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RESEARCH PAPER

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 (Fukaki *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; Czyzewicz *et al.*, 2013; Delay *et al.*, 2013*b*; Tavormina *et al.*, 2015). For example, CLE-LIKE (CLEL)/GOLVEN (GLV)/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.*, 2013*a*; 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 do 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 (RALFL34) 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 *RALFL34* expression.

Materials and methods

Plant materials

We used the following lines in our research: Columbia-0 (Col-0), Landsberg *erecta* (L*er*), *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), $pRALFL34_{869bp}$*::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, dH_2O 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^{-} MES, 10 g l^{-} bacteriological 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 TGACTAACCAAAAA GTCCACG; REV primer, ACGGGACCTCTAGCTCTGAAG; and T-DNA primer, ATTTTGCCGATTTCGGAAC; and *ralfl34-2* (JIC_SGT4223) using the following genotyping primers, FWprimer, ATGGCAGCTTCGTCTCTC; REV primer, CTATCTCCGGCATCGAGT; and T-DNA primer, ACGGTCGGGAAACTAGCTCTAC.

Constructs

The *pRALFL34::LUC* was generated as follows: the 400 bp *RALFL34* promoter fragment was PCR amplified using the *RALFL34* forward (CAACTGGACCCATCCGAA) and reverse primer (CGGCGATTGTTGGGGGA) and cloned into the pGem-T-easy vector. After sequencing confirmation, the 416 bp *RAFL34* promoter fragment was released from the pGem®-T Easy vector with restriction enzymes *Pst*I and *Nco*I 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. *ERF9* and *ERF4* were cloned into *pJIT60* as follows: the *ERF9* and *ERF4* coding sequences were PCR amplified using *ERF9* (ATGGCTCCAAGACAGGCG and CTAAACGTCCACCACCGGT) and *ERF4* primers (ATGGCCAAGATGGGCTTG and TCAGGCCTGTTCCGA

TGG), and were cloned into the pGem®-T-Easy vector. The sequences of the plasmids containing either *ERF9* or *ERF4* were confirmed, and the *ERF9* and *ERF4* coding sequences were released by the restriction enzyme *Eco*RI and cloned into the pJIT60 vector (Schwechheimer *et al.*, 1998; Lau *et al.*, 2011; De Smet *et al.*, 2013). The sequences of the final constructs were confirmed. For the yeast one-hybid (Y1H) experiments, *pRALFL34* fragments were cloned as follows: 416 bp and 869 bp *RALFL34* promoter fragments were PCR amplified using *RALFL34* forward (CAACTGGACCCATCCGAA or CAGTATCAGGCTTGTGTTCA) and reverse primer (CGGCGATT GTTGGGGGA or GGCGATTGTTGGGGGAAA) and then cloned into the entry vector pENTR™ 5'-TOPO (Invitrogen). The entry clones containing the 416 bp or 869 bp *RALFL34* promoters were then recombined to yield *promoter::HIS3* and *promoter::LacZ* reporter constructs (Deplancke *et al.*, 2004; Brady *et al.*, 2011) and their sequences confirmed.

Y1H

Y1H assays were performed as previously described (Gaudinier *et al.*, 2011). Interactions were called for transcription factors that activated at least one reporter assay.

Transgenic lines

We generated *pRALFL34::n3xRFP*, using a previously published vector backbone (Slane *et al.*, 2014) and a *RALFL34* promoter fragment of 869 bp (counting from ATG). To generate the *p35S::ERF9-GR* line, the coding sequence of ERF9 without a STOP codon, a glucocorticoid domain (GR), and the constitutive 35S promoter were cloned in pDONR221, pDONRP2RP3, and pDONRP4P1R, respectively, with Gateway Cloning®. A multisite LR recombination combined the entry vectors into the destination vector pK8m34GW-FAST. The sequences of both entry vectors and the expression vector were confirmed. Both *pRALFL34::n3xRFP* and *p35S::ERF9-GR* 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 (Péret *et al.*, 2012; Lavenus *et al.*, 2015; Voβ *et al.*, 2015), as previously described. Quantification of *pGATA23::NLS:GFP* fluorescence was analysed on Col-0 (wild type) and *ralfl34-1* seedlings. Total fluorescing 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^{-} 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^{-} 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 *ralfl34-1* and *ralfl34-2* mutants was performed on a Leica DMRB microsystem 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 2 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 RNeasy 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; dH_2O, 0.8 µl per reaction] and template cDNA (diluted 1/100 in dH_2O, 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* (CTGGAGGTTTTGAGGCTGGTAT and CCAAGGGTGAAAGCAAGAAGA) and/or *AT3G 60830* (ACTCTTCCTGATGGACAGGTG and CTCAACGATT CCATGCTCCT) and/or *AT4G16100* (GGAGATTAAGC AACCTGAGGAGTG and GTGGTGGTGGTGGAGGA GAC). Primers used for qPCR: *RALFL34* (CGATGCCGG AGATAGATGAAG or TGGCAGCTTCGTCTCTCAA and CAATCCACCCCTCACGACT or TGGAGAGAAATGAAA GTGAGAAGAG), *GATA23* (AGTGAGAATGAAAGAAGA GAAGGG or GGGGACAATTAGGTGTTGCA and GTGGCT GCGAATAATATGAATACC or CTCCTTCGTTTCTTCA CCGC), *ERF4* (TTTCTCGAGCTGAGTGACCA and GTCGGAGGAGAAGCACAGTC), and *ERF9* (AGAGAGTTT CGTGGCTCCAA and CCACCGTCGTTAACCGTAGT). 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.

