggesting that ethylene synthesis is necessary for SL-mediated RH elongation.

Application of SL leads to an increase in At-ACS2 transcription

Ethylene synthesis is associated with increased *acs* gene transcription (e.g. Yamamoto *et al.*, 1995; Barry *et al.*, 2000). Hence, the transcription level of *At-acs2*, which encodes an enzymatically active ACS enzyme and is expressed in *Arabidopsis* roots (Yamagami *et al.*, 2003 and references within), was examined under GR24 treatment and in controls. Results showed that *At-acs2* transcription was induced 13.4±2.8-fold in roots treated with 3×10^{-6} M GR24 relative to controls.

Fig. 1. Examples of primary root segments of WT, *max2-1*, and *etr1-1* mutants at 48 h of seedling growth in the presence of GR24 (3×10^{-6} M), ACC (4×10^{-10} M), IAA (2.5×10^{-8} M), AVG (2×10^{-7} M), or control (0). Scale bars 500 µm.

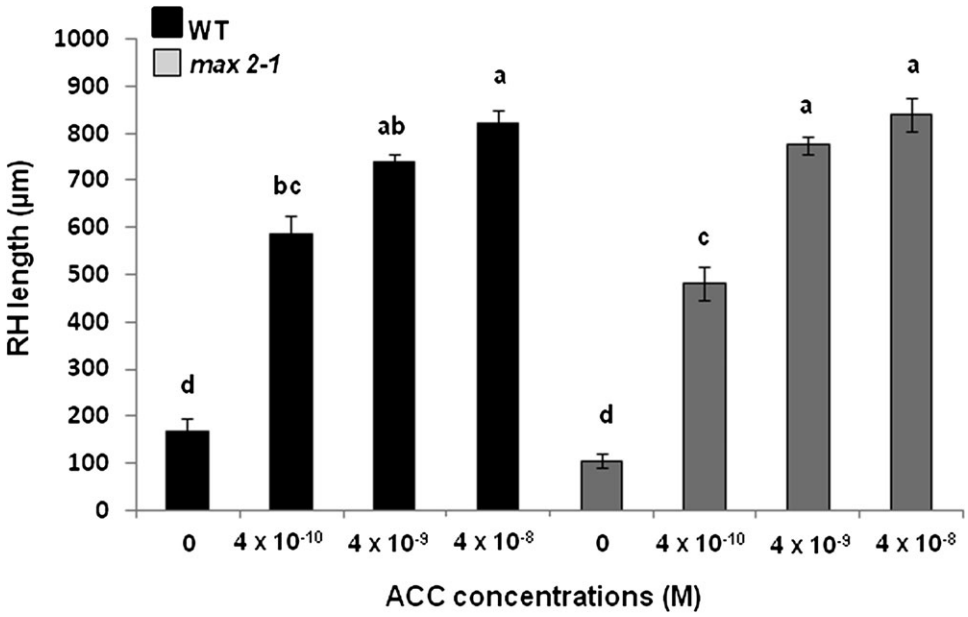

Fig. 2. Effect of ACC on RH length in WT and *max2-1* mutant at 48 h of seedling growth in the presence of ACC or control (0). Average±standard error is shown; different lower-case letters (a–e) above bars represent significantly different means ($P \leqslant 0.05$).

