RALFL34 regulates formative cell divisions in Arabidopsis pericycle during lateral root initiation

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Abstract

In plants, many signalling molecules, such as phytohormones, miRNAs, transcription factors, and small signalling peptides, drive growth and development. However, very few small signalling peptides have been shown to be necessary for lateral root development. Here, we describe the role of the peptide RALFL34 during early events in lateral root development, and demonstrate its specific importance in orchestrating formative cell divisions in the pericycle. Our results further suggest that this small signalling peptide acts on the transcriptional cascade leading to a new lateral root upstream of GATA23, an important player in lateral root formation. In addition, we describe a role for ETHYLENE RESPONSE FACTORS (ERFs) in regulating RALFL34 expression. Taken together, we put forward RALFL34 as a new, important player in lateral root initiation.

Key words: Arabidopsis thaliana, ERF, GATA23, lateral root initiation, RAPID ALKALINIZATION FACTOR (RALF).

Introduction

Root plasticity is one of the main adaptive traits enabling plants to cope with an ever-changing environment. Formation and positioning of lateral roots along the longitudinal primary root axis plays a vital role in nutrient acquisition and water uptake. Lateral roots are formed post-embryonically from the pericycle cells adjacent to the xylem poles (Malamy and Benfey, 1997; Dubrovsky et al., 2001; De Smet et al., 2006). Their initiation and development occur in a regular way, and depend largely on the plant hormone auxin (Laskowski et al., 1995; Malamy and Benfey, 1997; Beeckman et al., 2001; Casimiro et al., 2001; Lavenus et al., 2013). The development of lateral root primordia goes through several well-described...
stages, with the first stages being essential for proper lateral root primordium development (Malamy and Benfey, 1997; De Smet et al., 2008, 2010; Lucas et al., 2013; von Wangenheim et al., 2016). Typically, stage 1 comprises two rounds of asymmetric cell divisions of a small set of pericycle founder cells, forming smaller daughter cells with distinct cell fates. At stage 2, a rotation in the plane of division occurs; the cells divide periclinally toward the outer tissues forming an outer layer and an inner layer. This division normally occurs first in the two most central cells, followed by the adjacent cells. The most peripheral cells do not divide periclinally, so, as the central cells expand radially, the establishment of the lateral root primordia dome shape materializes. In stages 3–7, numerous rounds of anticlinal and periclinal cell divisions occur, establishing distinct tissue layers eventually mimicking the organization of the primary root tip. Stage 8 involves few cell divisions; however, rapid cell expansion results in penetration of the overlying tissue, emerging from the primary root. Several of the underlying genes and proteins that are involved in priming, founder cell specification or activation, and initiating and advancing lateral root development have been identified through transcript profiling and the use of gain-of-function and loss-of-function mutants, such as SOLITARY ROOT (SLR)/IAA14, AUXIN RESPONSE FACTOR7 (ARF7), ARF19, LATERAL ORGAN BOUNDARIES-DOMAIN16 (LBD16), LBD29, GATA23, ARABIDOPSIS CRINKLY4 (ACR4), and several others (Fukakai et al., 2002; Tatematsu et al., 2004; Okushima et al., 2005, 2007; De Smet et al., 2008; De Rybel et al., 2010; Lavenus et al., 2013). Less information, however, is known on cellular communication during lateral root development, specifically, through the relatively recently discovered small signalling peptides.