In silico *analyses*

CORNET analyses were done using standard settings in the TF tool on https://bioinformatics.psb.ugent.be/cornet/versions/cornet3.0/main/tf (De Bodt *et al.*, 2010, 2012; De Bodt and Inzé, 2013). We used SignalP 4.1 (Petersen *et al.*, 2011) with standard settings to predict the presence and location of a signal peptide cleavage site in RALFL34. Genevestigator (Zimmermann *et al.*, 2004, 2005; Hruz *et al.*, 2008) was probed using the standard application. For the identification of transcription factor-binding sites, we used CIS-BP (Weirauch *et al.*, 2014), and to search for the identified motif matrices in promoter sequences we used Regulatory Sequence Analysis Tools (RSAT) (Medina-Rivera *et al.*, 2015). We used Arabidopsis eFP Browser (Winter *et al.*, 2007) absolute or relative values to gain insight into expression patterns of selected genes.

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

RALFL34 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), *RALFL34* (*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). *RALFL34* 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.

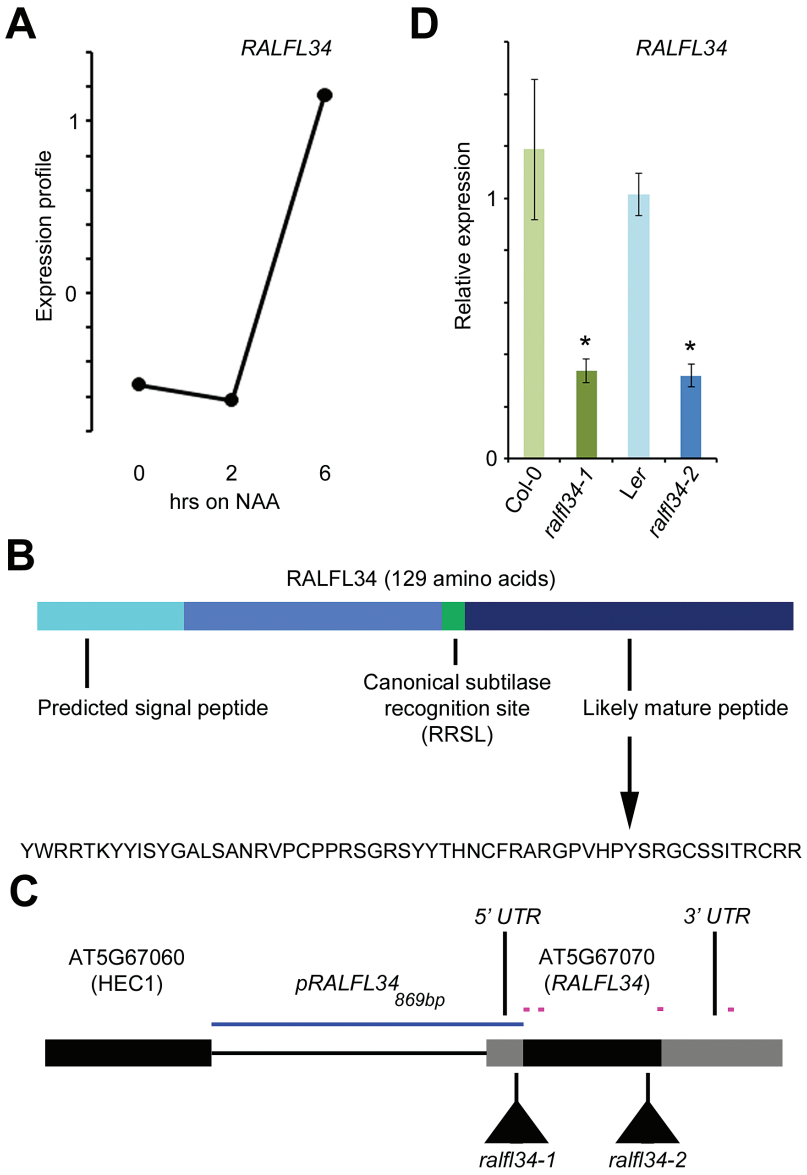

Fig. 1. *RALFL34* expression and gene/protein topology. (A) Expression profile of *RALFL34* in the pericycle of seedlings subjected to lateral root inducible system [after mixed model analysis; data from De Smet *et al.*, (2008)]. Seedlings were grown on NPA (1-*N*-naphthylphthalamic acid; 0 h) for 3 d. NAA, 1-naphthaleneacetic acid. (B) Schematic of RALFL34 protein, highlighting the putative signal peptide and likely mature peptide region and sequence. (C) Schematic of the *RALFL34* gene, with indication of qPCR primers (pink lines), position of T-DNA insertion, and promoterregion (blue line). (D) *RALFL34* 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 L*er*. 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 *ralfl34-1* (in Col-0) and *ralfl34-2* (in L*er*), with significantly reduced *RALFL34* expression (Fig. 1C,D). Both *ralfl34-1* and *ralfl34-2* were analysed with respect to overall lateral root density, lateral root stage distribution, and pericycle division patterns. Overall, *ralfl34-1* and *ralfl34-2* 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 *ralfl34-1* and *ralfl34-2* 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 *ralfl34-1* and *ralfl34-2* 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*$_{869bp}$*::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