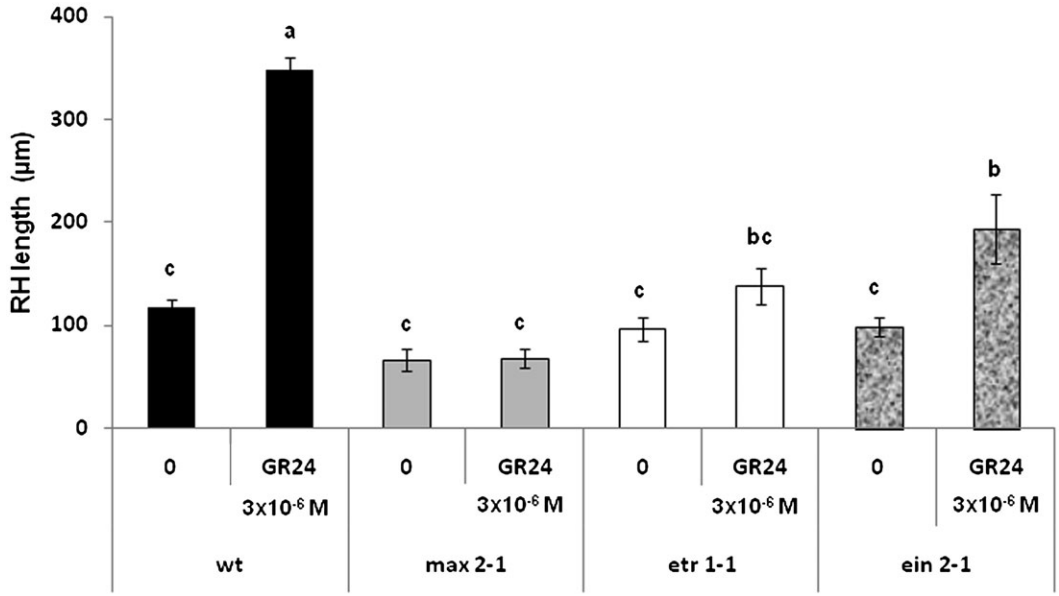

Fig. 3. Effect of GR24 on RH length in WT, *max2-1*, *etr1-1*, and *ein2-1* mutants at 48 h of seedling growth in the presence of GR24 or control (0). Average±standard error is shown; different lower-case letters (a–c) above bars represent significantly different means ($P \leqslant 0.05$).

SL mutants are sensitive to auxin

In the shoot, SLs have been suggested to regulate polar auxin transport capacity or to be secondary messengers of auxin (reviewed by Dun *et al.*, 2009). To better clarify the epistatic relations between SLs and auxin in their effect on RH elongation, the response of *max2-1* to IAA was examined. The results showed that the response of the *max2-1* plants to IAA was similar to that of the WT: IAA at concentrations of 2.5×10^{-8} and 5×10^{-8} M resulted in a significant increase in RH length in both the WT and *max2-1* (Figs 1, 5), suggesting that SL signalling is not necessary for the auxin response.

A mutant in auxin perception is reduced in its sensitivity to low SL concentrations compared with the WT

Next, the requirement of auxin perception for the RH response to SLs was examined. The results suggested that mutant *tir1-1*, flawed in the auxin receptor TIR1 (Dharmasiri *et al.*, 2005), is responsive to GR24 with respect to RH elongation. However, under low GR24

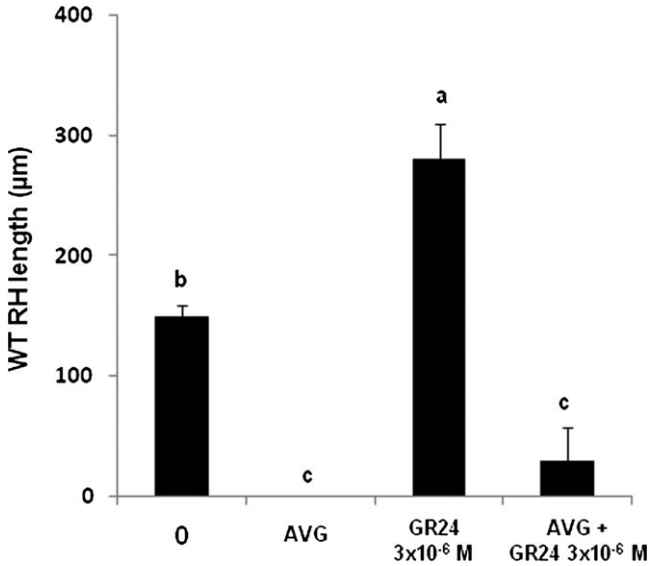

Fig. 4. Effect of AVG and/or GR24 on RH length in WT at 48 h of seedling growth in the presence of AVG (2×10^{-7} M) and/or GR24 or control (0). Average±standard error is shown; different lower-case letters (a–c) above bars represent significantly different means ($P\leqslant0.05$).