Small signalling peptides have been shown to play a wide variety of roles in the plant, with recent evidence also showing their involvement in lateral root development (Murphy et al., 2012; Cyzewicz et al., 2013; Delay et al., 2013b; Tavormina et al., 2015). For example, CLE-LIKE (CLEL)/GOLVEN (GLY)/ROOT GROWTH FACTOR (RGF) peptides inhibit pericycle cell divisions when overexpressed (Matsuzaki et al., 2010; Meng et al., 2012; Whitford et al., 2012; Fernandez et al., 2013, 2015). INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) and its receptors HAESA (HAE) and HAESA-LIKE2 (HSL2) play a role in lateral root emergence (Kumpf et al., 2013). Various C-TERMINALLY ENCODED PEPTIDES (CEPs) reduce (emerged) lateral root density when the synthetic peptide is exogenously applied or endogenously overexpressed (Delay et al., 2013a; Roberts et al., 2016). CLAVATA3/EMBRYO SURROUNDING REGION (CLE) peptides inhibit lateral root emergence when overexpressed (Araya et al., 2014) and also regulate lateral root development through BIN2-mediated phosphorylation of ARFs (Cho et al., 2014). A recently discovered peptide, AUXIN-RESPONSIVE ENDOGENOUS POLYPEPTIDE 1 (AREP1), promotes lateral root organogenesis in the presence of auxin (Yang et al., 2014). Finally, RAPID ALKALINIZATION FACTOR (RALF) peptides in Arabidopsis regulate various processes predominantly through regulating cell expansion (Pearce et al., 2001; Srivastava et al., 2009; Mingossi et al., 2010; Atkinson et al., 2013; Bergonci et al., 2014; Morato de Canto et al., 2014). Some RALF peptides, such as RALF1, RALF19, and RALF23, increase emerged lateral root densities as demonstrated by RALF-silenced transgenic lines and decrease densities as shown by the use of RALF-overexpressing lines (Atkinson et al., 2013; Bergonci et al., 2014). Taken together, it is peculiar that the majority of the so far characterized small signalling peptides have a negative impact on root architecture. Speculatively, this might imply that these small signalling peptides act as specific, negative regulators of, for example, the dominant, promoting effect of auxin.

Here, we describe the role of RALF-LIKE 34 (RALF34) in lateral root initiation, and position this small signalling peptide in the transcriptional cascade leading to a new lateral root. In addition, we describe a role for ETHYLENE RESPONSE FACTORS (ERFs) in regulating RALF34 expression.

Materials and methods

Plant materials

We used the following lines in our research: Columbia-0 (Col-0), Landsberg erecta (Ler), ralfl34-1 (SALK_004441) (Alonso et al., 2003), ralfl34-2 (JIC_SGT4223) (Parinov et al., 1999), pGATA23::NLS:GFP (De Rybel et al., 2010), WAVE131Y (Geldner et al., 2009), pRALF34_nosig:~n3xRFP (see below), and p35S::ERF9-GR (see below).

Growth of Arabidopsis thaliana seedlings

Seeds were surface sterilized (70% ethanol for 2 min, 10% bleach for 15 min, dH₂O for five washes) and then stratified at 4 °C for 2 d. Seeds were then plated on half-strength Murashige and Skoog (1/2 MS) agar plates (2.154 g l⁻¹ MS, 0.1 g l⁻¹ myo-inositol, 0.5 g l⁻¹ MES, 0.1 g l⁻¹ bacterial agar, pH 5.7 with 1 M KOH) and germinated vertically under constant white light at 21 °C.

Genotyping of T-DNA insertion lines

The following T-DNA insertion mutant lines were used and genotyped in our investigations: ralfl34-1 (SALK_004441) using the following genotyping primers, FW primer TGACTAACCCTTTAAGGTCCACG; REV primer, ACGGGACCTCTAGTCTCAGAG; and T-DNA primer, ATTATTGCCAGATTTGCGGAC; and ralfl34-2 (JIC_SGT4223) using the following genotyping primers, FW primer, ATTTTCGCTCTGCTCTC; REV primer, CATCTCCGGCATCGAG; and T-DNA primer, ACGGTCCGGAAACTGCTTAC.

Constructs

The pRALF34::LUC was generated as follows: the 400 bp RALF34 promoter fragment was PCR amplified using the RALF34 forward (CAACTGGACCCATCTGCTGAAG); and reverse primer (CGGGCATTTGGTGGGGA) and cloned into the pGemE-T-easy vector. After sequencing confirmation, the 400 bp promoter fragment was released from the pGemE vector using the primer (GCTCTAC ACTTC). The sequence of the final promoter fragment was released from the pGemE vector using the primer (CGGCGATTGTTGGGGGA) and cloned into the pJIT60 construct was confirmed. The 416 bp pRALF34 promoter fragment was released from the pGemE-T Easy vector with restriction enzymes PstI and NcoI and then cloned into the LucTrap vector (Calderon-Villalobos et al., 2006; Lau et al., 2011; De Smet et al., 2013). The sequence of the final construct was confirmed. The pERF9 and pERF4 were cloned into the pJIT60 vector (ATGGCTCTAGAAGCAGGCG and GCTACTAACCCTTTAAGGTCCACG) and then cloned into the luciferase vector (pERF9 and pERF4 coding sequences were PCR amplified using pERF9 (ATGGCCCAAGATGGCCTTGG and ATGGCCCAAGATGGCCTTGG) and pERF4 (ATGGCCCAAGATGGCCTTGG and ATGGCCCAAGATGGCCTTGG))
For dexamethasone (DEX) treatments, seedlings were grown on 1/2 MS plates (7.5 μM DEX) and were cloned into the pDONR221, pDONRP2RP3, and pDONRP4P1R, respectively, with Gateway Cloning® vector backbone (Invitrogen). Transgenic lines were confirmed. Both pRALFL34::n3xRFP and pS5m34GW-FAST constructs were transformed into Agrobacterium sp. and then floral dipped into Col-0 plants (Clough and Bent, 1998).

