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ORIGINAL PAPER

GA_3 enhances root responsiveness to exogenous IAA by modulating auxin transport and signalling in Arabidopsis

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Abstract

Key message **We used auxin-signalling mutants, auxin transport mutants, and auxin-related marker lines to show that exogenously applied GA enhances auxin-induced root inhibition by affecting auxin signalling and transport.**

Abstract Variation in root elongation is valuable when studying the interactions of phytohormones. Auxins influence the biosynthesis and signalling of gibberellins (GAs), but the influence of GAs on auxins in root elongation is poorly understood. This study was conducted to investigate the effect of GA_3 on *Arabidopsis* root elongation in the presence of auxin. Root elongation was inhibited in roots treated with both IAA and GA_3, compared to IAA alone, and the effect was dose dependent. Further experiments showed that GA_3 could modulate auxin signalling based on root elongation in auxin-signalling mutants and the expression of auxin-responsive reporters. The GA_3-enhanced inhibition of root elongation observed in the wild type was not found in the auxin-signalling mutants *tir1-1* and *axr1-3*. GA_3 increased *DR5::GUS* expression in the root meristem and elongation zones, and *IAA2::GUS* in the columella. The DR5rev::GFP signal was enhanced in columella cells of the root caps and in the elongation zone in GA_3-treated seedling roots. A reduction was observed in the stele of PAC-treated roots. We also examined the effect of GA_3 on auxin transport. The enhanced responsiveness caused by GA_3 was not observed in the auxin influx mutant *aux1-7* or the efflux mutant *eir1-1*. Additional molecular data demonstrated that GA_3 could promote auxin transport via AUX1 and PIN proteins. However, GA_3-induced *PIN* gene expression did not fully explain GA-enhanced PIN protein accumulation. These results suggest that GA_3 is involved in auxin-mediated primary root elongation by modulating auxin signalling and transport, and thus enhances root responsiveness to exogenous IAA.

Keywords Auxin · Root elongation · Gibberellins · Auxin transport · Auxin signalling · Crosstalk

Introduction

Root elongation is an important process during plant development. After germination in the soil, the developing seedling must reach a state of autotrophy through hypocotyl elongation before the nutrients stored in the seed are exhausted. When the hypocotyl is exposed to light, its growth stops and the first true leaf appears. Meanwhile, root elongation is induced, followed by exploration of the soil for water and minerals (Kircher and Schopfer 2012).

A classical physiological auxin response is its effect on cell elongation. Since the days of Sachs and Darwin, scientists have focused on this effect (Mockaitis and Estelle 2008). Auxin rapidly increases shoot and coleoptile elongation after a lag phase of 10–20 min (Dela Fuente and Leopold 1970). The mechanisms responsible for elongation in response to auxin have been described. Auxin stimulates the activity of H^+-ATPase, leading to acidification of the cell wall, which in turn enhances the activity of cell wall-

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loosening enzymes (Rayle and Cleland 1992). Recently, Takahashi et al. (2012) reported that auxin activates H^+-ATPase in etiolated hypocotyls of *Arabidopsis* (*Arabidopsis thaliana*) through phosphorylation of the penultimate threonine during early phase hypocotyl elongation.

Primary root elongation is a well-characterised auxin-associated phenotype. Since the level of auxin in planta is optimal, root elongation is exceptionally sensitive to changes in auxin content. Application of exogenous auxin may have positive or negative effects on root growth, depending on the concentration. Growth is promoted at a very low concentration (e.g., 10^{-10} M), but at higher concentrations (e.g., 10^{-6} M) elongation is strongly inhibited (Lincoln et al. 1990; Evans et al. 1994). In roots, the auxin gradient is clearly associated with patterns of cell proliferation and elongation observed at the apical-basal axis (Grieneisen et al. 2007). Auxin levels are highly regulated through changes in auxin biosynthesis, conjugation and storage, degradation, and polar transport. Auxin levels are then interpreted by the auxin perception machinery, resulting in tissue- and cell -type-specific changes in gene expression, thereby contributing to the regulation of elongation growth (Woodward and Bartel 2005; Chapman and Estelle 2009; Ljung 2013).

Alteration of root elongation in *Arabidopsis* mutants is related to auxin biosynthesis, transport, and signalling. The gain-of-function mutant *yucca* contains higher levels of IAA than the wild type, resulting in longer hypocotyls and shorter primary roots (Zhao et al. 2001). Roots of the mutant *aux1–7*, with impaired auxin influx transport, were resistant to the inhibition of elongation by IAA (Evans et al. 1994). The *transport inhibitor response 3* (*tir3*) mutants were isolated for resistance to the inhibitory effects of 1-*N*-naphthylphthalamic acid (NPA, a specific inhibitor of polar auxin transport) on root elongation. They displayed decreased inflorescence height, and decreased petiole and root length (Ruegger et al. 1997). The TIR1 and AFB proteins constitute a family of F box protein/auxin receptors that collectively mediate auxin-regulated transcription (Dharmasiri et al. 2005a). The triple mutant *tir1-1afb2-1afb3-1* and the quadruple *tir1-1 afb1-1afb2-1afb3-1* auxin receptor mutant display various root phenotypes, with some plants having shortened or no roots (Dharmasiri et al. 2005a, b). Scheitz et al. (2013) demonstrate that rapid auxin-induced root growth inhibition requires the TIR and AFB auxin receptors.

Gibberellins (GAs) are tetracyclic diterpenoid phytohormones that are also involved in cell elongation, which they strongly promote in many plants. They were discovered in studies of rice seedlings showing abnormal elongation due to fungal infection with *Gibberella fujikuroi* (Yamaguchi 2008). The role of exogenous GAs in root elongation has not been studied in detail. Reports show that exogenous GAs exert an indeterminate effect on root development (Inada and Shimmen 2000; Inada et al. 2000; Yaxley et al. 2001; Müssig et al. 2003; Desgagné-Penix and Sponsel 2008). The effects of exogenous GA and GA inhibitors on root development vary depending on the plant species and experiment (Tanimoto 2012). The requirement of GA for root elongation has been elucidated by GA depletion experiments in GA-deficient mutants and treatment with GA biosynthesis inhibitors. GA-deficient mutants and treatments with GA biosynthesis inhibitors indicate that GAs have a positive effect on primary root growth (Fu and Harberd 2003; Ueguchi-Tanaka et al. 2007; Ubeda-Tomás et al. 2008; Tanimoto 2012). GAs also control root cell proliferation (Achard et al. 2009; Ubeda-Tomás et al. 2009). Gibberellins signal the degradation of the DELLA growth repressor proteins GAI and RGA, promoting cell division activity in the root meristem, thereby contributing to the regulation of root growth (Ubeda-Tomás et al. 2009). Responses to GA dilution explain root growth dynamics, which is a multi-scale model of GA dynamics in the *Arabidopsis* root elongation zone (Band et al. 2012).