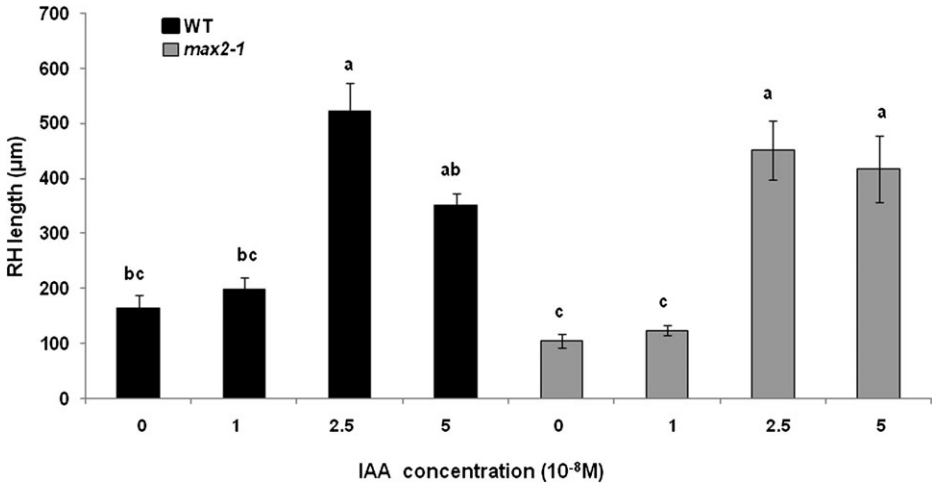

Fig. 5. Effect of IAA on RH length in WT and *max2-1* mutant at 48 h of seedling growth in the presence of IAA or control (0). Average±standard error is shown; different lower-case letters (a–e) above bars represent significantly different means ($P\leqslant0.05$).

concentrations (<3×10^{-6} M), its response was reduced in comparison with the WT (Fig. 6A). The reduction in the effect of SLs under low GR24 concentrations in *tir1-1* may be a result of an only moderate reduction in the sensitivity of *tir1-1* to auxin (i.e. reduction of IAA sensitivity in *tir1-1* only at low IAA concentrations). To further examine this point, the sensitivity of *tir1-1* to auxin was determined. The results suggested that *tir1-1* has lower sensitivity to auxin than the WT, under all WT-effective IAA concentrations (i.e. >1×10^{-8} M; Fig. 6B). Hence, auxin perception is only needed to some extent for the SL response.

tir1-1 shows reduced sensitivity to ACC relative to the WT

The reduced response of *tir1-1* to GR24 may result from reduced sensitivity to ethylene, rather than from a direct connection between auxin perception and the SL response. Hence, the sensitivity of *tir1-1* to ethylene was examined. Under low ACC concentration (4×10^{-11} M), *tir1-1* showed reduced sensitivity to ACC in comparison with the WT (RH length of 725±15 and 810±11 μm for *tir1-1* and WT, respectively); these results are in line with the findings of Alonso *et al.* (2003), suggesting that *tir1-1* has slightly reduced ACC sensitivity. However, under higher ACC concentrations (⩾4×10^{-10}), the RH elongation response in *tir1-1* was similar to that in the WT (not shown). Hence, *tir1-1* displayed reduced sensitivity to ACC under low ACC concentration, suggesting that the reduced sensitivity of *tir1-1* to GR24 (Fig. 6A) may have resulted from its reduced sensitivity to ACC, rather than from direct involvement of auxin perception in the SL response.

