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GNOM/FEWER ROOTS is Required for the Establishment of an Auxin Response Maximum for Arabidopsis Lateral Root Initiation

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Lateral root (LR) formation in vascular plants is regulated by auxin. The mechanisms of LR formation are not fully understood. Here, we have identified a novel recessive mutation in *Arabidopsis thaliana*, named *fewer roots* (*fwr*), that drastically reduces the number of LRs. Expression analyses of *DR5::GUS*, an auxin response reporter, and *pLBD16::GUS*, an LR initiation marker, suggested that *FWR* is necessary for the establishment of an auxin response maximum in LR initiation sites. We further identified that the *fwr* phenotypes are caused by a missense mutation in the *GNOM* gene, encoding an Arf-GEF (ADP ribosylation factor-GDP/GTP exchange factor), which regulates the recycling of PINs, the auxin efflux carriers. The *fwr* roots showed enhanced sensitivity to brefeldin A in a root growth inhibition assay, indicating that the *fwr* mutation reduces the Arf-GEF activity of GNOM. However, the other developmental processes except for LR formation appeared to be unaffected in the *fwr* mutant, indicating that *fwr* is a weaker allele of *gnom* compared with the other *gnom* alleles with pleiotropic phenotypes. The localization of PIN1–green fluorescent protein (GFP) appeared to be unaffected in the *fwr* roots but the levels of endogenous IAA were actually higher in the *fwr* roots than in the wild type. These results indicate that LR initiation is one of the most sensitive processes among GNOM-dependent developmental processes, strongly suggesting that GNOM is required for the establishment of the auxin response maximum for LR initiation, probably through the regulation of local and global auxin distribution in the root.

Keywords: *Arabidopsis thaliana* • Auxin • GNOM • Lateral root formation.

Abbreviations: ARF, AUXIN RESPONSE FACTOR; Arf, ADP ribosylation factor; Aux/IAA, Auxin/IAA; BAC, bacterial artificial chromosome; BFA, brefeldin A; EMS, ethyl methanesulfonate; *fwr*, *fewer roots*; GEF, GDP/GTP exchange factor; GFP, green fluorescent protein; GUS, β-glucuronidase; LC-ESI-MS/MS, liquid chromatography-electrospray ionization-tandem mass spectroscopy; *LBD16/ASL18*, *LATERAL ORGAN BOUNDARIES DOMAIN16/ASYMMETRIC LEAVES2-LIKE18*; LR, lateral root; MS, Murashige and Skoog; NAA, naphthylphthalamic acid; PIN, PIN-FORMED; RT–PCR, reverse transcription–PCR; SLR, SOLITARY-ROOT.

Introduction

Lateral root (LR) formation is one of the post-embryonic developmental processes in vascular plants, which contributes to the establishment of the root system for efficient water and nutrient uptake from the soil, to support shoot development above the ground. Developmental events in LR formation include the priming and specification of LR founder cells, LR initiation (asymmetric cell divisions of LR founder cells), LR primordium development and LR emergence (Péret et al. 2009, De Rybel et al. 2010). In most dicot plants, LRs are initiated by the asymmetric cell divisions in the pericycle cells adjacent to the xylem pole. These newly divided cells develop as an LR primordium containing the LR meristem. The LR primordium emerges from the parent root tissues as a new LR. These developmental processes of LR formation are regulated by both endogenous and environmental signals (Malamy 2005, Péret et al. 2009).

Many physiological and genetic studies have shown that LR formation is regulated by several plant hormones, mainly by auxins (Fukaki et al. 2007, Fukaki and Tasaka 2009). Auxin biosynthesis, transport and signaling are important for many aspects in plant growth and developments, as well as for LR formation. Specifically, the mutations affecting endogenous auxin biosynthesis are known to affect LR formation. For example, the *superroot1* (*sur1*)/*rooty* (*rty*)/*aberrant lateral root formation1* (*alf1*) and *sur2* mutants, which overproduce IAA, produce higher numbers of LRs (Boerjan et al. 1995,

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Celenza et al. 1995, King et al. 1995, Barlier et al. 2000), whereas the *transport inhibitor response2* (*tir2*) and *anthranilate synthase alpha subunit1* (*asa1*) mutants, which reduce IAA biosynthesis and contain less IAA, have decreased numbers of LRs (Sun et al. 2009, Yamada et al. 2009). In addition, mutants that are defective in auxin transport such as the *auxin resistant1* (*aux1*), *like-aux13* (*lax3*) and *transport inhibitor response3* (*tir3*)/*dark overexpression of CAB1* (*doc1*)/*big*/*corymbosa* (*crm1*) show the formation of fewer LRs (Ruegger et al. 1997, Casimiro et al. 2001, Gil et al. 2001, Swarup et al. 2001, Marchant et al. 2002, Paciorek et al. 2005, De Smet et al., 2007, Yamaguchi et al. 2007, Swarup et al. 2008). Furthermore, mutants that are defective in auxin signaling such as *tir1* (*transport inhibitor response1*) in the auxin receptor F-box also have reduced LR formation (Ruegger et al. 1998).

