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RESEARCH PAPER

Strigolactones spatially influence lateral root development through the cytokinin signaling network

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Abstract

Strigolactones are important rhizosphere signals that act as phytohormones and have multiple functions, including modulation of lateral root (LR) development. Here, we show that treatment with the strigolactone analog GR24 did not affect LR initiation, but negatively influenced LR priming and emergence, the latter especially near the root–shoot junction. The cytokinin module *ARABIDOPSIS HISTIDINE KINASE3* (*AHK3*)/*ARABIDOPSIS RESPONSE REGULATOR1* (*ARR1*)/*ARR12* was found to interact with the GR24-dependent reduction in LR development, because mutants in this pathway rendered LR development insensitive to GR24. Additionally, pharmacological analyses, mutant analyses, and gene expression analyses indicated that the affected polar auxin transport stream in mutants of the *AHK3*/*ARR1*/*ARR12* module could be the underlying cause. Altogether, the data reveal that the GR24 effect on LR development depends on the hormonal landscape that results from the intimate connection with auxins and cytokinins, two main players in LR development.

Key words: *Arabidopsis thaliana*, cytokinin signaling, lateral root development, polar auxin transport, strigolactones.

Introduction

Strigolactones (SLs) are phytohormones that affect lateral branching of the shoot (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008) and many other processes, such as photomorphogenesis, drought tolerance, leaf senescence, and secondary growth, among others (Woo *et al.*, 2001; Snowden *et al.*, 2005; Shen *et al.*, 2007, 2012; Tsuchiya *et al.*, 2010;

Abbreviations: AHK, ARABIDOPSIS HISTIDINE KINASE; ARR, ARABIDOPSIS RESPONSE REGULATOR; BAP, 6-benzylaminopurine; BES, BRASSINOSTEROID INSENSITIVE--EMS-SUPPRESSOR; BRC, BRANCHED; CRE, CYTOKININ RESPONSE; D14, DWARF14; DAG, days after germination; EMS, ethyl methanesulfonate; GUS, β-glucuronidase; IAA, indole-3-acetic acid; LR, lateral root; LRD, lateral root density; MAX, MORE AXILLARY GROWTH; NPA, 1-*N*-naphthylphthalamic acid; PIN, PIN-FORMED; SCF, Skp-Cullin-F-box; SHY, SHORT HYPOCOTYL; SL, strigolactone; WT, wild-type; XPP, xylem pole pericycle.

Agusti *et al.*, 2011; Bu *et al.*, 2014). In the rhizosphere, SLs influence interactions of the host plant with neighboring organisms, such as root-parasitic plants, mycorrrhizal fungi, and rhizobia (for review, see Xie *et al.*, 2010; Rasmussen *et al.*, 2013a). The root system architecture itself is also affected by SLs, because SLs influence adventitious root development, main root growth, root hair development, and lateral root (LR) development (Kapulnik *et al.*, 2011a, 2011b; Ruyter-Spira *et al.*, 2011; Mayzlish-Gati *et al.*, 2012; Rasmussen *et al.*, 2012, 2013a; Sun *et al.*, 2014). The ontogenesis of LRs consists of several successive steps that are highly regulated (reviewed by Péret *et al.*, 2009). The first step is priming of the LR that occurs in the xylem pole pericycle (XPP) cells in the basal meristem zone of the root tip. These primed XPP cells, also designated prebranch sites, have acquired the developmental program to become an LR. As the root grows, the primed XPP cells enter the elongation zone, where they undergo asymmetric cell division, a process designated LR initiation. Through further well controlled division patterns, an LR primordium will be formed that will ultimately develop into a typical dome-shaped primordium that will pierce through the main root and will form an emerged LR.

Regarding LR development, addition of the SL analog GR24 was found to reduce the LR density (LRD), because of a diminished LR initiation and LR outgrowth (Koltai *et al.*, 2010; Kapulnik *et al.*, 2011b; Ruyter-Spira *et al.*, 2011). In *Arabidopsis thaliana*, mutants in the F-box protein MORE AXILLARY GROWTH2 (MAX2) are perturbed in SL perception and display higher LRDs than the wild-type (WT) plants (Kapulnik *et al.*, 2011b; Kohlen *et al.*, 2011; Ruyter-Spira *et al.*, 2011). When MAX2 function was restored specifically in the root endodermis of *max2* mutants, their insensitivity could be partially complemented (Koren *et al.*, 2013). SLs are perceived by an α/β-hydrolase, DWARF14 (D14), that binds and hydrolyzes SLs and plays a central role in downstream signaling activation (Hamiaux *et al.*, 2012; Zhao *et al.*, 2013). In petunia (*Petunia hybrida*) and rice (*Oryza sativa*), D14 interacts with MAX2/D3, a nuclear-localized F-box protein that participates in the Skp-Cullin-F-box (SCF) complexes and, thus, can mediate the ubiquitin-dependent degradation of signaling proteins (Hamiaux *et al.*, 2012; Zhao *et al.*, 2013).

The interaction of SLs with auxins and cytokinins in regulation of shoot lateral branching has been thoroughly studied mainly in pea (*Pisum sativum*) and Arabidopsis (for a review, see Stirnberg *et al.*, 2010; Cheng *et al.*, 2013; Rasmussen *et al.*, 2013a). Indeed, SL biosynthesis and signaling are intimately connected with auxin transport regulation (Foo *et al.*, 2005; Bennett *et al.*, 2006; Brewer *et al.*, 2009; Ferguson and Beveridge, 2009; Hayward *et al.*, 2009; Crawford *et al.*, 2010; Koltai *et al.*, 2010; Shinohara *et al.*, 2013; Pandya-Kumar *et al.*, 2014). The application of GR24 reduces the basipetal auxin transport and the accumulation of PIN-FORMED1 (PIN1) in the plasma membrane of xylem parenchyma cells in the shoot in a MAX2-dependent manner (Crawford *et al.*, 2010). Moreover, in buds, SLs promote PIN1 endocytosis through a clathrin-dependent mechanism that occurs independently of *de novo* protein synthesis (Shinohara *et al.*, 2013). In pea, SLs have been demonstrated to act also independently of auxin (Brewer *et al.*, 2015). Interestingly, SLs could inhibit shoot lateral branching only when a competing auxin source was available (Crawford *et al.*, 2010; Liang *et al.*, 2010). The auxin landscape also influences the SL control on branching, because the negative effect on shoot lateral branching disappeared and even became positive when the auxin homeostasis was changed (Shinohara *et al.*, 2013). In buds, SLs and cytokinins are known to interact antagonistically and locally (Dun *et al.*, 2012; Zhang *et al.*, 2010; Hu *et al.*, 2014), probably through their common target, BRANCHED1 (BRC1) in Arabidopsis (Minakuchi *et al.*, 2010; Braun *et al.*, 2012; Dun *et al.*, 2012).