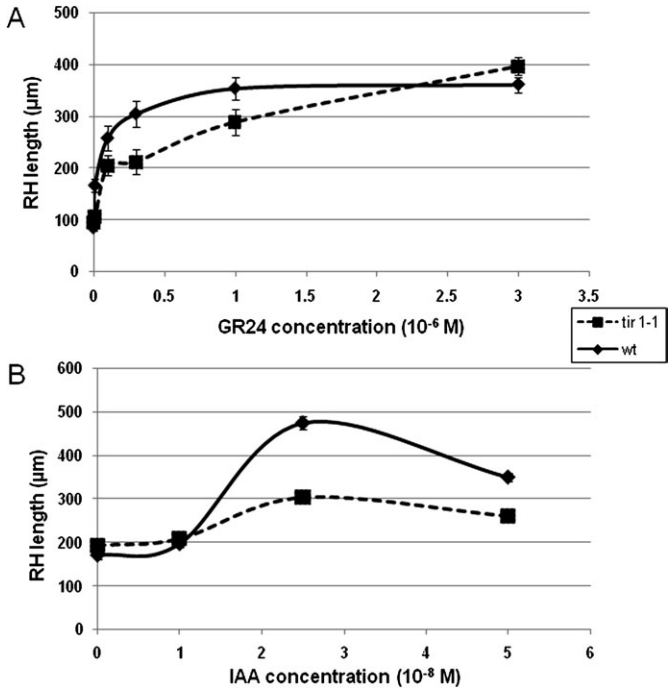

Fig. 6. (A) Effect of GR24 on RH length in WT and *tir1-1* mutant at 48 h of seedling growth in the presence of GR24. Vertical lines indicate standard errors of the means. (B) Effect of IAA on RH length in WT and *tir1-1* mutant at 48 h of seedling growth in the presence of IAA. Vertical lines indicate standard errors of means.

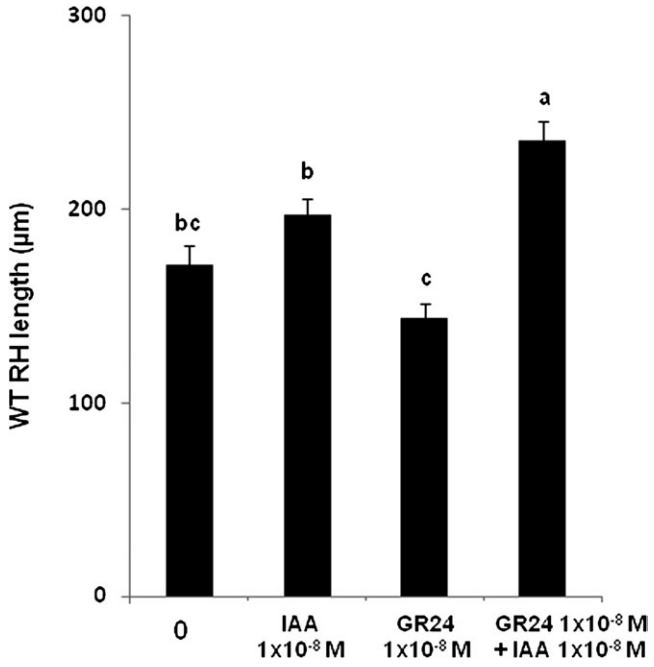

Fig. 7. Effect of IAA and/or GR24 on RH length in WT at 48 h of seedling growth in the presence of IAA and/or GR24 or control (0). Average±standard error is shown; different lower-case letters (a–c) above bars represent significantly different means ($P \leqslant 0.05$).

SLs and auxin have an additive effect on RH elongation

Since auxin signalling altered the RH response to SLs, SLs and auxin pathways may converge to regulate RH elongation. Hence, a possible additive effect of SLs and auxin on RH elongation was explored, using sub-effective concentrations of GR24 (Kapulnik *et al.*, 2011) and IAA (Fig. 6B). Sub-effective concentrations of either IAA or GR24 did not lead to an increase in RH length. However, combined application of both IAA and GR24 at their sub-effective concentrations led to a significant increase in RH length (Fig. 7). The results suggest that SLs and auxin have an additive effect on RH elongation.