The interaction between GAs and auxin and its effects on elongation have been investigated intensively, including in stems. There are numerous reports of GA biosynthesis induction by auxin. For example, in *Arabidopsis*, peas, tobacco, and rice, active GA levels in stems of decapitated plants deprived of the apical source of auxins are lower than those in stems of intact plants; however, application of auxin to the apex restores wild-type levels (Jouve et al. 1999; Ross et al. 2000; Wolbang and Ross 2001; Yin et al. 2007). In root elongation, auxin signalling has been shown to induce the degradation of the negative GA-signalling element RGA, thereby promoting GA signalling and root growth (Fu and Harberd 2003).

Auxins influence GA biosynthesis and signalling, but how GA influences auxin is poorly understood. In *Populus*, GAs negatively affect lateral root formation and adventitious rooting, which is due at least in part to the modification of polar auxin transport (Gou et al. 2010; Mauriat et al. 2014). Proper GA biosynthesis and signalling are required for auxin transport in *Arabidopsis* stems and root tips (Willige et al. 2011). Proper root gravitropism also requires GA biosynthesis and DELLA repressor-mediated GA signalling, which regulate vacuolar trafficking of PIN auxin transporters during root gravitropism (Willige et al. 2011; Löfke et al. 2013a, b).

To investigate the involvement of GA in auxin-mediated primary root elongation, we used auxin-related mutants and transgenic plants to determine whether GA is involved in auxin signalling and transport. Our results demonstrated that exogenous GA has a positive impact on auxin signalling and transport, and thus enhances the response of *Arabidopsis* roots to exogenous auxin. The results presented here provide more evidence of the interaction between gibberellin and auxin.

Materials and methods

Plant materials and growth conditions

The auxin transporter mutants used in this study were *aux1-7* (Pickett et al. 1990) and *eir1-1* (Luschnig et al. 1998). The auxin signalling mutants were *axr1-3* (Lincoln et al. 1990) and *tir1-1* (Ruegger et al. 1998), and the auxin-responsive reporter transgenic lines were *DR5rev-GFP* (Benková et al. 2003), *DR5::GUS* (Ulmasov et al. 1997), and *IAA2::GUS* (Swarup et al. 2001). The auxin transporter reporter transgenic lines were *PIN1, 2, 3, 4, 7::GUS* (Vieten et al. 2005), *AUX1::AUX1-YFP* (Swarup et al. 2001), *PIN1::PIN1-GFP* (Benková et al. 2003), *PIN2::PIN2-GFP* (Abas et al. 2006), and *PIN3::PIN3-GFP* (Zádníková et al. 2010). They were described previously and obtained from the Arabidopsis Biological Resource Center. The *AUX1::GUS* line was obtained from Dr. Weiming Shi (Institute of Soil Science, Chinese Academy of Sciences, China). The *A. thaliana* mutants and transgenic lines described were all in the Col-0 background.

Seeds were sterilised by incubation in freshly prepared 30 % bleach plus 0.01 % (v/v) Tween 20 for 10 min and then washed three times with sterile water. The surface-sterilised seeds were stratified at 4 °C for 5 days to synchronise germination, then were grown on 0.8 % agar with half-strength Murashige and Skoog medium, 1 % sucrose, and 0.1 % (w/v) MES supplemented with IAA, GA_3, and paclobutrazol (PAC). The plates were sealed with surgical tape and held vertically in a growth chamber at 23 ± 1 °C under a light intensity of 100 μmol photons m^{-2} s^{-1}, with a photoperiod of 16-h light and 8-h dark.

The IAA, GA_3, and PAC were purchased from Sigma-Aldrich (St Louis, MO, USA), wherein the PAC was used as a GA biosynthesis inhibitor. All chemicals were dissolved in 100 % ethanol and the stock solutions were stored at 4 °C. When used, the stock solutions were diluted to the required concentrations with sterile distilled water. The final concentration of ethanol did not exceed 0.01 %. Mock treatments were applied using equal amounts of solvent (ethanol). For the root elongation test, GA_3 (1 μM) or paclobutrazol (1 μM) was combined with various IAA concentrations (0, 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 nM). For GUS staining and confocal scanning, seedlings were grown in the presence of 1 μM GA_3 or 1 μM PAC for 5 days.

Measurement of root length

The lengths of primary roots of individual seedlings were measured from root tip to base of hypocotyls using the Image J software (National Institutes of Health; http://rsb.info.nih.gov/ij) from digital images captured with a Canon G7 camera. For all experiments, phenotypic data analysis was performed at least three times and the data represent one independent experiment.

GUS staining and quantitative measurement of GUS activity

For GUS staining, 5-day-old seedlings were fixed in 90 % acetone at −20 °C for 1 h, washed twice in 50 mM sodium phosphate buffer (pH 7.0), and then incubated in GUS-staining buffer containing 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc), 50 mM sodium phosphate buffer (pH 7.0), 1 mM $K_4Fe(CN)_6$, 1 mM $K_3Fe(CN)_6$, 1 mM EDTA, and 0.1 % Triton X-100. The seedlings were incubated at 37 °C overnight and subsequently de-stained in 70 % ethanol until the tissue became clear. Individual representative seedlings were photographed using a light microscope equipped with an imaging camera (model Stemi 2000-C; Zeiss, Germany).

A quantitative GUS activity assay was performed as described by Jefferson et al. (1987). Root samples were homogenised in GUS extraction buffer (50 mM potassium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1 % Triton X-100, and 0.1 % SDS). The extract was centrifuged at 12,000*g* for 15 min at 4 °C. The reaction was carried out in a reaction mixture containing 2 mM 4-methylum-belliferyld-glucuronide (MUG; Sigma-Aldrich) as a substrate. The extracts were kept at 37 °C for 30 min, and then the reaction was terminated by the addition of 0.2 M Na_2CO_3. Fluorescence was measured with excitation at 365 nm and emission at 455 nm using a multidetection Tecan Infinite M200 microplate reader (Tecan, Männedorf, Switzerland). A standard curve corresponding to 0, 0.1, 0.25, 0.5, 1, 5, and 10 μM 4-methylumbelliferone (4-MU; Sigma-Aldrich) was included with every plate and used to calculate the amount of MU liberated by each sample. Protein content was normalised according to the method of Bradford (1976).

Confocal microscopy

For confocal microscopy, roots were fixed for 1 h with 4 % paraformaldehyde, mounted in 5 % glycerol, and inspected using confocal microscopy. Imaging was performed with a Zeiss LSM 780 confocal microscope (Zeiss, Germany), and fluorescence images were digitised using the Zeiss LSM image browser. Images were representative of at least 10 individual plants from each treatment. Experiments were repeated at least three times.