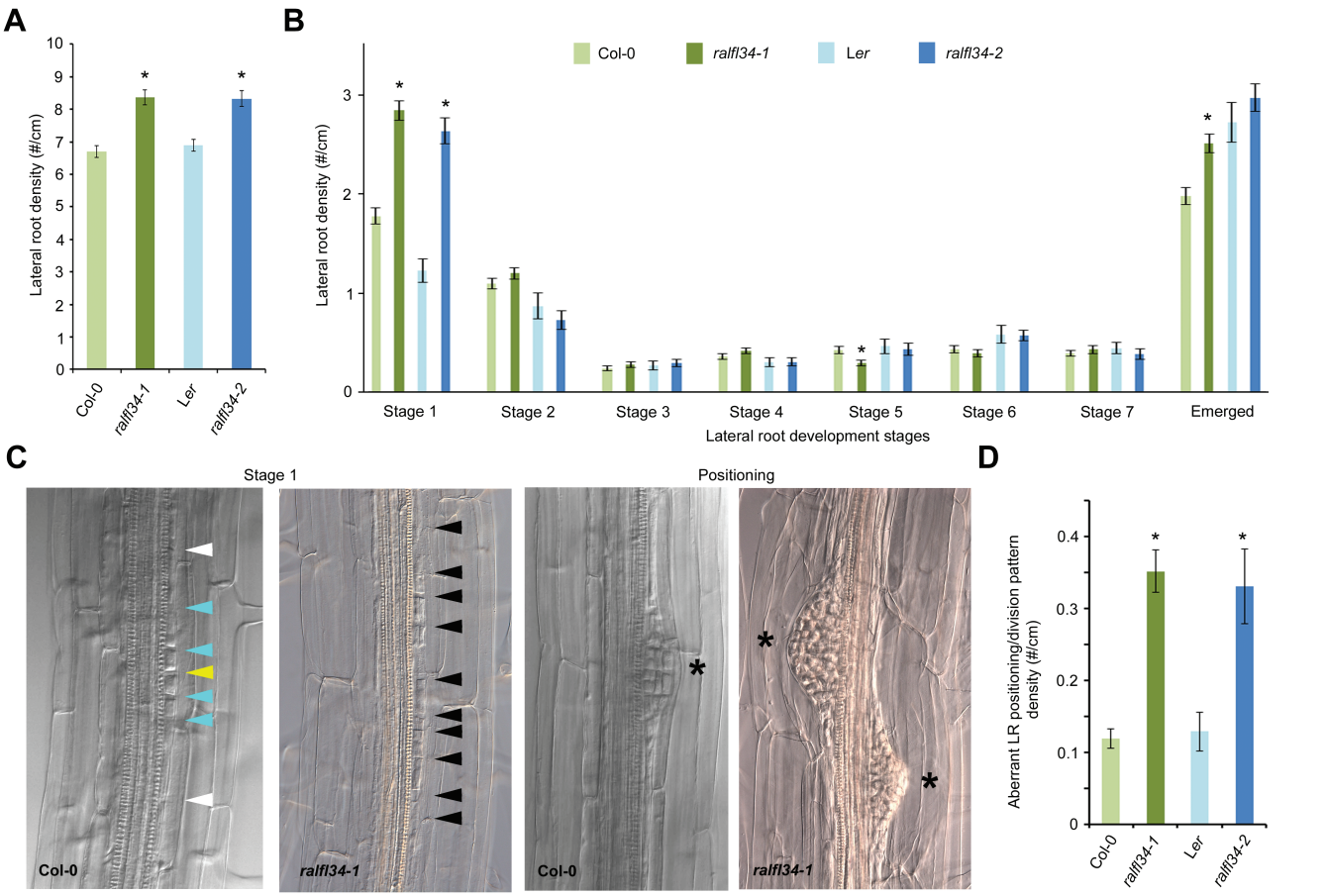

Fig. 2. Lateral root phenotypes in *ralfl34* mutants. (A, B) Lateral root density in *ralfl34-1* (*n*=69) and *ralfl34-2* (*n*=29) compared with their respective controls, Col-0 (*n*=79) and L*er* (*n*=20) depicted as total lateral root density, including all stages (A) and split into lateral root stages 1–7 and emerged lateral roots (according to Malamy and Benfey, 1997) (B) at 7 d after germination. Stage 1 includes regions with extra pericycle cell divisions. (C, D) Representative DIC pictures (C) and quantification (D) of aberrant stage 1, additional pericycle divisions, or unusually positioned lateral roots in *ralfl34-1* (*n*=69) and *ralfl34-2* (*n*=29) compared with their respective controls, Col-0 (*n*=79) and L*er* (*n*=20). An asterisk