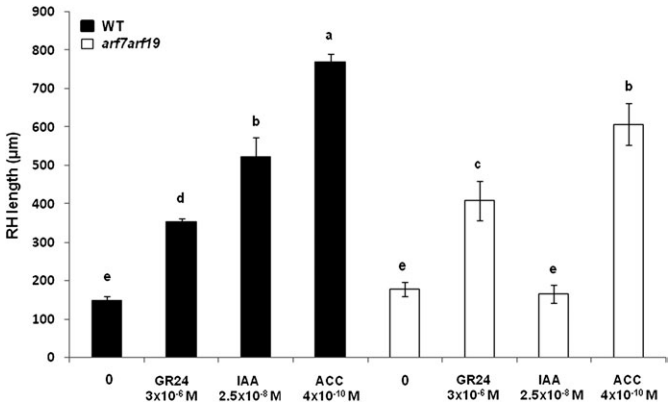

Fig. 8. Effect of GR24, IAA, or ACC on RH length in WT and *arf7arf19* mutant at 48 h of seedling growth in the presence of GR24, IAA, ACC, or control (0). Average ± standard error is shown; different lower-case letters (a–e) above bars represent significantly different means ($P \leqslant 0.05$).

arf7arf19 mutant is responsive to SLs to some extent

The double mutant *arf7arf19* is flawed in two auxin-responsive elements and displays severely reduced auxin sensitivity (Okushima *et al.*, 2005; Fig. 8). In addition, ARF19 and ARF7 have been suggested to participate in ethylene responses in *Arabidopsis* roots (Li *et al.*, 2006). Looking at RH elongation, *arf7arf19* was found to be more responsive to GR24 than the WT. However, it was less responsive to ACC than the WT and not responsive to IAA, in contrast to the significant WT response to this hormone (Fig. 8). These results suggest that *arf7arf19* sensitivity to SL is not mediated via the auxin signalling pathway, whereas its lower-than-WT sensitivity to ethylene may not entirely account for its higher-than-WT SL sensitivity.

aux1-7ein2-1 mutant is insensitive to SLs

The double mutant *aux1-7ein2-1* is resistant to auxin due to the mutation in *aux1-7*, and is insensitive to ethylene due to the mutation in *ein2-1* (Guzmán and Ecker, 1990; Pickett *et al.*, 1990; Rahman *et al.*, 2002). Looking at RH elongation, *aux1-7ein2-1* showed reduced sensitivity to GR24 relative to the WT (Fig. 9). These results suggest that SL's effect on RH elongation is dependent on both auxin and ethylene signalling.

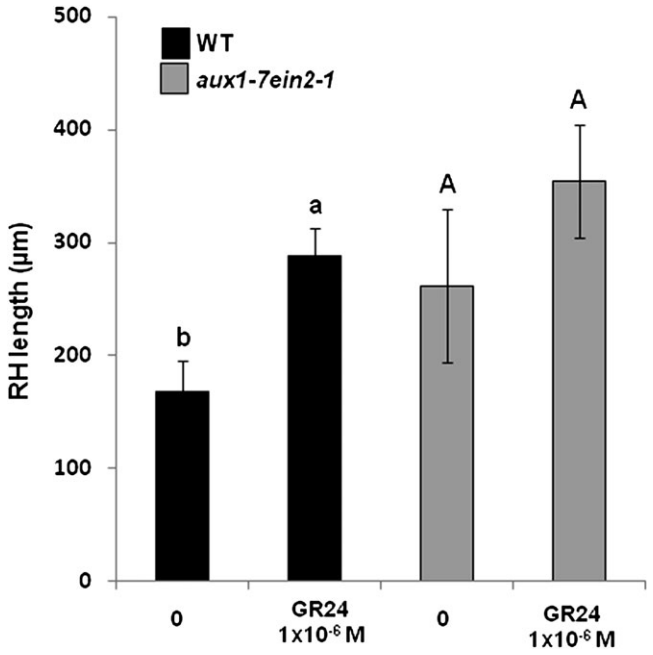

Fig. 9. Effect of GR24 on RH length in WT and *aux1-7ein2-1* mutant at 48 h of seedling growth in the presence of GR24 or control (0). Average±standard error is shown; different lower-case letters (a–b) above bars represent significantly different means ($P \leqslant 0.05$).

Discussion

Epistatic relations were determined between three phytohormones—SLs, ethylene, and auxin—in the regulation of RH elongation. The responsiveness of the SL-signalling mutant *max2-1* (Stirnberg *et al.*, 2007; Umehara *et al.*, 2008) to ethylene precursor suggested that SL signalling is not necessary for the ethylene response. However, the markedly reduced SL response in the ethylene-signalling mutants *etr* and *ein* (reviewed by Stepanova and Alonso, 2009) suggested that ethylene signalling is involved in the SL response.