Induction of lateral roots
Lateral root induction was performed through mechanical (Ditengou et al., 2008) or gravitropic bending (Peret et al., 2012; Lavenus et al., 2015; Voß et al., 2015), as previously described. Quantification of pGATA23:::NLS:FP fluorescence was analysed on Col-0 (wild type) and ralf34-l seedlings. Total fluorescent nuclei (in all tissues) were counted from the quiescent centre (QC) moving up the root for ~2500 μm and density measurements were calculated as ‘fluorescing nuclei in n μm’. 

DEX treatment
For dexamethasone (DEX) treatments, seedlings were grown in vitro on 1/2 MS plates (7.5 g l−1 agar) overlaid with a nylon mesh (Prosep, 20 μm pore size). At 7 d after sowing, the mesh with seedlings was transferred to plates with 1/2 MS medium (7.5 g l−1 agar) and plates with 1/2 MS medium containing 5 μM DEX (Sigma).

Microscopic analysis
Phenotyping was analysed under a Leica stereo dissection microscope at varying magnifications to observe and count the emerged lateral roots. Lateral roots were counted, marked, and then photographed. Root lengths were measured from the bottom of the hypocotyl to the root tip, using ImageJ software (http://imagej.nih.gov/ij/). Lateral root staging of the ralf34-l and ralf34-2 mutants was performed on a Leica DMRB microscopy using differential interference contrast (DIC). Fluorescent seedlings were analysed on a Nikon confocal microscope utilizing both an Argon 488 laser and a 514 HeNe laser at ×20 and ×40 magnification.

RALFL34 auxin response quantification
Arabidopsis (ecotype Col-0) seeds were surface sterilized, stratified at 4 °C for 3 d, then plated on 1/2 MS medium and grown vertically under constant white light at 21 °C for 4 d post-germination. Seedlings were transferred to 1/2 MS liquid medium on the fourth day to acclimate overnight. Seedlings were transferred to liquid 1/2 MS containing 1-naphthaleneacetic acid (NAA) at a concentration of 10 μM (or ethanol control) and were grown for a further 0, 1, 2, 4, 6, 8, 12, and 24 h; at each time point, the roots of 10 seedlings were excised, frozen in liquid nitrogen, and then total RNA extracted as below.

Quantitative real-time PCR (qRT-PCR)
Total RNA was extracted from lines of interest using an RNAsesy kit (Qiagen) as per the manufacturer’s instructions. cDNA was generated from 1 μg or 200 ng of total RNA via oligo(dT) primers using iScript (BioRad) or Superscript II (Invitrogen), respectively, as per the instructions. Quantitative PCRs were performed in at least two technical repeats, using the SYBR Green QPCR Master Mix (Quanta Biosciences), in our modified reaction protocol: Master mix, 7 μl per reaction (forward primer (100 μM), 0.1 μl per reaction; reverse primer (100 μM), 0.1 μl per reaction; SYBR (2×), 6 μl per reaction; dH2O, 0.8 μl per reaction) and template cDNA (diluted 1/100 in dH2O, 5 μl per reaction. Alternatively, the LightCycler® 480 SYBR Green I Master (2×) in Light Cycler 480 (Roche) was used, plates were filled with the JANUS Automated Workstation Perkin Elmer, primers were used at 0.5 μM, and cDNA was diluted 8× (total volume =5 μl =0.5 μl of template, 2 μl of primers, 2.5 μl; 2× mix). Gene-specific quantification was performed with intron-spanning primer pairs (where possible) designed using Primer3® software (http://bioinfo.ut.ee/primer3/). Expression was normalized using AT5G60390 (CTGGAGGTGTTGTGCGTGGT and CCAAGGGTGAGAAAGAAGA) and or AT3G60830 (ACTCTCTCTAGTGGACAGGTG and CTCAAGGTTTCCATGCT) and or AT4G16100 (GGGAGATTAGACGACGCAGTG and ATGCGCCGAGATGAGTGAAG) as reference in all qRT-PCR experiments were performed in three biological replicates (unless otherwise specified) and 2–3 technical repeats, and the data presented represent means ±SE.
Transient activity assays