Also in the root, the interaction of SLs with auxins has been investigated. PIN1, PIN3, and PIN7 protein levels are reduced upon prolonged treatment with GR24 (Ruyter-Spira *et al.*, 2011). Additionally, during GR24-induced root hair elongation, PIN2 abundance increases at the apical plasma membrane of epidermal cells, suggesting that SLs affect PIN2 endocytosis and endosomal trafficking via actin dynamics in a MAX2-dependent manner (Pandya-Kumar *et al.*, 2014). The inhibitory effect of GR24 on LR development can be reverted to an induction rather than a reduction of LRD by applying a high dose of auxin, or under low phosphate conditions that may increase the auxin sensitivity (Pérez-Torres *et al.*, 2008; Ruyter-Spira *et al.*, 2011). These observations suggest that, just as for branching, changes in the auxin landscape could modulate the impact of GR24 (Ruyter-Spira *et al.*, 2011).

Cytokinins are also well known to influence the root architecture (reviewed in Vanstraelen and Benková, 2012). Cytokinin signaling negatively affects LR development by impinging on PIN-dependent auxin transport (Laplaze *et al.*, 2007; Bishopp *et al.*, 2011; Marhavý *et al.*, 2011, 2014; Bielach *et al.*, 2012; Chang *et al.*, 2013; Moreira *et al.*, 2013). Interaction of SLs with cytokinins during LR development has been poorly studied, but *max2-1* mutants have been reported to have a reduced sensitivity to the synthetic cytokinin 6-benzylaminopurine (BAP) (Koren *et al.*, 2013).

Here, LR priming as well as outgrowth are shown to be modulated by treatment with GR24, the latter in a spatiotemporal manner, mainly affecting the emergence of LRs, which are closest to the root–shoot junction. In addition, the *ARABIDOPSIS HISTIDINE KINASE3* (*AHK3*)/*ARABIDOPSIS RESPONSE REGULATOR1* (*ARR1*)/*ARR12* cytokinin signaling module interacts with SLs to affect LR development, probably through changes in polar auxin transport. Altogether, the results place the SL action on LR development in the auxin landscape context via cross-talk mechanisms with cytokinin signaling.

Materials and methods

Plant material and growth conditions

The *pin7-1* mutant from *Arabidopsis thaliana* (L.) Heyhn. is in Landsberg *erecta* (L*er*) background, whereas the other lines described are in Columbia-0 (Col-0) background. The plant material used has been described previously: *ahk2-2*, *cre1-12*, and *ahk3-3* (Higuchi *et al.*, 2004); *ahk2;ahk3*, *ahk2;ahk4*, and *ahk3;ahk4*

(Riefler *et al.*, 2006); *arr1*, *arr12*, and *arr1;arr12* (Mason *et al.*, 2005); *arr3;arr4;arr5;arr6* and *arr3;arr4;arr5;arr6;arr8;arr9* (To *et al.*, 2004); *pin1-613* (Bennett *et al.*, 2006); *35S:PIN1-GFP* (Růžička *et al.*, 2007); *pin3-3* (Friml *et al.*, 2002); *pin5-3* (Mravec *et al.*, 2009); *pin7-1* (Friml *et al.*, 2003); *shy2-24* (Tian and Reed, 1999); *proAHK3:GUS* (Higuchi *et al.*, 2004); *proPIN1:GUS* and *pGATA23:NLS-GFP-GUS* (De Rybel *et al.*, 2010); and *YUCCA1-D* (Zhao *et al.*, 2001).

Seeds were surface-sterilized for 5 min in 70% (v/v) ethanol, 0.05% (v/v) SDS solution, then incubated in 95% (v/v) ethanol for 5 min, and plated on half-strength Murashige and Skoog (½MS) medium [1% (w/v) sucrose and 0.8% (w/v) agar]. Plants were stratified at 4 °C for 2 d, transferred to a growth chamber at 21 °C (16-h light/8-h dark photoperiod). A racemic mixture of GR24 was supplemented to the growth medium at the start of the experiment and plants were grown for the indicated time. All the experiments were repeated three times. Chemical compounds were added in the following concentrations, except indicated otherwise: 1 µM GR24 and 0.1 µM 1-*N*-naphthylphthalamic acid (NPA).

Phenotypic analysis and statistics

After 9 d of growth, LRs were counted under a binocular S4E microscope (Leica Microsystems) and root length was measured with ImageJ (http://rsb.info.nih.gov/ij). Both values were used to calculate the LRD. For the decapitation experiments, seedlings were grown for 6 d, whereafter the shoot was removed as described (Forsyth and Van Staden, 1981). The bottom part was transferred to ½MS medium with or without 1 µM GR24. For the complementation with indole-3-acetic acid (IAA), agar blocks (0.5 cm^3) containing solidified growth medium with and without 10 µM IAA were added to the decapitated site and the LRD was analyzed 5 d later. Replicate means were subjected to statistics by analysis of variance (ANOVA; SAS Institute Inc., Cary, NC, USA).

Stage determination of GATA23 *expression analysis*

pGATA23:NLS-GFP-GUS seeds were put on medium supplemented with 1 µM GR24 or with the same volume of acetone as control and were stratified for 2 d at 4 °C. Seedlings were grown vertically under continuous white light at 21 °C. At 4 d after germination (DAG), half the seedlings were harvested for analysis, whereas for the remaining seedlings, the position of the main root tip was marked and the plates were transferred back to the growth room. At 9 DAG, the root parts above the mark were harvested. Samples were stained with β-glucuronidase (GUS), cleared as described (Malamy and Benfey, 1997), and analyzed under the microscope (see below). To calculate the percentage of initiated sites, the average of initiations at 9 DAG was divided by the average sites present at 4 DAG. Likewise for the calculations of the percentage of emerged sites, the average of emerged LRs at 9 DAG was divided by that of the sites present at 4 DAG.

Histochemical analysis of GUS activity

Whole seedlings were stained in multiwell plates as described (Jefferson *et al.*, 1987). Samples were cleared as described (Malamy and Benfey, 1997) and were analyzed by a differential interference contrast BX51 microscope (Olympus). Alternatively, samples were mounted directly in chloral hydrate solution (chloral hydrate:water:glycerol, 8:3:1) and microscopically analyzed.

RNA isolation, quantitative RT-PCR, and statistical analysis of PIN1 *expression*

Arabidopsis *pPIN1:GUS* seeds were sown on ½MS medium with or without 1 µM GR24. Seeds were stratified for 2 d at 4 °C and then grown in vertical position at 21 °C (16-h light/8-h dark photoperiod). After 7 d, root material was harvested and flash-frozen in liquid nitrogen. The region between the root–shoot junction and the first emerged LR was harvested separately from the remaining root system. Approximately 100 seedlings were used for each treatment and the experiment was repeated three times.