Nevertheless, the significant response of the ethylene mutant *ein2-1* to GR24 in terms of RH elongation might suggest that RH elongation by SLs is mediated through both ethylene-dependent and -independent pathways; this residual response of *ein2-1* to GR24 could be due to the effect of SLs on RH elongation via the auxin-response pathway.

The observed WT-like RH length in the *etr1-1* and *ein2-1* mutants was in contrast to previously published results in which both *ein2-1* and *etr1* were shown to have reduced RH length (Pitts *et al.*, 1998; Rahman *et al.*, 2002). This discrepancy may be explained by differences in the growth conditions: Pitts *et al.* (1998) and Rahman *et al.* (2002) used minimal media, whereas here a relatively rich medium was used. Under the latter conditions, RH elongation is suppressed in the WT, reducing the ability to distinguish between WT and mutant with respect to RH elongation under control conditions.

Blockage of ethylene biosynthesis by AVG abolished the effect of SLs on RH elongation, suggesting that this effect is mediated via ethylene biosynthesis. Moreover, GR24 treatment elevated *At-acs2* transcription. The transcription level of *acs* genes, encoding key rate-determining enzymes in ethylene synthesis, has been shown to be correlated with the level of ethylene synthesis (e.g. Yamamoto *et al.*, 1995; Barry *et al.*, 2000). In *Arabidopsis* seedlings, auxin was shown to positively regulate ethylene biosynthesis (Woeste *et al.*, 1999) via induction of the transcription of *acs* genes, including *At-acs2* (Yamagami *et al.*, 2003). Moreover, *At-acs2* transcription has been shown to be induced in *Arabidopsis* roots upon gravity stimulation (Kimbrough *et al.*, 2004), whereas the root response to gravity stimulation was shown to be associated with ethylene biosynthesis (Lee *et al.*, 1990). Taken together, the induction of *At-acs2* by GR24 treatment may further support the suggestion that SLs induce ethylene biosynthesis.

Together, the results suggest that ethylene and SLs are in the same pathway regulating RH elongation, and that ethylene may be epistatic to SLs in this hormonal pathway.

Support for the suggestion that the effect of SLs on RH elongation involves ethylene biosynthesis comes from evidence suggesting that SLs induce ethylene biosynthesis in seeds of the parasitic plant *Striga*, leading to seed germination (Sugimoto *et al.*, 2003). These findings imply a general effect of SLs on ethylene synthesis, which may affect plant development.

However, as previously described, even in the double mutant *aux1-7ein2-1*, RH initiation and elongation are not completely suppressed (Rahman *et al.*, 2002). Since both auxin and ethylene are required for RH elongation, lack of ethylene response (or biosynthesis, as inhibition of biosynthesis would reduce the ethylene response) alone should not completely suppress RH elongation and initiation. Therefore, it might be that AVG, in addition to inhibiting ethylene biosynthesis, affects other signalling pathways regulating RH development.

The *aux1-7ein2-1* mutant showed reduced sensitivity to GR24 relative to the WT. On the other hand, Rahman *et al.* (2002) suggested that endogenous auxin acts together with ethylene in RH outgrowth. Hence, it might be that SL signalling is dependent on both auxin and ethylene signalling (or, for the latter, production), thereby affecting RH elongation.

The double mutant *arf7arf19* is flawed in two auxin-response factors (ARFs), and is known to exhibit severely reduced sensitivity to auxin (Okushima *et al.*, 2005). In addition to their role in auxin signalling, ARF19 and ARF7 have been suggested to participate in ethylene responses in *Arabidopsis* roots, implying that these ARFs serve as a junction between the auxin and ethylene pathways (Li *et al.*, 2006). However, Růzicka *et al.* (2007) found single and double *arf* mutants to be resistant to auxin but not to ethylene in the roots, with respect to primary root elongation. Here, *arf7arf19* indeed exhibited severely reduced sensitivity to auxin in terms of RH elongation; however, it responded significantly to both

ethylene and SL treatments—less in the former and more in the latter—in comparison with the WT. The sensitivity of *arf7arf19* to SLs in terms of RH elongation may be mediated through its sensitivity to ethylene. Nevertheless, its reduced sensitivity to ethylene and increased sensitivity to SLs in comparison with the WT suggest the existence of other signalling pathways that regulate RH development; these may be highly active in the *arf7arf19* mutant, allowing a higher-than-WT response to SL. These results support the suggestion of Růzicka *et al.* (2007) that in addition to the auxin-response-dependent pathway, there is also an auxin-independent pathway for ethylene regulation of root growth.