BY-2 protoplast assays were performed as previously described (Vanden Bossche et al., 2013), using reporter (LucTrap, encoding firefly luciferase) and effector constructs (pJIT60).

Results and Discussion
RALF34 regulates pericycle cell division patterns during lateral root initiation

In a transcriptome analysis focusing on early stages of lateral root initiation and utilizing a lateral root inducible system based on auxin treatment leading to synchronous induction of lateral roots (Himanen et al., 2002, 2004; De Rybel et al., 2010), RALF34 (AT5G67070) expression was up-regulated, together with a small set of 14 potential key regulatory genes for asymmetric cell division and/or cell fate specification during lateral root initiation, including ARABIDOPSIS CRINKLY4 (ACR4) (De Smet et al., 2008) (Fig. 1A). RALF34 encodes a member of a family of cysteine-rich small signalling peptides (Murphy and De Smet, 2014) and probably gives rise to a 56 amino acid mature peptide (Fig. 1B). While members of the RALF family have been functionally characterized (Pearce et al., 2001; Olsen et al., 2002; Haruta et al., 2008, 2014; Matos et al., 2008; Srivastava et al., 2009; Atkinson et al., 2013; Bergonci et al., 2014; Morato do Canto et al., 2014; Li et al., 2015), no data are so far available for RALFs in the context of lateral root initiation.

Fig. 1. RALF34 expression and gene/protein topology. (A) Expression profile of RALF34 in the pericycle of seedlings subjected to lateral root inducible system [after mixed model analysis; data from De Smet et al., (2008)]. Seedlings were grown on NPA (1-N-naphthylphthalamic acid; 0 h) for 3 d. NAA, 1-naphthaleneacetic acid. (B) Schematic of RALF34 protein, highlighting the putative signal peptide and likely mature peptide region and sequence. (C) Schematic of the RALF34 gene, with indication of qPCR primers (pink lines), position of T-DNA insertion, and promoter region (blue line). (D) RALF34 expression as monitored through qPCR in ralfl34-1 and ralfl34-2 roots at 5 d after germination and their respective control lines, Col-0 and Ler. The graph depicts the average of three biological repeats (and 3–6 technical repeats) ±SE. Student’s t-test with P-value <0.01.
To validate a role for RALFL34 in lateral root initiation, we identified two T-DNA lines, namely ralf34-1 (in Col-0) and ralf34-2 (in Ler), with significantly reduced RALFL34 expression (Fig. 1C,D). Both ralf34-1 and ralf34-2 were analysed with respect to overall lateral root density, lateral root stage distribution, and pericycle division patterns. Overall, ralf34-1 and ralf34-2 displayed, respectively, a 25% and 21% increase in total lateral root density (emerged and non-emerged) compared with their control line (Fig. 2A; Supplementary Fig. S1 at JXB online). A more detailed analysis quantifying the different stages of lateral root development in ralf34-1 and ralf34-2 revealed that this is probably due to an enrichment of stage 1 lateral root primordia (which also included regions with divisions that did not fully resemble a typical stage 1 primordium (as shown in Fig. 2D)) compared with the control (Fig. 2B). Furthermore, we observed a 3-fold increase in ‘aberrant’ pericycle division patterns (extra cell divisions flanking the primordia) or unusually positioned (defined as lateral root primordia being closer to each other than 400 µm) in ralf34-1 and ralf34-2 compared with their control line (Fig. 2C, D; Supplementary Fig. S1). Based on these observations, we concluded that RALFL34 plays a role during lateral root initiation and probably acts to restrict (proliferative) cell divisions and/or mediate the spatial distribution of asymmetric founder cell divisions in the pericycle along the root’s longitudinal axis.