RNA preparation, cDNA synthesis, real-time quantitative (q) RT-PCR, and statistical analysis of expression profiling were done as described (Rasmussen *et al.*, 2013b). The primers used are the following: *PIN1*_forward GGCATGGCTATGTTCAGTCTTGGG and *PIN1*_reverse ACGGCAGGTCCAACGACAAATC; *ACTIN*_forward CGCCATCCAAGCTGTTCTC and *ACTIN*_reverse TCACGTCCAGCAAGGTCAAG.

Accession numbers

The Arabidopsis Genome Initiative locus identifiers for the genes characterized in this study are: *AHK3* (AT1G27320), *SHY2* (AT1G04240), *PIN1* (AT1G73590), *PIN7* (AT1G23080), and *YUCCA1* (AT4G32540). Germplasm identification numbers for the seeds are: *ahk2* (*ahk2-2tk*), *ahk3-3* (SALK_069269), *cre1-12* (SALK_048970), *ahk2;ahk3* (*ahk2-5ahk3-7*), *ahk2;ahk4* (*ahk2-5cre1-12*), *ahk3;ahk4* (*ahk3-7;cre1-2*), *arr1-2* (N6368), *arr12-1* (CS6978), *arr1;arr12* (*arr1-3;arr12-1*), *pin1-613* (SALK_047613), and *pin5-3* (SALK_021738).

Results

GR24 reduces lateral rooting in Arabidopsis *by affecting LR emergence, especially near the root–shoot junction in a MAX2-dependent manner*

The overall MAX2-dependent reduction in LRD caused by GR24 application had already been reported (Kapulnik *et al.*, 2011b; Kohlen *et al.*, 2011; Ruyter-Spira *et al.*, 2011), but phenotypical insights into this event are still lacking. Upon GR24 treatment, the first emerged LR had an altered position and this effect was abolished in the *max2-1* mutant. When plants were grown without GR24 (mock), the distance from the hypocotyl to the first emerged LR was on average 3.37 mm, whereas when grown in the presence of GR24 it increased to 6.27 mm in WT plants (Fig. 1A).

To understand this effect, the LR development was spatiotemporally followed, with specific focus on the upper root zone. Therefore, the expression of the early LR marker GATA23 that indicates prebranch sites (De Rybel *et al.*, 2010) was used and combined with the staging of the LR primordia (Malamy and Benfey, 1997), in both WT and *max2-1* plants, under mock and GR24 treatments (Fig. 1E, F). As such, all sites in which an LR could develop were visualized from the root–shoot junction down to the root meristem at 4 DAG (Fig. 1E; Supplementary Fig. S1A at *JXB* online). The progression in LR development was subsequently analyzed at 9 DAG (Fig. 1F; Supplementary Fig. S1B at *JXB* online) to obtain a spatiotemporal view of how the LR primordium development was affected by GR24 treatment. Fewer GATA23-marked sites were observed at 9 DAG than at 4 DAG, implying that not all primed sites developed into an LR primordium. When the number of LR sites between mock and GR24-grown plants was compared, slightly, but significantly, fewer sites were counted upon GR24 treatment, both at 4 and 9 DAG (Fig. 1B), indicating that GR24 treatment reduced the total number of prebranch sites in WT, but not in *max2-1*, seedlings (Fig. 1B). Concerning initiated