Statistical and graphical analyses

For all experiments, data were analysed using SPSS v. 17.0 (SPSS, Chicago, USA). Additional detail is presented in

the figure legends. Graphs were produced using Origin 8.0 (OriginLab Corp., Northhampton, MA, USA). All graphs and images were arranged using Adobe Photoshop 7.0 (Adobe Systems, USA).

Results

GA_3 enhances the responsiveness of *Arabidopsis* roots to exogenous IAA

Gibberellin has been reported to play a role in root elongation (Fu and Harberd 2003). Thus, to examine the involvement of exogenous GA in root growth, the effect of exogenous GA_3 on root elongation was analysed. GA_3 treatment alone did not inhibit *Arabidopsis* primary root elongation compared with the control (Fig. 1a). At 50 μM, GA_3 slightly promoted root growth, but there was no statistical significance to this trend.

A low concentration of IAA (0.1 nM) stimulated root elongation, while root elongation was inhibited when the IAA concentration exceeded 10 nM (Fig. 1b), consistent with earlier reports (Lincoln et al. 1990; Evans et al. 1994). The roots exhibited an increase in elongation (~40 %) in response to 0.1 nM auxin and 1 μM GA_3, compared to the auxin-only treatment (~26.5 %). However, roots treated with a high concentration of auxin (5 nM) and 1 μM GA_3 were significantly inhibited compared to those in the auxin-only treatment ($P < 0.05$; Fig. 1b, e). This suggests that GA might result in a suboptimal auxin level, affecting primary root growth. To determine if the enhanced inhibition caused by exogenous GA_3 was concentration dependent, plants were co-treated with GA_3 and 5 nM IAA. Root length was inhibited by 2.5, 8.4, and 13.9 % in response to application of 0.5, 1, and 5 μM GA_3, respectively, combined with 5 nM IAA and compared to 5 nM IAA alone, indicating that the GA_3-enhanced inhibition of root growth was concentration dependent (Fig. 1c).

The role of GA_3 in auxin-mediated primary root elongation was further investigated using the GA biosynthesis inhibitor PAC. This treatment inhibits the *ent*-kaurene oxidation reactions of GA biosynthesis, which causes a reduction in root length and root growth rate (Band et al. 2012). A concentration of 1 μM PAC was used for our experiments. The relative length of the primary root increased significantly in response to IAA concentrations of 1, 5, and 10 nM with PAC when compared to a treatment without PAC (Fig. 1d). The inhibition of root elongation caused by IAA was partially alleviated when IAA was co-applied with PAC, suggesting that PAC decreases auxin activity. These data suggest that GA_3 regulates root elongation through modulating root responsiveness to auxin.

GA_3 enhances root responsiveness to exogenous IAA through auxin signalling

To investigate the mechanism by which GA_3 mediates or promotes the auxin response in roots, we compared the primary root growth of the *tir1-1* and *axr1-3 Arabidopsis* mutants. TIR1 is an auxin receptor, and the *tir1-1* mutant showed reduced sensitivity to auxin (Ruegger et al. 1998). The *Arabidopsis* auxin-resistance gene *AXR1* encodes a protein related to the ubiquitin-activating enzyme E1 that, primarily but not exclusively, affects the auxin signal (Leyser et al. 1993; Timpte et al. 1995). The *axr1*-3 (*auxin-resistant 1*) mutants also exhibited a reduction in auxin response (Lincoln et al. 1990). The enhanced responsiveness caused by GA_3 was not observed in the auxin-signalling mutants *tir1-1* and *axr1-3* (Fig. 2a and b). Both TIR1 and AXR1 are regulatory components of auxin signal transduction (De Smet et al. 2011), suggesting that the effect of GA_3 on *Arabidopsis* root responsiveness to exogenous IAA might be involved in the process of auxin signalling.

To gain additional insight into the role of GA_3 in auxin signalling responses, the auxin-responsive reporters *DR5::GUS* (Ulmasov et al. 1997), *DR5rev::GFP* (green fluorescent protein) (Benková et al. 2003), and *IAA2::GUS* (Swarup et al. 2001) were employed. In untreated roots, *DR5* expression was observed mainly in the quiescent zones and surrounding columella cells of the root cap and *IAA2::GUS* was expressed in the stele and columella, as reported previously (Ulmasov et al. 1997; Swarup et al. 2001). GA_3 changed the expression of *DR5::GUS* by increasing its activity in the root meristem and elongation zones (Fig. 3a, c). Increased expression of *IAA2::GUS* in the columella was observed in GA_3-treated seedlings (Fig. 3b, d). Confocal sections revealed that the DR5 signal was enhanced in the columella cells of the root caps and the elongation zone in GA_3-treated seedling roots, and a reduction in DR5rev::GFP signal was observed in the stele of PAC-treated roots (Fig. 3e, f). The genetic and molecular data indicated that GA_3 enhances auxin-responsive gene expression, as indicated by increases in auxin signalling and the response to auxin in the GA_3-treated roots.

GA_3 promotes auxin transport in root tips

Auxin transport regulates root elongation by controlling local auxin distribution in roots, which is mediated by the influx carrier of the AUX/LAX family and efflux carriers of the PIN family (Grieneisen et al. 2007; Band and King 2012; Boot et al. 2012). Auxin transport was examined using mutants with impaired auxin transport [import into (*aux1*) and export from (*eir1/pin2*) cells). Both *aux1-7* and *eir1-1* are less sensitive to auxin than the wild type (Evans