RALFL34 is expressed before asymmetric pericycle cell division

To investigate at which stage during lateral root development RALFL34 is expressed, we generated a pRALFL34::n3xRFP line (referred to as pRALFL34::n3xRFP) and combined this with a yellow fluorescent protein-NPSN12 plasma membrane marker line (WAVE131Y) (Geldner et al., 2009). RALFL34 is expressed in xylem pole pericycle cells before any visible sign of asymmetric cell division (Fig. 3A). At a later stage, RALFL34 is strongly expressed in the small central cells of the lateral root initiation site, but also in the larger flanking cells (Fig. 3B, C). In addition, we detected RALFL34 expression in the epidermis (Fig. 3A). Given that lateral root development is
controlled by auxin at various levels (Lavenus et al., 2013), we explored if RALFL34 expression is regulated by auxin in the root. Treatment with the synthetic auxin NAA (1-naphthaleneacetic acid) showed minor, but significant, concentration-dependent down-regulation after 6 h and minor, but significant, concentration-dependent transcriptional up-regulation following a 24 h treatment (Fig. 4A). Primary auxin-responsive genes are generally significantly differentially regulated within 2 h after exposure to auxin (Abel et al., 1994; Oeller and Theologis, 1995). This suggests that RALFL34 is probably not a (positively regulated) primary auxin response gene, but acts nevertheless downstream of an auxin response module. It should be noted that these results are obtained from auxin-treated seedlings grown on control medium before treatment, while the initial identification of RALFL34 (see above; Fig. 1A) was based on auxin treatment synchronously inducing asymmetric cell divisions in the pericycle following growth on the auxin transport inhibitor NPA. Furthermore, this change in RALFL34 expression is likely to be due to initiation of multiple formative asymmetric divisions, as an increased number of lateral root primordia induced by the NAA treatment are observed (Fig. 4B). Interestingly, RALFL34 was recently identified as having a mobile mRNA in a root to shoot direction (Thieme et al., 2015), suggesting that the RALFL34 domain—also in the root—is likely to be broader than reported by our pRALFL34::n3xRFP line. In this context, Arabidopsis eFP Browser data suggested the presence of RALFL34 mRNA in above-ground plant parts (Supplementary Fig. S2), but this can be derived from mobile mRNA and/or local expression.

‘Flanking’ RALFL34 expression is associated with lateral root initiation

Interestingly, the pRALFL34::n3xRFP expression around the lateral root initiation site often showed a ‘flanking’ expression profile, which appears to be contained within the pericycle, that extended from the lateral root initiation site shootward and/or rootward (25/30 lateral root primordia) (Fig. 5A). We established that this ‘flanking’ expression is indeed associated with the formation of a lateral root. For this, we subjected seedlings that do not display lateral root initiation, namely grown on NPA or in the slr-1 background, to mechanical root tip bending to induce a lateral root, as was previously described (Ditengou et al., 2008). Under these experimental conditions (and 20 h after the bending), we did not observe any lateral root initiation-associated (and thus probably no lateral root formation) or ‘flanking’ RALFL34 expression in the pericycle (Fig. 5B). Since RALFL34 seems to affect the number of lateral roots along the longitudinal primary root axis, we speculated that this ‘flanking’ expression might play a role in regularly positioning these lateral roots.

A shoot-derived signal affects RALFL34 expression around the lateral root primordium

Since shoot-derived signals are involved in various steps of lateral root development (Reed et al., 1998; Bhalerao et al., 2002; McAdam et al., 2016), we explored the involvement of the shoot (as a source of auxin or another signal) in establishing the ‘flanking’ pRALFL34::n3xRFP expression in the root. Therefore, we removed the aerial tissues (hypocotyl and upward) and subjected pRALFL34::n3xRFP plants to the gravitropic bending assay (Péret et al., 2012; Lavenus et al., 2015; Voß et al., 2015) to induce the formation of a lateral root. pRALFL34::n3xRFP expression was assessed for 20 h after the removal of their aerial tissues and following mechanical root tip bending. Seedlings grown under control conditions (no aerial tissues removed) showed >70% pRALFL34::n3xRFP flanking expression after 20 h gravitimulation (Fig. 5C). In contrast, seedlings with aerial tissues removed had only 10% with pRALFL34::n3xRFP flanking expression (Fig. 5C). These data strongly suggest that
a shoot-derived signal is required for pRalfl34::n3xRFP ‘flanking’ expression.