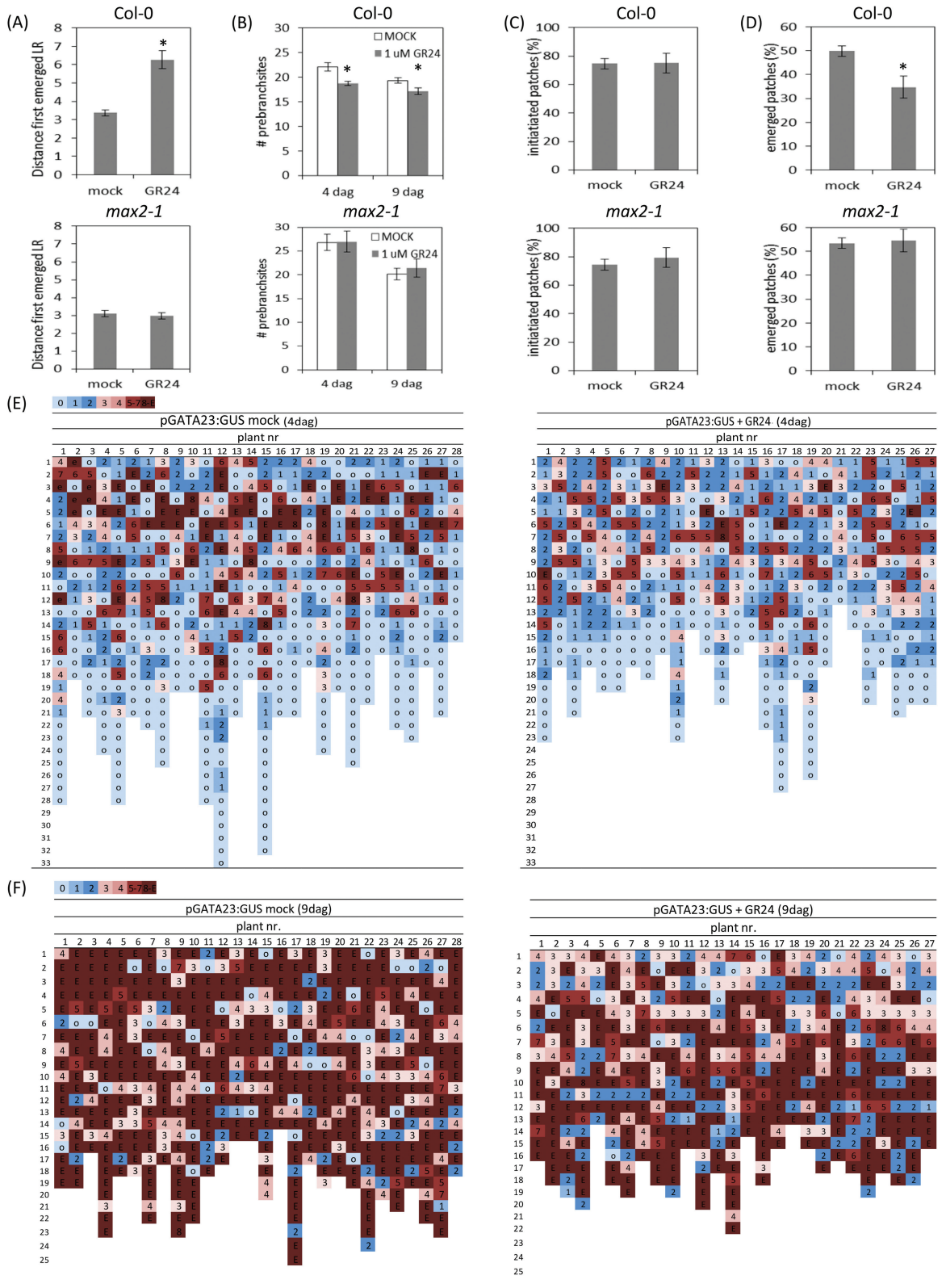

Fig. 1. Effect of exogenous GR24 on LR development near the root–shoot junction.(A) Distance to the first emerged LR in Col-0 (top) and *max2-1* (bottom). (B) Total number of prebranch sites under mock (white bars) and GR24 treatment (gray bars), 4 and 9 DAG in Col-0 (top) and *max2-1* (bottom). (C) Percentage of initiated patches under mock and 1 μM GR24 treatments in Col-0 (top) and *max2-1* (bottom) at 9 DAG. (D) Percentage of emerged patches under mock and GR24 treatment in Col-0 (top) and *max2-1* (bottom). (A–D) Data presented are means ± standard error (SE) of three biological repeats (n>20). *P<0.001, according to the Student's t-test. (E, F) Stages of LR primordia via *GATA23:GUS* staining in Col-0 under mock (left) and GR24 treatment (right) at 4 DAG (E) and 9 DAG (F). All events, possibly leading to emerged LRs, were scored in individual plants, color-coded, and for each plant, vertically ordered from the closest to the hypocotyl (up) downward to the meristem (down). The root fragments used for analysis were comparable in length. Data of one representative experiment are shown. The experiments were repeated three times with similar results.

patches (see Materials and Methods), mock and GR24-grown roots of both WT and *max2-1* seedlings did not differ, suggesting that GR24 had no effect on LR initiation, once the prebranch site had been formed (Fig. 1C). GR24 treatment also affected LR outgrowth (Kapulnik *et al.*, 2011b; Kohlen *et al.*, 2011; Ruyter-Spira *et al.*, 2011). When the percentage of emerged patches was calculated, significantly fewer sites were counted on GR24-grown roots than on control roots, but again not on *max2-1* roots (Fig. 1D). Interestingly, when the emergence pattern was analyzed at 9 DAG (Fig. 1F), the LR outgrowth inhibition was most pronounced at positions 1–8, corresponding to the LR primordia closest to the root–shoot junction, but did not occur in the *max2-1* mutant (see Supplementary Fig. S1B at *JXB* online). These data indicate that mainly the first formed LR primordia, thus those near the root–shoot junction, do not develop when plants are grown in the presence of GR24 and that this effect depends on MAX2.

The cytokinin signaling components AHK3, ARR1, *and* ARR12 *mediate the effect of GR24 on LR development*

Both cytokinins and SLs have been described as negative regulators of LR development in Arabidopsis (Benková *et al.*, 2003; Li *et al.*, 2006; Laplaze *et al.*, 2007; Kapulnik *et al.*, 2011b; Ruyter-Spira *et al.*, 2011). Therefore, the link between the GR24-mediated LRD reduction and the cytokinin-mediated LRD inhibition was investigated in further detail. First, the LRD of several cytokinin signaling mutants, single and higher-order mutants affected in the cytokinin receptors *CYTOKININ RESPONSE1 (CRE1)/AHK4*, *AHK2*, and/or *AHK3* (see Materials and Methods) was examined upon treatment with 1 µM GR24 (Fig. 2A, B). For all tested genotypes, GR24 treatment did not significantly affect the main root length (see Supplementary Fig. S2 at *JXB* online). For Col-0, *cre1/ahk4*, and *ahk2*, the LRD was significantly reduced upon GR24 treatment, but not for the *ahk3* mutant (Fig. 2A). In the double cytokinin receptor mutant *ahk2;ahk4*, the LRD decreased significantly upon GR24 treatment, whereas no significant changes in LRD were observed for *ahk2;ahk3* and *ahk3;ahk4* between mock and GR24 treatment (Fig. 2B). Taken together, these data show that in mutants specifically affected in one member of the cytokinin receptor family, i.e. *AHK3* (*ahk3*, *ahk2;ahk3*, and *ahk3;ahk4*), the GR24 impact on LRD was abolished, whereas other cytokinin receptor mutants responded as WT plants. The *AHK3* expression was unaffected by GR24 treatment (see Supplementary Fig. S3 at *JXB* online).

These observations prompted the investigation of the downstream signaling components of the cytokinin perception machinery. As the B-type response regulators *ARR1* and *ARR12* are involved in mediating the *AHK3*-dependent effects in the root elongation zone (Dello Ioio *et al.*, 2007, 2008), the GR24 impact on the LRD was tested in mutants of these response regulators. The single mutants *arr1* and *arr12* displayed a sensitivity to GR24 similar to that of Col-0 (Fig. 2C), but the double mutant *arr1;arr12* did not, indicating that both ARRs need to be disrupted to interfere with the GR24 effect on LR development (Fig. 2C).