indicates a lateral root primordium. Arrowheads separate pericycle cells: central (yellow) and outer position (white) for adjacent pericycle cells undergoing two rounds of asymmetric cell divisions (blue) and extra rounds of divisions (black). All graphs show the average ±SE of the indicated sample numbers. *P<0.05 according to Student's *t*-test compared with control.

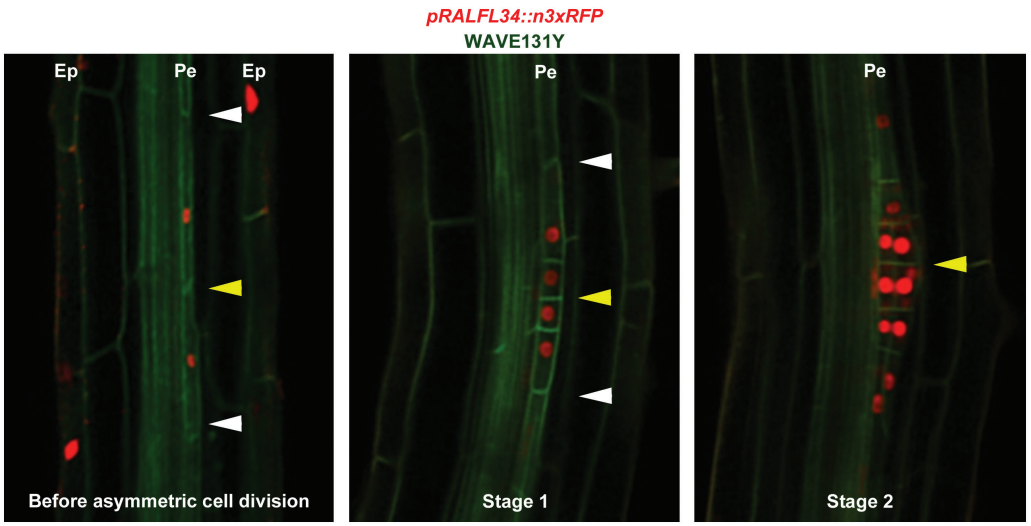

Fig. 3. *RALFL34* expression during lateral root initiation as visualized through *pRALFL34::n3xRFP* (red) and WAVE 131Y (green). Arrowheads separate pericycle cells: central (yellow) and outer position (white) for adjacent pericycle cells undergoing two rounds of asymmetric cell divisions. Lateral root development stages are indicated. Ep, epidermis. Pe, pericycle (at xylem pole).