To observe precisely the impact of removing shoot-derived signals on RALFL34-mediated lateral root formation, we utilized the gravitropic bending assay in combination with aerial tissue (hypocotyl and upward) removal on ralfl34 mutants. Without removing aerial tissues, there was no significant difference between any of the controls or mutant lines 20h post-bending, as all lateral roots progressed beyond stage 1 (Fig. 5D). However, 20h post-bending and having removed the aerial tissues, we observed a reduction in the percentage of seedlings with lateral root initiation events in ralfl34-1 (64%) and ralfl34-2 (43%), compared with the Col-0 (100%) and Ler (93%) control, respectively (Fig. 5D). In addition, we also observed a reduction in the percentage of lateral root primordia that progressed beyond stage 1 in ralfl34-1 (56%) and ralfl34-2 (0%), compared with the Col-0 (64%) and Ler (31%) control, respectively (Fig. 5D). Taken together, our results suggest that RALFL34 is required for lateral root initiation (and progression beyond this stage) and appears to interpret a shoot-derived signal that is possibly not auxin. In addition, these results and the RALFL34 ‘flanking’ expression pattern might indicate that RALFL34 is part of a lateral inhibition mechanism based on positive and negative feedback.

RALFL34 expression is negatively regulated by ERFs

To gain insight into the regulation of RALFL34 expression (and possibly the shoot-derived signal), we initially performed an in silico analysis using CORNET, which allows the integration of regulatory interaction data sets accessible through the transcription factor tool (De Bodt et al., 2010, 2012; De Bodt and Inzé, 2013). This revealed a set of 21 transcription factors potentially regulating RALFL34 expression (Fig. 6A), of which one was also differentially expressed in the pericycle during lateral root initiation (De Smet et al., 2008) and/or all those that could be checked were to some extent differentially expressed in the Arabidopsis Lateral Root initiation eFP Browser (Supplementary Table S1). Next, we performed an enhanced yeast one-hybrid (eY1H) analysis (Gaudinier et al., 2011) on two RALFL34 upstream regulatory regions of different length (869bp or 416bp; with the original idea to narrow down the regulatory region arbitrarily), with a collection of transcription factors expressed in the root stele which includes pericycle cells (Supplementary Table S1). This revealed a set of 34 transcription factors potentially regulating RALFL34 expression, of which four were also differentially expressed in the pericycle during lateral root initiation (De Smet et al., 2008) and/or all were to some extent differentially expressed in the Arabidopsis Lateral Root initiation eFP Browser (Supplementary Table S1). This list of interacting transcription factors showed limited overlap with the CORNET data, namely only AGAMOUS-LIKE 15 (AGL15) (Supplementary Table 1). AGL15 has already been characterized extensively (Perry et al., 1999; Fernandez et al., 2000, 2014; Wang et al., 2002, 2004; Harding et al., 2003; Tang and Perry, 2003; Adamczyk et al., 2007; Hill et al., 2008; Zheng et al., 2009; Patharkar and Walker, 2015), and RALFL34 was also listed in a genome-wide identification of in vivo AGL15-binding sites (Zheng et al., 2009). To determine if AGL15 is sufficient to regulate RALFL34 expression in planta, we performed experiments using a heterologous in vivo protoplast system which monitors gene expression quantitatively by activation of a luciferase (LUC) reporter (Lau et al., 2011; Vanden Bossche et al., 2013). Based on the results (data not shown), we concluded that although AGL15 can physically bind the promoter, it was not able to reproducibly regulate the expression of pRalfl34::LUC in this transient system. We therefore decided to focus on members of the ERF protein family, as this family was represented by seven out of 34 Y1H
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Fig. 5. *RALFL34* ‘flanking’ expression. (A) Representative image of *pRALFL34::n3xRFP* expression with expression in the lateral root primordium (between white arrowheads) and flanking the lateral root primordium (brown line) upon bending. (B, C) Percentage of seedlings showing *pRALFL34::n3xRFP* ‘flanking’ expression in (B) the wild type (Col-0) versus slr and mock (DMSO) versus NPA-treated seedlings upon bending and in (C) seedlings with aerial tissues removed. (D) Percentage of seedlings, with aerial tissues removed, which failed to initiate lateral roots (red), developed a stage 1 primordium (khaki), or progressed beyond stage 1 (purple), 20h post-mechanical bending.