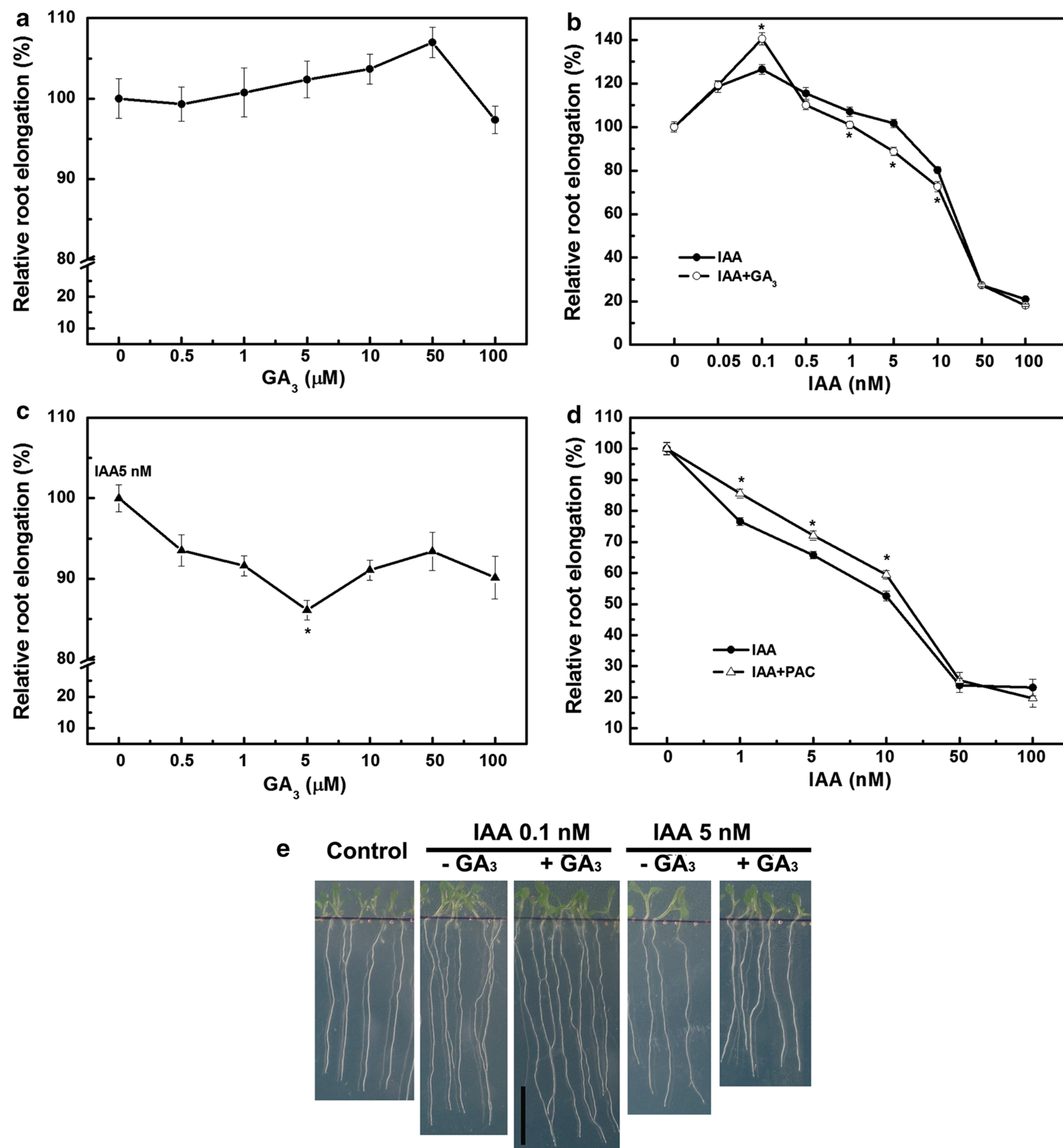

Fig. 1 GA_3 enhanced the responsiveness of *Arabidopsis* root to exogenous IAA. **a** Dose responsiveness of root elongation to exogenous GA_3. *Arabidopsis* seedlings were grown in the presence of various concentrations of GA_3 for 10 days. **b** Effects of GA_3 co-applied with various concentrations of IAA on primary root elongation in WT seedlings. *Arabidopsis* seedlings were grown in the presence of various concentrations of IAA supplemented with (*open circle*) or without (*solid circle*) 1 μM GA_3 for 10 days. **c** Dose responsiveness of root elongation to GA_3 supplemented with 5 nM IAA. *Arabidopsis* seedlings were grown in the presence of various concentrations of GA_3 supplemented with 5 nM IAA for 10 days. Mean values for 100 % root elongation were 25.28 ± 0.44 mm (5 nM IAA). **d** Dose responsiveness of root elongation to IAA with (*open triangle*) or without PAC (*solid circle*). *Arabidopsis* seedlings were grown in the presence of various concentrations of IAA supplemented with or without 1 μM PAC for 10 days. Mean values for 100 % root elongation were 36.59 ± 0.72 mm (Control) and 22.05 ± 0.42 mm (PAC 1 μM). **e** Photographs of seedlings grown in the presence of 0.1 and 5 nM IAA with or without 1 μM GA_3. *Scale bar* 1 cm. **b**, **e** were from the same experiment. Data are the averages of 40–50 seedlings ±SE. Data in **a**, **c** were analysed by one-way ANOVA procedure, and the *asterisk* in **c** represents means that differ significantly between the control and treatment conditions (Duncan's multiple range test, $P < 0.05$). The *asterisk* in **b**, **d** represents means that differ significantly between the control (*IAA*) and treatment (IAA + GA_3 or IAA + PAC) conditions (Student's *t* test, $P < 0.05$)

et al. 1994; Rahman et al. 2001). The enhanced responsiveness caused by GA_3 was not observed in the auxin influx mutant *aux1-7* (Fig. 4a) or the auxin efflux mutant *eir1-1* (Fig. 4b), which indicates that auxin transport might be involved in the enhanced root responsiveness to IAA caused by GA_3.

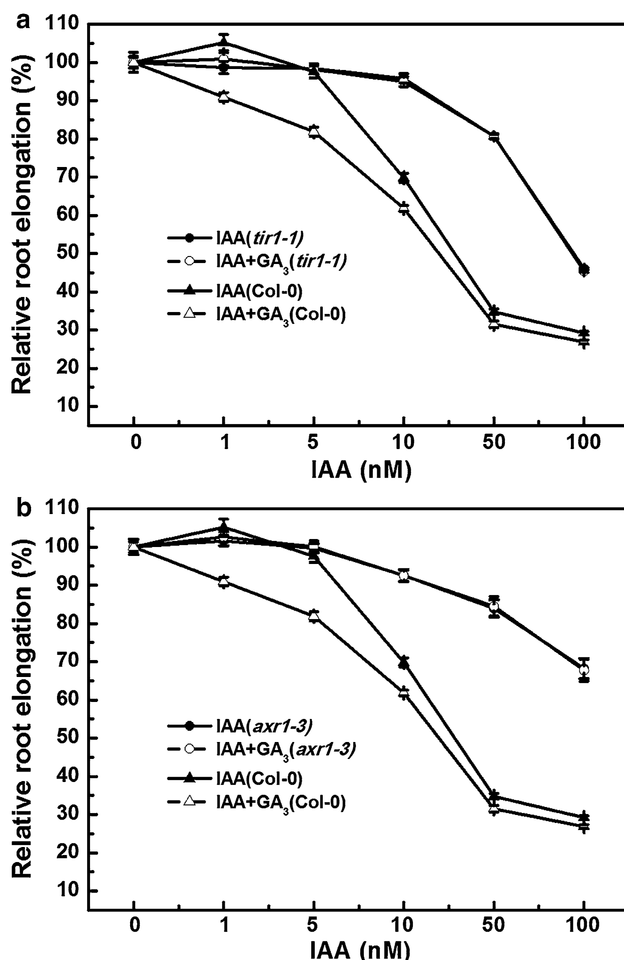

Fig. 2 Effects of GA_3 co-applied with IAA on primary root elongation in the *Arabidopsis* auxin signalling mutants *tir1-1* (**a**) and *axr1-3* (**b**). Ten-day-old seedlings were grown in the presence of various concentrations of IAA supplemented with (*open circle* for mutants and *open triangle* for wild-type) or without (*solid circle* for mutants and *solid triangle* for wild-type) 1 μM GA_3. Data are the averages of 40–50 seedlings ± SE. Mean values for 100 % root elongation were 30.55 ± 0.77 mm (Col-0), 29.53 ± 1.74 mm (*tir1-1*) and 32.81 ± 1.42 mm (*axr1-3*)