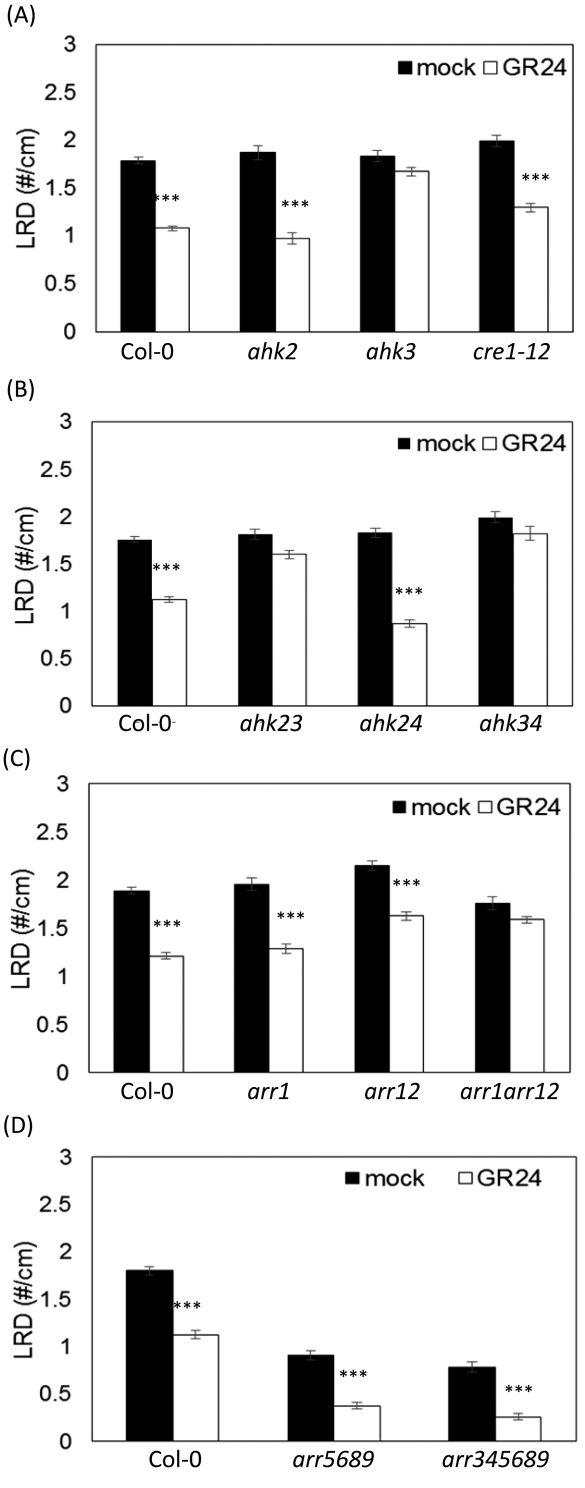

Fig. 2. Effects of GR24 on cytokinin perception and signaling mutants. LRD of single cytokinin receptor mutants (A), double cytokinin receptor mutants (B), B-type response regulators *ARR1*, *ARR12*, and *ARR1;ARR12* (C), and mutants in higher-order A-type response regulators (D) upon GR24 treatment. Data presented are means ± SE of three biological repeats (n>20). ***P<0.001, according to ANOVA mixed-model statistical analyses.

Having established that *AHK3*, *ARR1*, and *ARR12* are involved in the GR24-mediated reduction of LRD, we analyzed whether mutants affected in A-type response regulators would affect the GR24-mediated LRD reduction. Therefore, the sensitivity was tested of higher-order A-type

ARR mutants to GR24, because these negative regulators of the cytokinin response are known to act redundantly in root architecture control (To *et al.*, 2004; Zhang *et al.*, 2011). The *arr5;arr6;arr8;arr9* and *arr3;arr4;arr5;arr6;arr8;arr9* mutants showed a significant increase in sensitivity to GR24: LRD decreased by 37% in WT and by 58% and 67% in *arr5;arr6;arr8;arr9* and *arr3;arr4;arr5;arr6;arr8;arr9*, respectively (Fig. 2D). Hence, these data support the hypothesis that an altered cytokinin responsiveness can enhance (A-type ARRs) or repress (B-type ARRs or AHK3) the GR24 effect on LR development. Taken together, these experiments demonstrate that specific cytokinin signaling components are needed for the GR24 action on LR development.

The modified sensitivity to GR24 of ahk3/arr1;arr12/shy2 *mutants is due to changes in the auxin landscape*

The AHK3/ARR1/ARR12 cytokinin signaling pathway has been shown to act upstream of *SHORT HYPOCOTYL2* (*SHY2*) to control root differentiation (Dello Ioio *et al.*, 2007, 2008) and, additionally, the *shy2* loss-of-function mutant to be insensitive to GR24 as well (Koren *et al.*, 2013). To elucidate why mutants in the *AHK3*/*ARR1*/*ARR12*/*SHY2* module are affected in their GR24 sensitivity, the GR24 phenotype of different *pin* mutants was examined, because *SHY2* specifically represses *PIN1*, *PIN3*, *PIN5*, and *PIN7* and cytokinin treatment downregulates *PIN1* and *PIN3*, but upregulates