hits (Supplementary Table 1). Next, we specifically tested an interaction between *RALFL34* expression and (the arbitrarily selected) ERF4 and ERF9 in protoplasts. This revealed a significant down-regulation of *LUC* expression (Fig. 6B), which is in agreement with ERF4 and ERF9 containing a repression domain (Licausi et al., 2013). In addition, we did an in silico search for ERF4 and ERF9 DNA-binding motifs. ERFs are known to bind directly to the *cis*-element called a GCC-box containing the core 5′-GCCGCC-3′ sequence (Ohme-Takagi and Shinshi, 1995). We could indeed find ERF4-binding motifs and ERF9 inferred binding motifs in the promoter of *RALFL34*. Interestingly, 92% of the identified motifs were found in the first 400 bp of the promoter (Fig. 6C). We further confirmed this interaction *in planta* using a transgenic *ERF9-GR* line. This revealed that in the roots, ERF9 could indeed (mildly) down-regulate *RALFL34* expression (Fig. 6E). The ERF-mediated down-regulation of *RALFL34* expression possibly links *RALFL34* expression to a regulation by ethylene. However, an analysis of available, general Arabidopsis Genevestigator and eFP Browser data did not reveal any significant differential regulation of *RALFL34* expression upon ACC (1-aminocyclopropane-1-carboxylic acid) treatment (data not shown). However, to conclude this convincingly, a more detailed and cell- or tissue-specific expression profiling would be needed to evaluate the influence of ACC on *RALFL34* expression. Interestingly, ERF4 and mainly ERF9 expression is up-regulated by auxin in the root (Fig. 6D). In addition, based on eFP Browser data, ERF4 and ERF9 appear to be ethylene inducible (Supplementary Fig. S3). In future, we will further look into this potential hormone crosstalk, but it remains possible that this ERF4– or ERF9–*RALFL34* network edge is not associated with...
ethylen- and/or lateral root development, as RALFL34 is more broadly expressed in the plant (Supplementary Fig. S2).

**RALFL34 acts upstream of GATA23**

Finally, we aimed to position RALFL34 with respect to known regulators in the cascade that leads to lateral root development (Atkinson et al., 2014; Behringer and Schwechheimer, 2015; Vermeer and Geldner, 2015). One of the earliest markers for lateral root initiation is GATA23, a transcription factor that controls founder cell identity (De Rybel et al., 2010), and we therefore analysed GATA23 expression in ralfl34-1 roots. qRT-PCR analyses on seedling roots revealed a significant down-regulation of GATA23 expression levels in ralfl34-1 compared with the control (Fig. 7A). To confirm this, we evaluated the pGATA23::NLS:GFP line (De Rybel et al., 2010) in the
ralfl34-1 background. This revealed overall less green fluorescent protein (GFP)-positive nuclei in the root (Fig. 7B, C). In addition, the location of the first GFP-positive nucleus, with respect to the QC, was higher up the root (Fig. 7D). Based on these results, we conclude that RALFL34 probably acts upstream and as a positive regulator of GATA23. However, the lateral root initiation phenotypes of ralfl34-1 (this work) and a GATA23RNAi line (fewer emerged lateral roots and fewer stage 1/2 lateral root primordia) (De Rybel et al., 2010) do not appear to match. There are potentially additional regulators of GATA23 or possibly altered expression of GATA23 in ralfl34-1 is an indirect effect, for example through a perturbed auxin balance, especially in view of GATA23 being a highly auxin-responsive gene (Supplementary Fig. S4) (De Rybel et al., 2010). Interestingly though, GATA23 expression seems also to be down-regulated upon DEX-induced ERF9 activity, suggesting that RALF34 might represent a component connecting ERF9 signalling with downstream transcriptional regulation of GATA23 (an indirect effect caused by the direct down-regulation of RALFL34) or, alternatively, ERF9 acts on GATA23 in a parallel pathway (a direct effect by the binding of ERF9 to the promoter of GATA23) (Fig. 6E).