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; Voβ *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 gravistimulation (Fig. 5C). In contrast, seedlings with aerial tissues removed had only 10% with *pRALFL34::n3xRFP* flanking expression (Fig. 5C). These data strongly suggest that

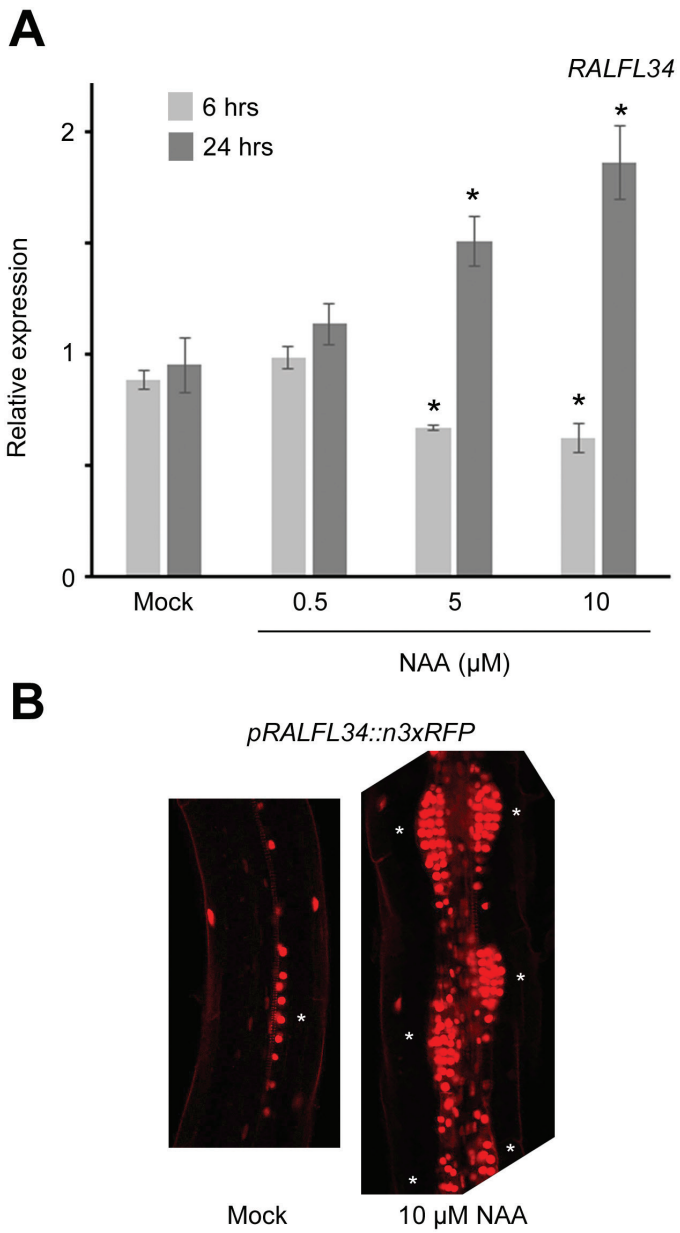

Fig. 4. Auxin-mediated regulation of *RALFL34* expression. (A) Expression of *RALFL34* after 6 h and 24 h treatment with NAA. The graph shows the average ±SE of three biological repeats. *P<0.05 according to Student's t-test compared with mock. (B) Representative images of *pRALFL34::n3xRFP* (red) expression upon 24 h of NAA treatment. An asterisk indicates the position of lateral root initiation/primordium and/or dividing pericycle.

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 20 h post-bending, as all lateral roots progressed beyond stage 1 (Fig. 5D). However, 20 h 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 L*er* (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 L*er* (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 (869 bp or 416 bp; 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 $pRALFL34_{416bp}$*::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