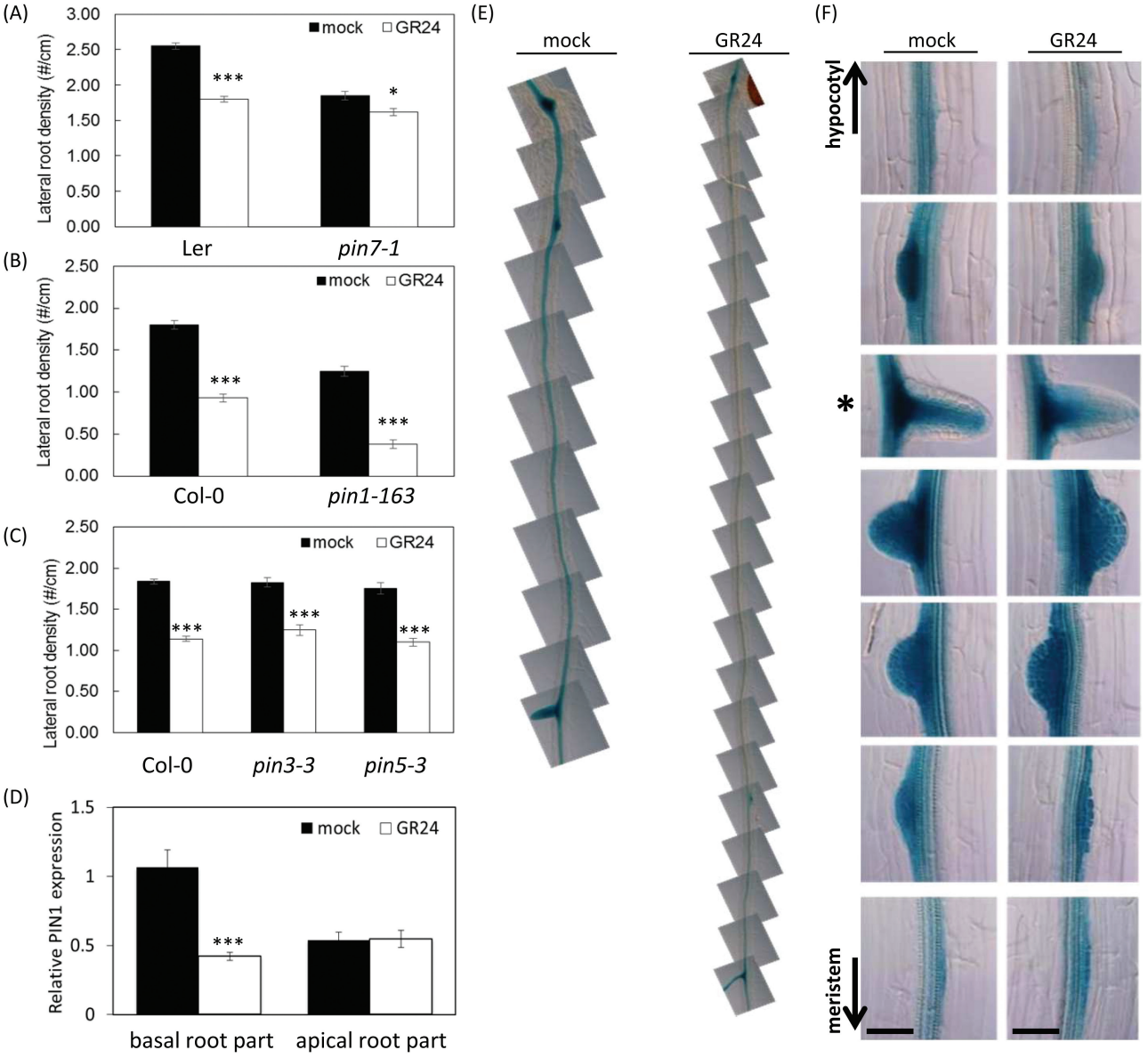

Fig. 3. Interrelation between the polar auxin transport and the GR24 effect on LR development. (A–C) LRD of *pin7-1*, *pin1-613*, *pin3-3*, and *pin5-3* mutants compared with WT grown in the presence or absence of GR24. Data presented are means ± SE of three biological repeats (n>20). (D) Relative *PIN1* expression in 5-d-old seedlings under mock and GR24 treatment as determined by qRT-PCR. Material was harvested separately from the upper part (old, above the first emerged LR) and the lower part (young) of the root. ***P<0.001, *P<0.05, according to ANOVA mixed-model statistical analyses. (E) p*PIN1:GUS* expression patterns of plants grown with and without *GR24*, 7 d after growth. Frames until the first emerged LR are shown. (F) Expression of *PIN1* with p*PIN1:GUS* plants during different stages of LR development under mock and GR24 treatment. The panels indicated by the asterisk display the first emerged LR and those above the asterisk correspond to the LR primordia near the root–shoot junction. Scale bars=40 μm.

PIN7 expression (Dello Ioio *et al.*, 2007; Růžička *et al.*, 2009). First, the GR24 effect on LRD of mutations in *PIN1*, *PIN3*, *PIN5*, or *PIN7* was analyzed. The decrease in LRD of the *pin7* mutants was only minor upon GR24 treatment, indicating that mutations in *PIN7* reduced the root sensitivity to GR24 (Fig. 3A); however, the LRD reduction of the *pin1-613* mutants was significantly higher than that in WT plants (Fig. 3B). For the *pin3-3* and *pin5-3* mutants, the LRD did not differ from that of WT plants (Fig. 3C).

Hence, these results provide the first genetic evidence that the LR response to exogenous GR24 is modulated by interference with the polar auxin transport through PIN1 and, to a lesser extent, PIN7. Previously, prolonged, but not short, GR24 treatments had been demonstrated to influence the expression of *PIN1*, *PIN3*, and *PIN7* in the root meristem, but the expression in root parts other than the meristem had not been assessed (Ruyter-Spira *et al.*, 2011; Shinohara *et al.*, 2013). Therefore, the GR24 effect was investigated on the transcription of *PIN1* in the mature root, at the hypocotyl–root junction, where LR emergence is most affected by the GR24 treatment (Fig. 3). The GR24 impact on *PIN1* expression was analyzed after 7 d of growth of p*PIN1:GUS* seedlings. Interestingly, *PIN1* expression was affected in a spatial way because, especially closest to the shoot, the expression in the vasculature was lower upon GR24 treatment than under mock conditions (Fig. 3E, F). This observation was confirmed by analyzing the *PIN1* gene expression by qRT-PCR of roots grown either in the presence or the absence of GR24 and by assessing the mature versus younger regions of the root (Fig. 3D). Moreover, *PIN1* expression was also lower in the developing LRs from the upper part of GR24-treated plants than that of mock-grown roots, in contrast to developing LRs at younger stages, i.e. near the root meristem (Fig. 3F).

Thus far, our data demonstrate that mutations in the *AHK3*/*ARR1*/*ARR12* cytokinin signaling module and in the auxin transport genes *PIN1* and *PIN7* affect the root sensitivity to GR24, and that GR24 influences auxin homeostasis by downregulating the expression of *PIN1* near the shoot–root junction, in agreement with the decreased PIN protein levels in the root upon prolonged treatments with high concentrations of GR24 (Ruyter-Spira *et al.*, 2011).

To further investigate how the auxin environment alters the GR24 effect, the GR24 response was examined in plants that overexpressed *YUCCA* with concomitantly increased free auxin levels (Zhao *et al.*, 2001). The LRD of *YUCCA1-D* plants did not decrease upon GR24 treatment, indicating that enhanced endogenous auxin levels also modulate the GR24 response in roots (Fig. 4A). Also *35S:PIN1*-overexpressing plants that have highly increased frequencies of root primordia with retarded growth were analyzed (Benková *et al.*, 2003). The typical GR24-mediated decrease in LRD was no longer visible, but rather an increase in LRD (Fig. 4B). Moreover, when the foliar auxin source that determines the outgrowth potential of LRs (Bhalerao *et al.*, 2002; Ljung *et al.*, 2005) was removed by decapitation after 6 d of growth and when