We next examined AUX1 and PIN protein distribution in untreated and treated roots using the auxin influx carrier AUX1 and the auxin efflux facilitators PIN1, PIN2, and PIN3. As shown previously (Swarup et al. 2001), AUX1 is expressed in the epidermis, lateral root cap, and columella of the untreated root tip. AUX1-YFP (yellow fluorescent protein) expression in GA-treated roots increased in the epidermis compared with untreated roots, and expression was decreased in PAC-treated roots (Fig. 5a, e). This is in agreement with physiological data showing that GA_3 did not enhance root responsiveness to IAA in the auxin influx mutant *aux1-7* (Fig. 4a). The GA_3 treatment enhanced PIN2-GFP expression in *PIN2::PIN2-GFP* plants, which was consistent with the results for the auxin efflux mutant *eir1-1* (Fig. 4b); in contrast, PAC treatment inhibited this expression (Fig. 5c, e). The GA_3-treated roots had considerably increased accumulation of PIN1-GFP and PIN3-GFP in the meristematic zone, compared with a reduction in PAC-treated roots (Fig. 5b, d, e). These results, combined with genetic data, suggest that GA_3 might promote the accumulation of auxin transport proteins in roots.

GA-induced transcriptional changes cannot explain protein accumulation

The increased AUX1 and PIN protein accumulation can be explained by increased *AUX1* and *PIN* transcription. We, therefore, examined *AUX1* and *PIN* gene expression using *AUX1::GUS* (Swarup et al. 2001) and *PIN::GUS* reporter lines (Vieten et al. 2005). Both GA_3 and PAC had no obvious effect on the expression of *AUX1::GUS* (Fig. 6a, g). The expression of *PIN1::GUS* in the root tips increased in the presence of PAC and was reduced in GA_3-treated seedlings (Fig. 6b, g). Thus, *PIN1::GUS* transcription does not explain the PIN1 protein levels, which show the opposite behaviour; i.e., an increase in GA-treated roots and a decrease in PAC-treated roots. The expression of *PIN2::GUS* in the root tips did not change in GA_3-treated roots, and a slight reduction was observed in PAC-treated roots (Fig. 6c, g). In the case of *PIN3::GUS,* we noted increased expression in the GA_3-treated root tips and a reduction in PAC-treated root tips (Fig. 6d, g). The expression of *PIN4::GUS* in the root tips increased in the presence of PAC, and was reduced in GA_3-treated seedlings (Fig. 6e, g). In the case of *PIN7::GUS*, GA_3 increased expression and PAC reduced expression (Fig. 6f, g). Since we did not analyse PIN4 and PIN7 distributions in the root, we cannot correlate this observation with the behaviours of PIN4 and PIN7 proteins. Therefore, we conclude that *AUX1* and *PIN* gene expression does not explain the GA-dependent accumulation of AUX1 and PIN proteins.

Discussion

Interactions between hormones, such as between auxin and cytokinin (El-Showk et al. 2013), and auxin and ethylene (Muday et al. 2012), are reciprocal in terms of their biosynthesis and responses. There are many reports of modulation by auxin of GA through biosynthesis and responses (Björklund et al. 2007; Frigerio et al. 2006; Fu and Harberd 2003; Jouve et al. 1999; Ross et al. 2000; Wolbang and Ross 2001; Yin et al. 2007). However, the mechanism underlying the effect of GA on auxin is poorly understood. In this work, we show that exogenous GA has a positive impact on auxin signalling and transport, and thus enhances the response of *Arabidopsis* roots to exogenous auxin.

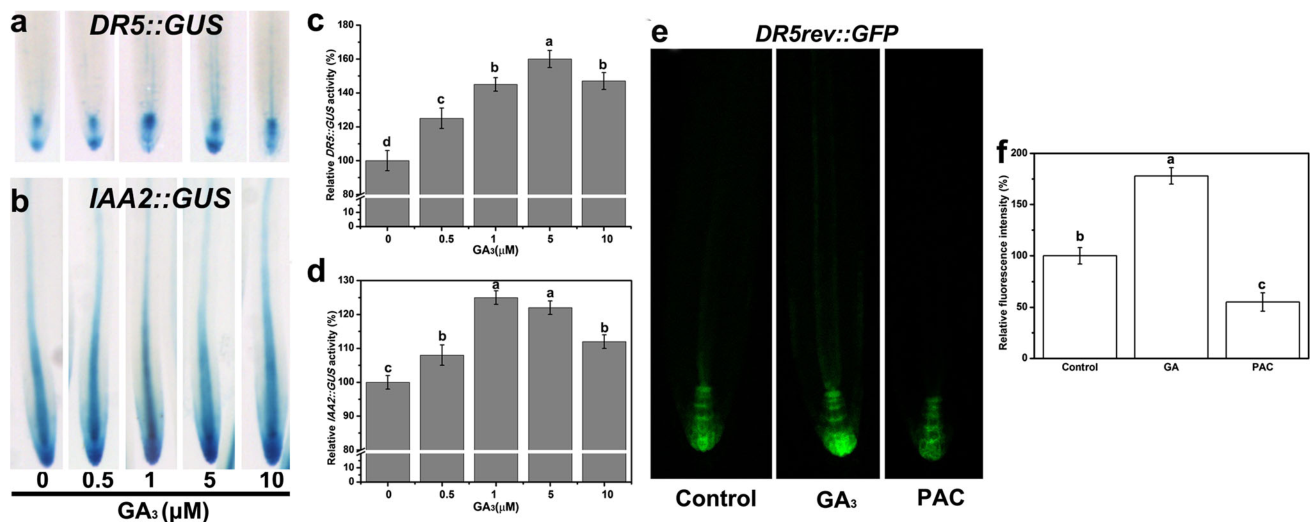

Fig. 3 GA_3 induced the expression of auxin-responsive reporter in the roots of transgenic lines. Seedlings of *DR5::GUS* (**a**) and *IAA2::GUS* (**b**) were grown in the presence of various concentrations of GA_3 for 5 days, and then stained for GUS expression. Quantification of the GUS activity in *DR5::GUS* and *IAA2::GUS* seedlings were showed in **c** and **d**. Seedlings of *DR5rev::GFP* (**e**) were grown in 1 μM GA_3 and 1 μM PAC for 5 days, and then *DR5* activities were assayed by means of *DR5-GFP* signals. Images were representatives of at least 15 individual plants from each treatment. **f** Quantification of the fluorescence signals from cells as shown and imaged in **e**. Data are expressed as the GUS activity or fluorescence intensity relative to untreated controls (100 %). Mean values and SE were calculated from three independent experiments ($n = 15$). Within each set of experiments, *bars* with *different letters* were significantly different at the 0.05 level