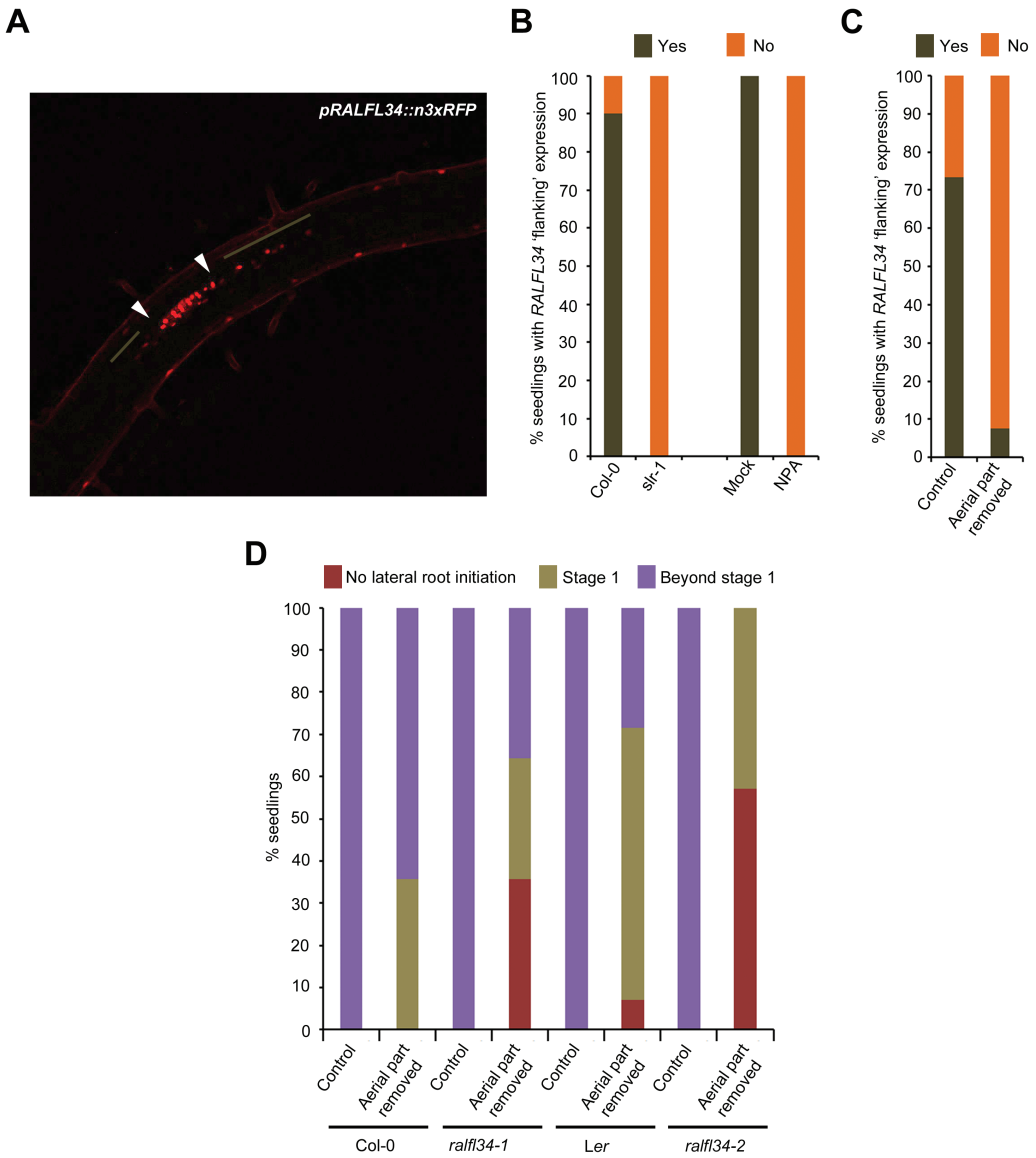

Fig. 5. *RALFL34* 'flanking' expression. (A) Representative image of *pRALFL34::n3xRFP* expression with expression in the lateral root primordium (between white arrowheads) and flanking the lateral root primordium (brown line) upon bending. (B, C) Percentage of seedlings showing *pRALFL34::n3xRFP* 'flanking' 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), 20 h 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