In the shoot, SLs have been suggested to either regulate polar auxin transport capacity or be secondary messengers of auxin; auxin and SLs have been suggested to regulate each other's level and distribution in a feedback loop (Leyser, 2009; reviewed by Dun *et al.*, 2009). Our results suggest that SL signalling is not necessary for the auxin response, but auxin signalling enhances the SL response: the *max2* mutant in SL signalling was responsive to auxin, whereas under low SL concentrations, the auxin receptor mutant *tir1-1* (Dharmasiri *et al.*, 2005) was less responsive to SLs than the WT.

Due to a measure of redundancy in auxin receptor activity (Dharmasiri *et al.*, 2005), triple and quadruple mutants of auxin receptors should be examined; nevertheless, our results suggest that the hormonal pathways of SLs and auxin converge: although auxin perception may not be essential for the RH response to SLs, it may promote it. The additive effect of SLs and auxin on RH elongation further supports this notion.

Several cross-talk junctions have been identified between auxin and ethylene pathways (e.g. Růzicka *et al.*, 2007; Stepanova *et al.*, 2007; Swarup *et al.*, 2007; Ivanchenko *et al.*, 2008; Negi *et al.*, 2008). Both hormonal pathways have been shown to be necessary for RH elongation (Pitts *et al.*, 1998), with insensitivity in the ethylene response affecting auxin-driven RH elongation (Rahman *et al.*, 2002). Ethylene has been shown to induce auxin synthesis in the root apex (Swarup *et al.*, 2007; Ivanchenko *et al.*, 2008), as well as auxin response and distribution (Růzicka *et al.*, 2007; Stepanova *et al.*, 2007; Swarup *et al.*, 2007; Ivanchenko *et al.*, 2008), and to positively regulate both acropetal and basipetal auxin transport in the root (e.g. Negi *et al.*, 2008, 2010). However, free IAA level in whole root tissues (which is a combination of IAA synthesis, conjugation, and transport) is reduced, rather than induced in tomato root tissue in the presence of ACC (Negi *et al.*, 2010).

Since on the one hand, ethylene was shown to be epistatic to SLs and SL signalling to be both auxin- and ethylene-signalling dependent (this study), and on the other, ethylene has been shown to regulate auxin synthesis, transport, response, and distribution in roots (e.g. Růzicka *et al.*, 2007; Swarup *et al.*, 2007; Ivanchenko *et al.*, 2008; Negi *et al.*, 2008, 2010; reviewed by Yoo *et al.*, 2009), one possibility is that both the SL and auxin pathways converge through that of ethylene.

Several lines of evidences in this study suggest that SLs' effect on RH elongation is mediated via the ethylene pathway, whereas the ethylene pathway interacts with that of auxin. These evidences include the observed lack of SL-induced RH elongation in the absence of ethylene synthesis, the findings suggesting increased *At-acs2* transcription upon GR24 treatment, and the reduced SL response in mutants of ethylene signalling. Further support for this notion comes from the reduced sensitivity of *tir1-1* to ethylene, as reported by Alonso *et al.* (2003) and as evident in our results. This reduced sensitivity may account for the reduced sensitivity of *tir1-1* to SLs, suggesting that ethylene, rather than auxin, is directly involved in the RH response to SLs.

To conclude, it is suggested that auxin, SLs, and ethylene undergo intimate cross-talk in the regulation of RH elongation. These putative cross-talk junctions between SLs, auxin, and ethylene may be valid for other SL-mediated regulation in plant development as well; this notion, however, remains to be explored.

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