Effect of GA on auxin responses

It has been documented that auxin responsiveness changes when auxin biosynthesis, transport, and signalling are disturbed (Lincoln et al. 1990; Evans et al. 1994; Rahman et al. 2001; Ruegger et al. 1998; Woodward and Bartel 2005; Zhao et al. 2001). Here, we show that GA_3 enhances the responsiveness of *Arabidopsis* roots to exogenous IAA. The gain-of-function mutant *yucca* contains higher levels of IAA than the wild type (Zhao et al. 2001), and Desgagné-Penix (2008) found that GA_4 (the major bioactive GA in *Arabidopsis*) decreases the length of the primary root in *yucca* seedlings. These observations suggest that GAs enhance the responsiveness of *Arabidopsis* roots to both endogenous and exogenous IAA. In addition, some studies report differences in the response of auxin to GAs in different systems. For example, in pea plants, joint application of GA_3 and IAA to excised stem segments results in a synergistic growth response (Ockerse and Galston 1967; Yang et al. 1996).

Effect of GA on auxin signalling

Previous reports have shown that auxin affects GA signalling (Fu and Harberd 2003) but whether GA impacts auxin signalling is unknown. The *Arabidopsis Aux/IAA* gene family plays a central role in auxin signal transduction (De Smet et al. 2011). Several auxin-response genes, such as *Aux/IAA19*, were identified as DELLA-down genes in young flower buds and etiolated seedlings (Cao et al. 2006; Gallego-Bartolomé et al. 2011a, b). In addition, transcriptional control of *Aux/IAA19* by GAI (a DELLA protein involved in GA signalling) is direct (Gallego-Bartolomé et al. 2011a, b). Additionally, microarray gene expression data show that GA might affect auxin signalling, but there is no direct physiological or genetic evidence to support this. In this study, we found that GA cannot enhance the responsiveness of *Arabidopsis* roots to IAA in the *tir1-1* and *axr1-3* mutants (Fig. 2). Furthermore, GA enhanced auxin-induced expression of the auxin reporter genes *DR5-GUS* and *IAA2::GUS* (Fig. 3a–d). Similarly, the GFP signal was increased in GA-treated *DR5rev::GFP* roots and reduced in PAC-treated roots (Fig. 3e, f). Thus, our results support the hypothesis that GA modulates auxin signalling. However, Desgagné-Penix and Sponsel (2008) proposed that *DR5::GUS* activity is not affected by GA_4 and PAC, as plants grown under a wide range of environmental conditions can produce different results. Therefore, even if GA affects auxin signalling, other factors such as light (Halliday et al. 2009), temperature (Gray et al. 1998), and sugar level (Le et al. 2010) also influence plant growth and development.

Effect of GA on auxin transport

Auxin transporter proteins have been identified and grouped into three families: AUXIN RESISTANT1/LIKE AUX1 (AUX1/LAX) influx carrier, PIN-FORMED (PIN)

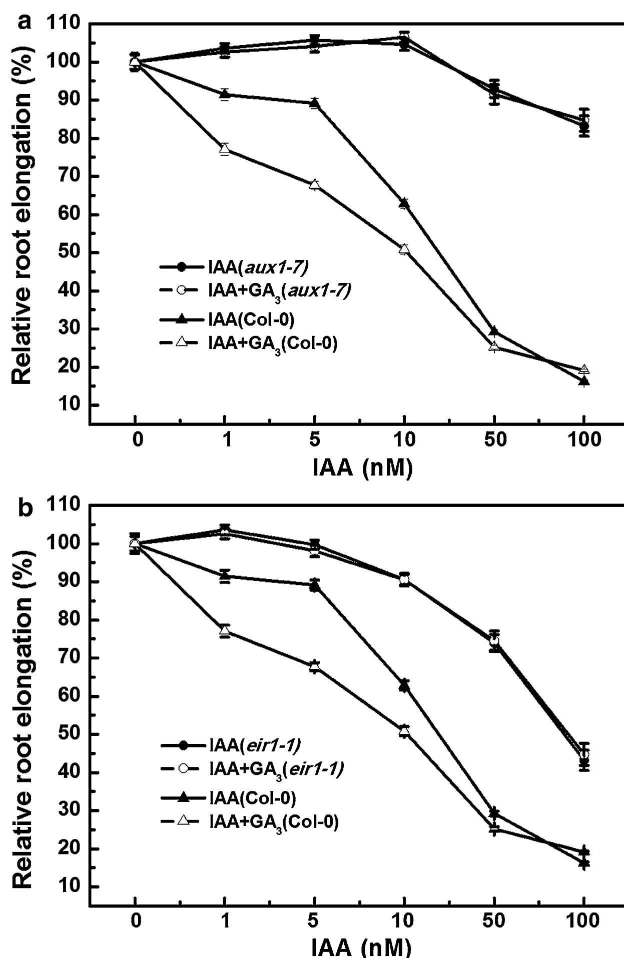

Fig. 4 Effects of GA_3 co-applied with IAA on primary root elongation in the *Arabidopsis* auxin transport mutant *aux1-7* (**a**) and *eir1-1* (**b**). Ten-day-old seedlings were grown in the presence of various concentrations of IAA supplemented with (*open circle* for mutants and *open triangle* for wild-type) or without (*solid circle* for mutants and *solid triangle* for wild-type) 1 μM GA_3. Data are the averages of 40–50 seedlings ±SE. Mean values for 100 % root elongation were 30.33 ± 0.94 mm (Col-0), 31.73 ± 1.74 mm (*aux1-7*) and 27.43 ± 1.02 mm (*eir1-1*)