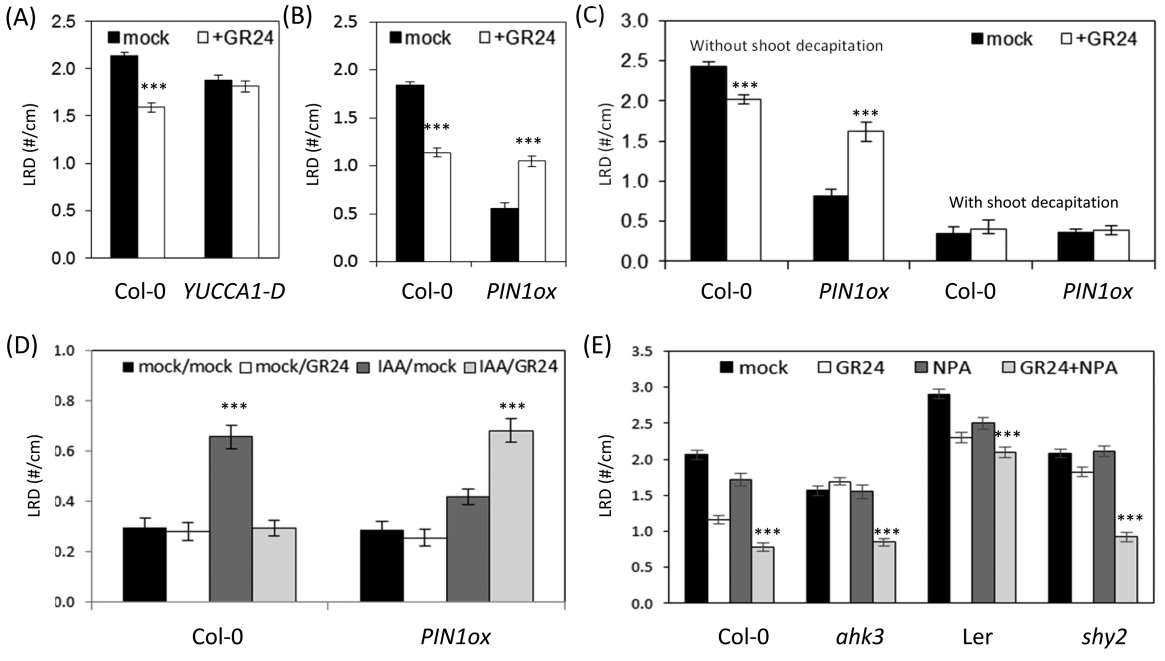

Fig. 4. Dependence of GR24 action on the plant auxin status. (A) LRD of WT and *YUCCA*-overexpressing (*YUCCA1-D*) plants, grown with and without GR24. (B) LRD of WT and *PIN1*-overexpressing (*PIN1ox*) plants, grown with and without GR24. (C) LRD of Col-0 and *35S:PIN1* (*PIN1ox*) plants with and without shoot decapitation, grown in the presence or absence of GR24. (D) LRD of Col-0 and *PIN1ox* plants with decapitation and with and without apically applied IAA grown in the presence or absence of GR24. Mock/mock: decapitated plants grown in the absence of GR24 and without applied IAA; mock/+GR24: decapitated plants grown in the presence of GR24 and without applied IAA; IAA/mock: decapitated plants grown in the absence of GR24 and with apically applied IAA; IAA/+GR24: decapitated plants grown in the presence of GR24 and with apically applied IAA. (E) LRD of Col-0, *ahk3*, L*er* and *shy2-24* mutants upon treatment with mock, GR24, NPA, or NPA+GR24. Data presented are means ± SE of three biological repeats (n>20). ***P<0.001, according to ANOVA mixed-model statistical analyses.

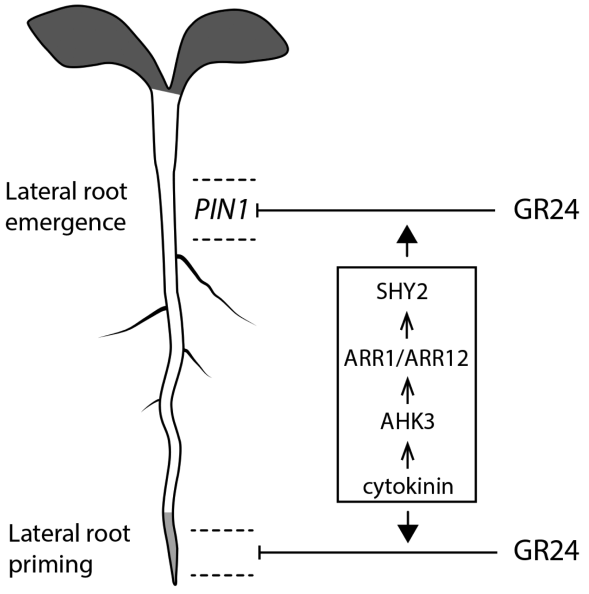

Fig. 5. Working model on the interaction of cytokinins with the SL analog GR24 to control LR development. GR24 treatment results in inhibition of LR emergence, mainly, but not exclusively, near the root–shoot junction and, to a minor extent, in inhibition of LR priming in the root meristem zone. In the root region near the root–shoot junction, this LR emergence inhibition coincides with a spatial downregulation of *PIN1* expression by GR24 treatment. The cytokinin module that signals via AHK3, through the response regulators ARR1/ARR12, and ultimately to SHY2, influences the effect of GR24 on LR development. Mutants in this pathway are insensitive to GR24, probably due to their reported increased PIN1 levels, because reduction of the auxin flux by NPA treatment renders the mutants sensitive again to GR24.

these plants were subsequently treated with GR24 for 5 d, the effects disappeared on both the *PIN1*-overpressing lines (increase in LRD) and the WT (decrease in LRD), indicating that shoot-derived auxin is important for the GR24 responses in roots (Fig. 4D). Application of IAA in these experiments (see Materials and Methods) revealed that shoot-derived auxin mediated the effect, because it complemented the phenotype of decapitated plants (Fig. 4D). Altogether, the functional data demonstrate that shoot-derived auxin controls the effect of GR24 on lateral rooting in Arabidopsis, as previously hypothesized (Ruyter-Spira *et al.*, 2011).

All mutants with GR24-insensitive root responses, i.e. *ahk3*, *arr1;arr12*, and *shy2-24*, display enhanced *PIN1* expression (Dello Ioio *et al.*, 2007, 2008; Zhang *et al.*, 2011) that might cause their insensitivity toward GR24. This hypothesis was tested by applying low concentrations (100 nM) of NPA, a polar auxin transport inhibitor (Himanen *et al.*, 2002). The LRD response was analyzed under mock and GR24 treatment after 9 d of growth (Fig. 4E). Addition of this low concentration of NPA had no impact on the LRD (Fig. 4E) and had no clear effect on *PIN1* expression in the main root, although a slight increase in *PIN1* gene expression was observed in the root tip (see Supplementary Fig. S4 at *JXB* online). However, when the *ahk3* and *shy2-24* mutants were grown on plates supplemented with NPA as well as GR24, the LRD was lower than that of roots grown under mock conditions or supplemented with GR24 or NPA alone, implying that treatment with NPA rendered the mutant plants responsive to GR24 again. For Col-0, no additional effect was seen when the roots were treated with both NPA and GR24.

Discussion

Several aspects of the root system architecture are modulated by SLs (for reviews, see Cheng *et al.*, 2013; Rasmussen *et al.*, 2013a; Koltai, 2014). Here, GR24 was found to control LR development spatiotemporally and to interplay with cytokinin that, just like SLs, regulates LR development. A summarizing model is presented (Fig. 5).

The method established to build a developmental map of all possible initiated LRs combines the *GATA23* marker gene for induction of prebranching sites, i.e. pericycle-derived LR founder cells that are predestined to start cell division for LR development and LR positioning (Malamy and Benfey, 1997; De Rybel *et al.*, 2010). Together with the determination of the position of each event along the main root, a precise developmental map provides location and developmental stage of each LR event, thereby revealing that the main effect of GR24 on the development of LRs concerns their emergence. This observation concurs with previously published work, although the proposed specific interruption at stage V of LR development was not detected (Ruyter-Spira *et al.*, 2011).