**Conclusion**

In this work, we used a candidate gene approach [based on differential expression in our transcriptomics data (De Smet et al., 2008)] to identify molecular components of lateral root initiation and associated asymmetric cell division. Our results indicate a strong developmental role for the small signalling peptide RALFL34 during the early stages of lateral root development. RALFL34 expression is down-regulated by auxin (Fig. 8). Interestingly, upstream of RALFL34 expression are auxin-inducible and ethylene-inducible ERFs that down-regulate RALFL34 expression (Fig. 8). Previously, ethylene has been shown to affect the ability of pericycle cells to undergo lateral root initiation, probably through interfering with auxin accumulation (Ivanchenko et al., 2008; Negi et al., 2008, 2010; Lewis et al., 2011). With respect to a shoot-derived signal, our data confirm that this is required for the normal progression of lateral root development from stage 1, and which acts downstream of primary auxin responsiveness (Bhalerao et al., 2002; Marchant et al., 2002; Ditengou et al., 2008). In addition, it appears that RALFL34 is interpreting a shoot-derived signal to drive the progression from founder cell to stage 1 primordia. Concurrently, RALFL34 expression in the flanking pericycle cells induced by a shoot-derived signal might be essential to restrain cell proliferation in the neighbouring pericycle cells because ralfl34 mutants are characterized by extra cell divisions in these regions. One possibility is that this shoot-derived signal
is auxin, as indeed this hormone plays a role in regulating lateral root development (Lavenus et al., 2013). However, given that the primary effect of auxin is repression of RALFL34 expression, we believe other signals are likely to be involved. For example, in view of lateral root positioning, a carotenoid-derived molecule was already shown to play a role (Van Norman et al., 2014). In addition, it was recently shown that ERF109 expression is strongly up-regulated by methyl jasmonate (MeJA), and that ERF109 regulates lateral root development in response to MeJA (Cai et al., 2014). In this context, however, it is interesting to note that in the leaves ERF9 is transcriptionally down-regulated following short-term MeJA and salicylic acid (SA) treatment (Maruyama et al., 2013). Finally, we have shown that RALFL34 acts genetically upstream of GATA23 (Fig. 8), but it is not clear if this is a direct regulation or indirect due to perturbed auxin accumulation and/or responsiveness. However, GATA23 expression seems also to be regulated downstream of ERF9 (Fig. 8). Taken together, our data revealed an as yet unreported role for RALF peptides in lateral root initiation, and position RALFL34 as a possible earlier marker for founder cell identity than GATA23. In future, the spatio-temporal regulation of and genetic interactions within this small network will need to be investigated in detail in the root in order to establish its biological role further. In addition, what the underlying mechanism and targets of RALFL34 action are remain to be explored. In this respect, RALF peptides have been shown to be linked to Ca²⁺ release (Haruta et al., 2008, 2014; Morato do Canto et al., 2014), which—in turn—is associated with mechanical stress (Richter et al., 2009). Interestingly, mechanistical stimulation of roots causes increases in Ca²⁺ in epidermal, cortical, endodermal, and pericycle cells of roots (potentially through stretch-activated Ca²⁺ channels) (Richter et al., 2009). Furthermore, lateral root initiation also requires root bending, which can be seen as a mechanical stimulus (Laskowski et al., 2008; Kircher and Schoepfer, 2016; Scheres and Laskowski, 2016). In future, it will be important to determine to what extent Ca²⁺ levels are perturbed in ralf34 roots under normal conditions and during (mechanical) root bending compared with wild-type roots. Another explanation might come from the recently identified RALF1 receptor, namely FERONIA (FER), a member of the highly expressed malcetin receptor-like kinase family (Haruta et al., 2014). It was shown that RALF1 binds to FER, causing downstream phosphorylation on a plasma membrane-associated H⁺-ATPase (AHAR), resulting in an increased apoplastic pH and a decrease in cell wall elongation. AHAR2 (aha2) has since been shown also to cause a decrease in lateral root density, correlating with a downstream mechanism as to why 35S::RALF34 lines have decreased lateral root density (Mlodzinska et al., 2015). Overall, RALFL34 possibly affects ion balance in the pericycle (and/or surrounding tissues) to regulate cell divisions associated with lateral root initiation.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Lateral root phenotypes in ralf34-1 and ralf34-2.

Figure S2. Absolute expression value for RALFL34 in above-ground organs.

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