efflux carrier, and P-GLYCOPROTEIN (MDR/PGP/ABCB) efflux/conditional transporters (Boot et al. 2012; Petrásek and Friml 2009; Peer et al. 2011; van Berkel et al. 2013). Our data show that GA_3 enhances *Arabidopsis* root responsiveness to exogenous IAA through modulating auxin transport via AUX1 and PINs. Löfke et al. (2013a, b) reported that GA had no effect on the auxin transporter P-glycoprotein19 (PGP19/ABCB19), and no study has revealed effects of GA on ABCB efflux transporters. The effect of GAs on the auxin transporter AUX1 and PINs has been observed in other species, such as *Populus* (Björklund et al. 2007; Gou et al. 2010; Mauriat et al. 2011, 2014) and peas (Chawla and DeMason 2004). In addition, GAs-induced changes in auxin transport have been observed in several developmental processes, such as cotyledon development (Willige et al. 2011), root gravitropism (Löfke et al. 2013a, b; Willige et al. 2011), lateral root formation (Gou et al. 2010), and adventitious rooting (Mauriat et al. 2014). Our data show that GA is also involved in auxin-mediated primary root elongation. These observations suggest that changes in auxin transport caused by GA may be a common mechanism underlying auxin-mediated development processes. However, auxin carriers involved in these processes are variable, perhaps because the *Arabidopsis* genome has at least three protein families that possess auxin-exporting or -importing activities (Petrásek and Friml 2009; Peer et al. 2011). It is common to find different auxin transporter(s) among developmental processes. For example, aluminium inhibited root length by affecting auxin distribution via modulation of AUX1 and PIN2 (Sun et al. 2010), while copper affected auxin distribution through PIN1 (Yuan et al. 2013), and boron affected auxin distribution via AUX1 (Martín-Rejano et al. 2011). However, how these carriers are modulated in different processes remains unclear.

The auxin transport protein can be regulated at both the transcriptional and post-transcriptional levels (Petrásek and Friml 2009; Titapiwatanakun and Murphy 2009; van Berkel et al. 2013). We also sought to understand the mechanisms by which GA increased the levels of auxin carrier proteins. To this end, we investigated *AUX1* and *PIN* gene promoter GUS lines and their response to treatments with GA and PAC. Our results indicate that the transcriptional regulation observed with the GUS reporters does not reflect differences in PIN protein abundance. However, Ogawa et al. (2003) found that GA_4 upregulates the expression of *PIN2* and *AUX1* in the gibberellin-deficient mutant *ga1-3*. In our study, all GFP and GUS reporter lines were controlled by their native promoters. It is, thus, possible that GA increased auxin carrier protein levels via post-transcriptional regulation, especially cellular trafficking. The cellular trafficking pathway of the PIN and AUX1 auxin transport proteins has been investigated extensively (Geldner et al. 2001, 2003; Kleine-Vehn et al. 2006). The amount of auxin transport protein at the plasma membrane is regulated by the balance between vacuolar-trafficking-mediated degradation and constitutive recycling back to the plasma membrane (Kleine-Vehn and Friml 2008; Löfke et al. 2013a, b). Two previous studies reported that GA increases the abundance of PIN2 protein by inhibiting PIN2 vacuolar trafficking (Löfke et al. 2013a, b; Willige et al. 2011). In *Arabidopsis* root gravitropism, GA stabilises PIN2 protein by inhibiting trafficking to the lytic vacuole, suggesting that GA contributes to the stabilisation of PIN2 at the lower side of the root, and thus promotes asymmetric auxin flow and distribution for gravitropic bending (Löfke et al. 2013a, b; Willige et al. 2011). In our study, GA_3 most likely enhanced the stability of PIN protein in the manner

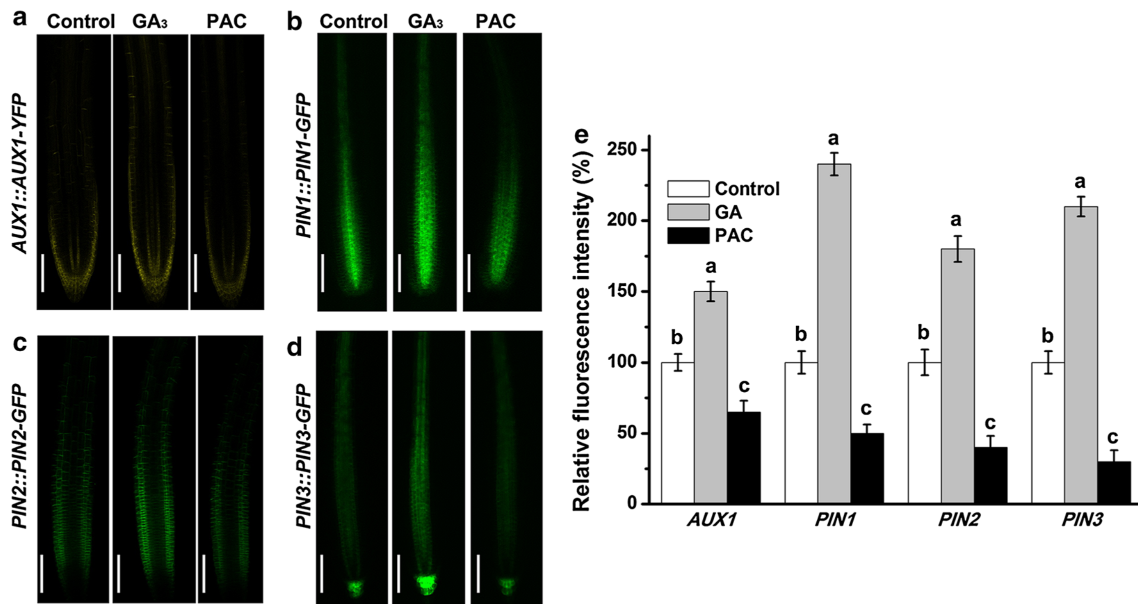

Fig. 5 GA_3 modulated the expression of auxin transport components. Effects of GA_3 and PAC on *AUX1-YFP*, *PIN1-GFP*, *PIN2-GFP*, and *PIN3-GFP* expression in *AUX1::AUX1-YFP* (**a**), *PIN1::PIN1-GFP* (**b**), *PIN2::PIN2-GFP* (**c**), and *PIN3::PIN3-GFP* (**d**) seedlings. **e** Quantification of the fluorescence signals from cells as shown and imaged in **a**–**d**. Five-day-old seedlings were grown in the presence of 1 μM GA_3 and 1 μM PAC. Data are expressed as the fluorescence intensity relative to untreated controls (100 %). Mean values and SE were calculated from three independent experiments ($n = 40$). Within each set of experiments, *bars* with *different letters* were significantly different at the 0.05 level. *Scale bar* 0.1 mm