On the 9-DAG map, the LRs were mainly, but not exclusively, situated close to the root–shoot junction that no longer emerged under GR24 treatment. Accordingly, the distance between the hypocotyl–shoot junction and the first emerged LR was longer in GR24-grown roots than in control roots. This MAX2-dependent effect concurs with its essential function in SL signaling. Hence, GR24 might affect specifically the emergence of the LRs that develop first and are positioned in the older part of the root. This spatiotemporal effect was also seen on the *PIN1* expression pattern in the root. Although the reason for this effect still needs to be investigated, the disappearance of the SL receptor might be the underlying cause, because GR24 treatment reduces D14 protein abundance in roots (Chevalier *et al.*, 2014).

Additionally, a small, but significant, decrease in prebranch sites was visible, whereas GR24 had no appreciable impact on LR initiation. The previously detected GR24 effect on LR initiation (Kapulnik *et al.*, 2011b; Ruyter-Spira *et al.*, 2011) might be due to an impact on prebranching. Prebranch sites are established by a periodic oscillation of auxin concentrations accompanied by fluctuations in specific gene expression (De Smet *et al.*, 2007; Moreno-Risueno *et al.*, 2010). This oscillating pattern has been found to be mediated by a carotenoid compound, distinct from SLs (Van Norman *et al.*, 2014). In agreement with the data presented, the *max2* mutants also exhibited an increased LR capacity (Van Norman *et al.*, 2014). It would be interesting to analyze whether GR24, as a mimic of SLs or related compounds, modulates the periodic oscillation of auxin to cause the small effect on prebranching. Furthermore, independently of SLs, at 9 DAG, fewer

LR events are observed on the same main root part than at 4 DAG, possibly indicating that not all primed sites develop into LRs.

Cytokinins have been identified as endogenous repressors of LR development in a close interplay with auxin (Benková *et al.*, 2003; Li *et al.*, 2006; Laplaze *et al.*, 2007). Here, the GR24 effect on LR development required the functional cytokinin receptor AHK3, but not AHK2 and AHK4/CRE1. The dependence on *AHK3* and not on *AHK4* is remarkable, because *AHK4* has been implicated in LR patterning along the main root (Marhavý *et al.*, 2011), whereas *AHK3* and the two immediately downstream B-type response regulator genes, *ARR1* and *ARR12*, play an important role in determining the root meristem size (Dello Ioio *et al.*, 2007, 2008). Also in the experimental setup, the double mutant *arr1;arr12* had no LR response toward GR24, implying that the same cytokinin module (*AHK3*/*ARR1*/*ARR12*) that determines the root meristem differentiation also governs the GR24 action on LR development. *AHK3* is involved in meristem differentiation by transcriptional control of the auxin-induced *SHY2*/*IAA3* gene (Dello Ioio *et al.*, 2007, 2008). The typical reduction in lateral rooting upon GR24 treatment was indeed not seen in the *shy2-24* loss-of-function mutants (Koren *et al.*, 2013), supporting the hypothesis that the *AHK3*/*ARR1*/*ARR12* module acts through *SHY2* to result in GR24 insensitivity.

The *AHK3*/*ARR1*/*ARR12*/*SHY2* module negatively influences *PIN1*/*PIN3*/*PIN5*/*PIN7* expression (Dello Ioio *et al.*, 2007, 2008), whereas cytokinin treatment downregulates *PIN1*/*PIN3*/*PIN5*, but upregulates *PIN7* expression (Laplaze *et al.*, 2007; Růžička *et al.*, 2009). These changes in *PIN* gene expression and their consequences on polar auxin transport might be the underlying cause for the GR24 insensitivity of the mutants. Several *PIN* mutants had a modified sensitivity to GR24: *pin3* and *pin5* mutants still displayed a reduced LR development upon GR24 treatment, whereas *pin7* mutants were only slightly responsive to GR24, and *pin1-613* mutants were hypersensitive, in agreement with the opposite influence of cytokinins on their expression. In addition, treatment of *ahk3* and *shy2-24* with NPA made them sensitive again to GR24. Hence, the changes in *PIN* gene expression, such as the *PIN1* overexpression observed in these mutants (Dello Ioio *et al.*, 2007, 2008; Zhang *et al.*, 2011) with an enhanced polar auxin transport as a result, might be the reason that GR24 does not reduce the LRD in these mutants.

Moreover, the data support the central role of auxin transport for SL action. Based on exogenous auxin and phosphate level modulation, the auxin content in roots has been shown to determine its responsiveness toward GR24 (Ruyter-Spira *et al.*, 2011). Indeed, endogenous overproduction of auxin via overexpression of *YUCCA* could make LR development unresponsive to GR24. As auxin is well known to positively regulate its own efflux from cells, PIN1 internalization in the *YUCCA1-D* mutant was reduced, resulting in the accumulation of PIN1 on the plasma membrane (Paciorek *et al.*, 2005), an observation fitting the theory that mutants with an enhanced *PIN1* expression are insensitive to GR24. Interestingly, *PIN1*-overexpressing plants no longer displayed a reduced LRD when treated with GR24, but an opposite phenotype with an increased LRD. The difference in phenotypes between the plants overexpressing *YUCCA1-D* and *PIN1* is intriguing, but might be due to differences in the severity of PIN1 accumulation. Also in the shoot, depending on the auxin transport landscape, GR24 could have positive or negative effects on the shoot lateral branching by depleting PIN1 from the membranes of xylem parenchyma cells of inflorescence stems (Shinohara *et al.*, 2013). In addition, GR24 has been shown to have a different impact on LR development that depends on the growth conditions: inhibition under sufficient and induction under low phosphate conditions or with exogenous IAA (Ruyter-Spira *et al.*, 2011). Hence, *PIN1* overexpression has an effect on GR24 responses similar to that of phosphate-limiting conditions: an increase, rather than a decrease, in LRD.

In conclusion, the data presented imply that GR24 regulates LR development in a spatiotemporal manner with the strongest effect on emergence of the first developed LR positioned close to the root–shoot junction. This effect is tightly integrated into the auxin–cytokinin network that rules the root architecture, with the polar auxin transport capacity as a central player on which both cytokinin and GR24 act.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Stages of LR primordia via p*GATA23:GUS* staining in *max2-1* under mock and GR24 treatment at 4 and 9 DAG.

Fig. S2. Main root lengths of WT and cytokinin receptor and signal transduction mutants under mock and GR24 treatment.

Fig. S3. p*AHK3:GUS* expression patterns of LR primordia at different developmental stages under mock and GR24 treatment.

Fig. S4. p*PIN1:GUS* expression pattern after treatment with 0.1 µM NPA around the root–shoot junction (left) and the root meristem zone (right).

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