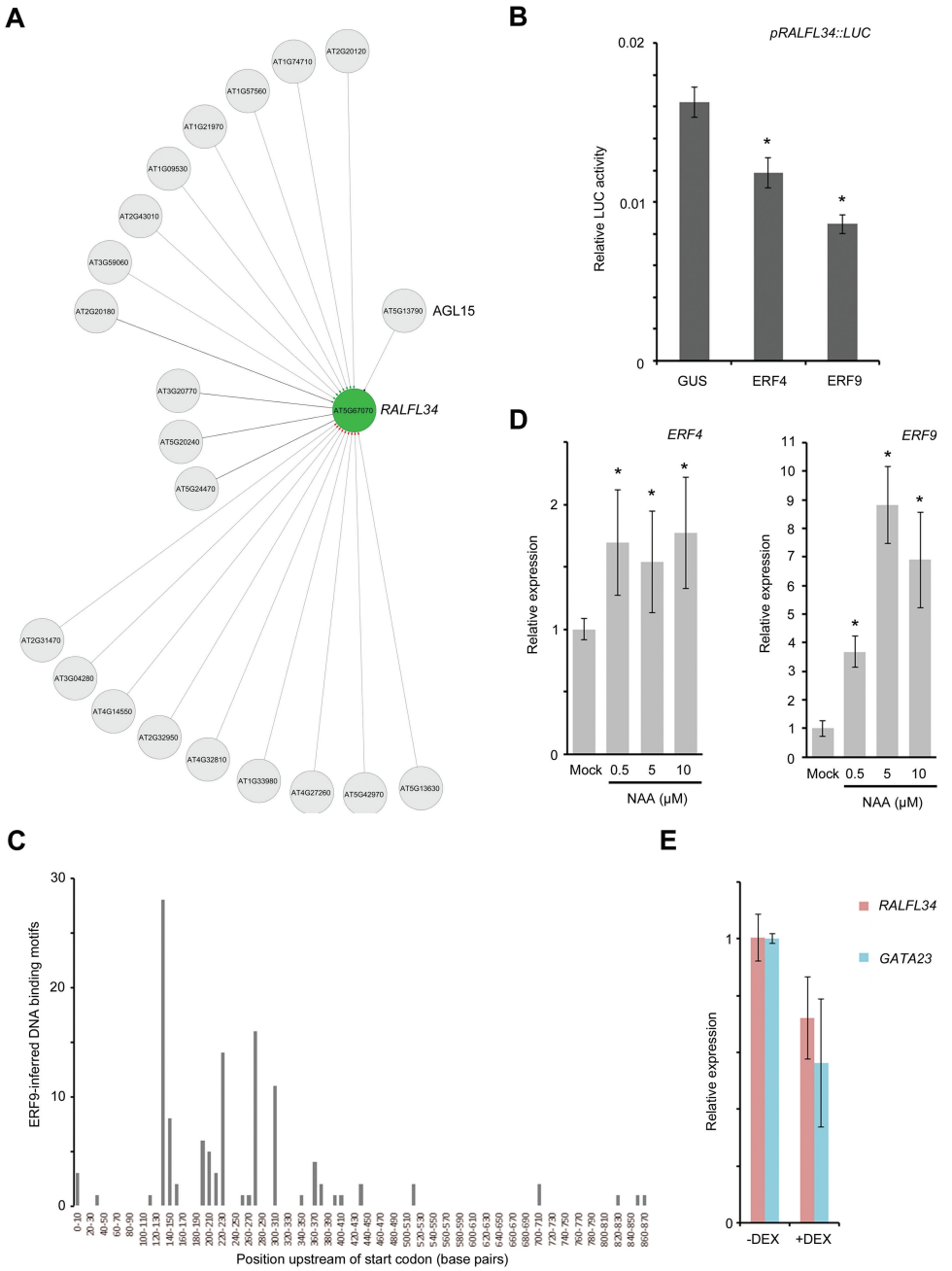

Fig. 6. ERFs regulated *RALFL34* expression. (A) *In silico* generated transcription factor network, highlighting putatively interacting proteins with *RALFL34* in CORNET. Interactions are as follows: confirmed (full line), unconfirmed (dotted line), indirect (diamond), direct+unknown (disc), direct+activation (arrowhead), direct+repression [line activation (green), repression (red), and unknown (black)]. (B) Luciferase (LUC) activity upon co-expression of pRALFL34::LUC and GUS (control) or ERF4/9 in protoplasts. Luciferase assay was performed three times, each time with eight biological replicates. The graph shows the average of all 24 data points ±SE. Student's *t*-test with *P*-value <0.01. (C) Number of ERF9-inferred DNA-binding motifs according to their position upstream of the coding sequence (start codon=position 0). (D) *ERF4* and *ERF9* expression upon 6 h of NAA treatment at the indicated concentrations. The graph shows the average ±SE error of three biological repeats. **P*<0.05 according to Student's *t*-test compared with mock. (E) Expression of *RALFL34* and *GATA23* in an inducible ERF9 overexpression line (*35S::ERF9-GR*) after 4 h of DEX-induced overexpression of *ERF9*.