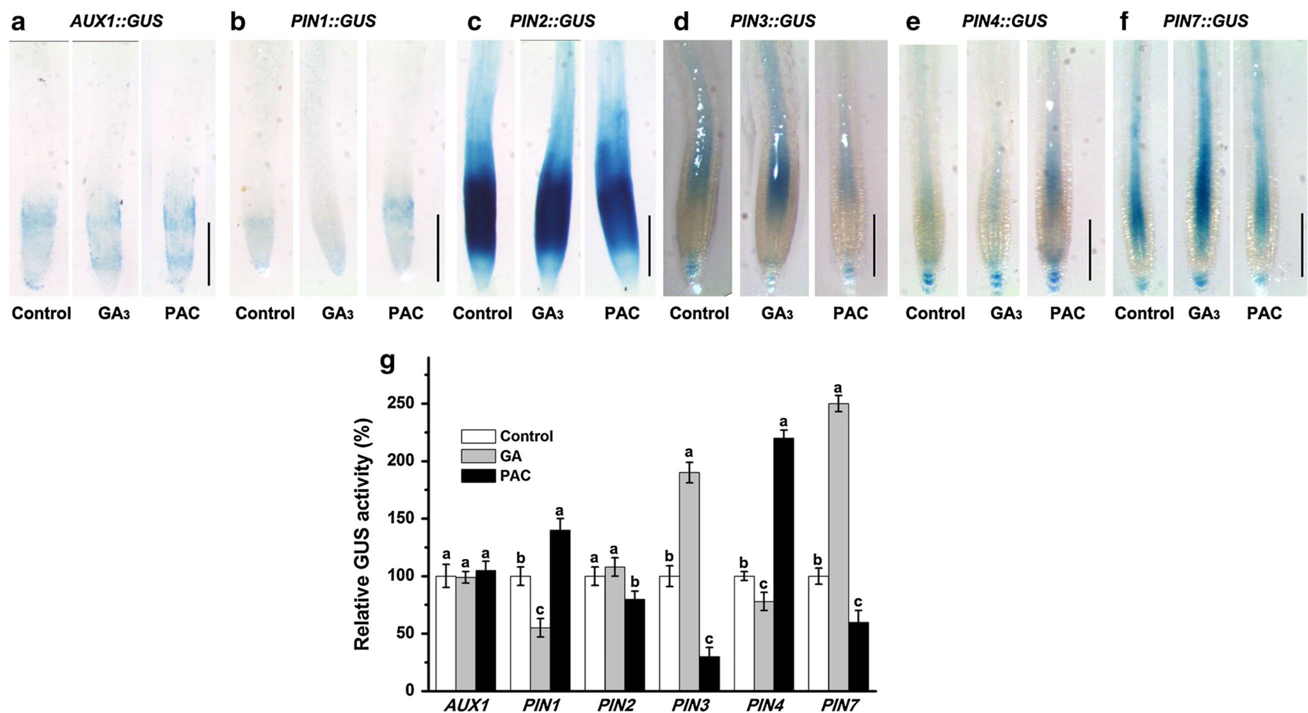

Fig. 6 The effects of increased GA_3 levels on AUX1 and PIN protein accumulation were not consistent with *AUX1* and *PIN* transcription levels. Photographs of seedlings of *AUX1::GUS* (**a**), *PIN1::GUS* (**b**), *PIN2::GUS* (**c**), *PIN3::GUS* (**d**), *PIN4::GUS* (**e**), and *PIN7::GUS* (**f**). **g** Quantification of the GUS activity in *AUX1::GUS*, *PIN1::GUS*, *PIN2::GUS*, *PIN3::GUS*, *PIN4::GUS*, and *PIN7::GUS* seedlings. Five-day-old seedlings were grown in the presence of 1 μM GA_3 and 1 μM PAC. Data are expressed as the GUS activity relative to untreated controls (100 %). Mean values and SE were calculated from three independent experiments ($n = 15$). Within each set of experiments, *bars* with *different letters* were significantly different at the 0.05 level. *Scale bar* 0.2 mm

reported by Löfke et al. (2013a, b) and Willige et al. (2011). However, how GA stabilises AUX1 remains unclear.

Effect of GA on auxin biosynthesis

It has been reported that auxin induces GA biosynthesis (Björklund et al. 2007; Frigerio et al. 2006; Fu and Harberd 2003; Jouve et al. 1999; Ross et al. 2000; Wolbang and Ross 2001; Yin et al. 2007). However, the opposite might also be true. Previous reports have shown that GA mediates the enhancement of the L-Tryptophan (Trp, an IAA precursor) to IAA conversion in the *Avena* coleoptile (Sastry and Muir 1965), and in peas (Law 1987) and garlic (Sata et al. 2002). In *Arabidopsis*, the conversion of Trp to indole-3-acetaldoxime is catalysed by the cytochrome P450 enzymes CYP79B2 and CYP79B3 (Zhao et al. 2002). Exogenous GA_4 upregulates the expression of *CYP79B2* and *CYP79B3* in the gibberellin-deficient mutant *ga1-3* (Ogawa et al. 2003). *INDOLE-3-ACETIC ACID METHYLTRANSFERASE1* (*IAMT1*) and *YUCCA3* (*YUC3*) are involved in IAA inactivation and biosynthesis, respectively (Qin et al. 2005; Li et al. 2008). *pGAI::gai-1-GR* seedlings express dominant versions of the DELLA proteins GIBBERELLIN INSENSITIVE (GAI) that constitutively block GA-induced growth (Peng et al. 1997). Downregulated expression of *YUC3* and upregulated expression of *IAMT1* were observed in etiolated *pGAI::gai-1-GR* seedlings (Gallego-Bartolomé et al. 2011a). Nemhauser et al. (2006) also reported a possible effect of the GA pathway on auxin metabolism through regulation of the *IAMT1* gene. Recently, it has been shown that the bioactive IAA content in *ga1-3* mutant seedlings is considerably lower than that of the wild type, possibly due to the significantly reduced expression of the auxin local synthesis gene *YUC* (Xiao 2011). In *Arabidopsis* roots, IAA accumulates in the root tips, the 2–3-mm region containing the root apical meristem. We were unable to obtain sufficient material for IAA quantification. Since we did not monitor IAA changes between roots treated with IAA alone and those treated with both IAA and GA_3, we cannot speculate on the role of auxin biosynthesis in GA-enhanced root responsiveness to exogenous IAA in *Arabidopsis*.

Conclusion

In summary, we propose that GA strengthens the response of the root to exogenous IAA application in *Arabidopsis* root elongation, and this process requires auxin transport mediated by both AUX1 auxin influx and PIN auxin efflux facilitators. Auxin signalling is also involved in GA-enhanced root responsiveness to exogenous IAA, providing another mechanism for the influence of GA on auxin. Furthermore, we cannot exclude the possibility that GA modulates auxin biosynthesis. Our findings provide additional evidence of the reciprocal interaction between GA and auxin. However, the mechanism underlying modulation by GA of auxin transport and signalling has yet to be determined.

Author contribution statement Conceived and designed the experiments: GL KX DN. Performed the experiments: GL CZ LG. Analysed the data: GL CZ LG. Wrote the manuscript: GL KX DN.

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Conflict of interest The authors declare that they have no conflict of interest.

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