ethylene 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

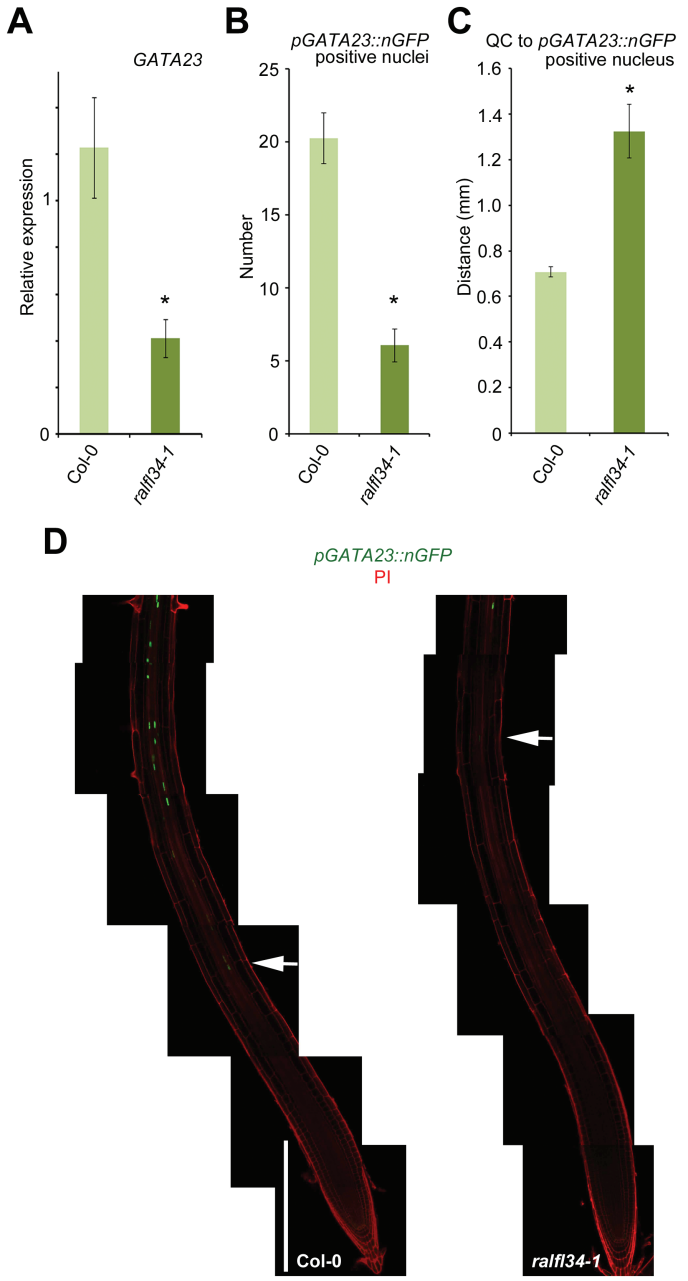

Fig. 7. *GATA23* expression downstream of RALFL34. (A) *GATA23* expression in wild-type (Col-0) and *ralfl34-1* seedling roots 5 d after germination as monitored through qPCR. The graph depicts the average of three biological repeats (and 3–6 technical repeats) ±SE. Student's *t*-test with *P*-value <0.01. (B) Representative images of *pGATA23::nGFP* expression in the wild type (Col-0) and *ralfl34-1*, white arrows indicating the first GFP expressing nucleus. White bar=300 µm. (C, D) Average number of *pGATA23::nGFP*-positive nuclei (C) and average length from the quiescent centre (QC) to the first nGFP-positive nucleus in the root tips of Col-0 and *ralfl34-1* (D). Graphs show the average ±SE of 30 seedlings. *P<0.05 according to Student's *t*-test compared with the wild type.

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 $GATA23^{RNAi}$ 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

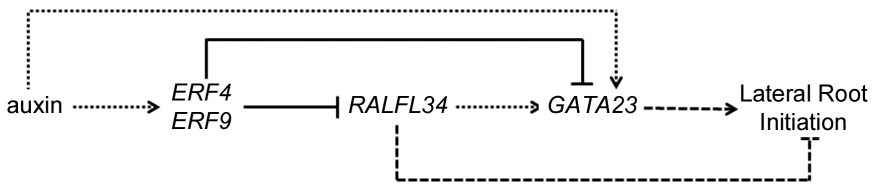

Fig. 8. Schematic representation of possible RALFL34-mediated regulation of lateral root initiation and regulation of *ERF4*, *ERF9*, *RALFL34*, and *GATA23* expression in the root. Dotted lines (possibly/likely) indirect effects; dashed lines, developmental impact; full line, (likely) direct effect.

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 Ca^{2+} 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, mechanical stimulation of roots causes increases in Ca^{2+} in epidermal, cortical, endodermal, and pericycle cells of roots (potentially through stretch-activated Ca^{2+} 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 Schopfer, 2016; Scheres and Laskowski, 2016). In future, it will be important to determine to what extent Ca^{2+} levels are perturbed in *ralfl34* 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 malectin 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 (AHA2), resulting in an increased apoplastic pH and a decrease in cell wall elongation. AHA2 (*aha2*) has since been shown also to cause a decrease in lateral root density, correlating with a downstream mechanism as to why *35S::RALF1* 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 *ralfl34-1* and *ralfl34-2*.

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

Figure S3. Relative expression values for *ERF4* and *ERF9* upon ACC treatment.

Figure S4. *GATA23* expression upon NAA treatment.

Table S1. CORNET and Y1H data.

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