In addition to these genes, several members of the AUXIN RESPONSE FACTOR (ARF) and Auxin/IAA (Aux/IAA) protein families, that regulate auxin-responsive transcription, also regulate LR formation. ARFs directly activate or repress the transcription of their target genes that contain the auxin response elements (AuxREs) in their promoter regions. In the absence of auxin, the Aux/IAA protein interacts with its partner ARF, thereby inactivating ARF activity. In the presence of auxin, the Aux/IAA protein is degraded through ubiquitination by the $SCF^{TIR1/AFBs}$ E3 ubiqutin ligase complex, thus permitting the activated ARF to regulate the target genes positively or negatively, resulting in the ARF-dependent auxin responses (reviewed in Hayashi 2012). Gain-of-function mutations in domain II of Aux/IAAs stabilize the protein in the presence of auxin, thereby constitutively inactivating ARF activity. Gain-of-function mutants in several Aux/IAA members were shown to inhibit LR formation (Fukaki et al. 2002, Uehara et al. 2008, reviewed in Overvoorde et al. 2010). Among them, *solitary-root* (*slr*), a gain-of-function mutant in IAA14, has no LR primordium initiation sites even in the presence of auxin, indicating that stabilized mutant IAA14 protein constitutively inactivates the ARFs responsible for LR initiation (Fukaki et al. 2002, Fukaki et al. 2005). On the other hand, the *arf7 arf19* double mutant has almost no LRs, indicating that ARF7 and ARF19 positively regulate LR formation (Okushima et al. 2005, Wilmoth et al. 2005, Okushima et al. 2007). Recent molecular genetic analysis showed that the SLR/IAA14–ARF7–ARF19 auxin signaling module regulates LR initiation via the activation of several *LATERAL ORGAN BOUNDARIES DOMAIN/ASYMMETRIC LEAVES2-LIKE* (*LBD/ASL*) genes including *LBD16/ASL18* (Okushima et al. 2007, Goh et al. 2012a). These LBD/ASL proteins regulate the establishment of asymmetry of LR founder cells prior to the asymmetric cell divisions for LR initiation (Goh et al. 2012a). In addition to the SLR/IAA14–ARF7–ARF19 module, other Aux/IAA–ARF signaling modules, including IAA28–ARFs, BODENLOS (BDL)/IAA12–MONOPTEROS (MP)/ARF5 and SUPPRESSOR OF HY2/SHORT HYPOCOTYL2 (SHY2)/IAA3–ARFs, take part in regulating the developmental steps during LR formation, from specification of LR founder cells (De Rybel et al. 2010), to establishment of asymmetry of LR founder cells (De Smet et al. 2010, Goh et al. 2012a), to LR primordium development and LR emergence (Swarup et al., 2008, Goh et al. 2012b). This suggests the existence of a rather complex feedback mechanism for LR formation through multiple Aux/IAA–ARF signaling modules.

The *GNOM/EMBRYO DEFECTIVE30* (*EMB30*) encoding an ADP ribosylation factor (Arf)-GDP/GTP exchange factor (GEF), that regulates the Arf GTPase acting in vesicle trafficking, was originally reported to be required for embryogenesis (Mayer et al. 1991, Mayer et al. 1993, Shevell et al. 1994, Busch et al. 1996, Richter et al. 2010). Loss of GNOM led to improper cellular localization of PIN-FORMED1 (PIN1), an auxin efflux carrier, resulting in an embryo-lethal phenotype, indicating that GNOM plays an essential role for proper localization of PIN1 protein to the plasma membrane during embryogenesis (Steinmann et al. 1999). Partial loss-of-function *gnom* mutants, which can grow after germination, also have severe defects in auxin-regulated root and shoot development (Geldner et al. 2004). For example, $gnom^{R5}$ has dwarf shoots, agravitropic roots, arrested root meristematic activity and defects in LR primordium initiation and development, suggesting that GNOM controls LR formation through the regulation of PIN1-dependent auxin transport as well as other auxin-regulated processes (Geldner et al. 2004). However, no *gnom* mutant allele has been reported to impair specifically LR initiation, and it remains unknown how GNOM regulates LR initiation, particularly in regards to the establishment of an auxin response maximum in LR initiation sites.

In this study, we have isolated a new LR mutant, *fewer roots* (*fwr*) in *Arabidopsis thaliana*, in which LR initiation is strongly inhibited. The *fwr* mutation dramatically decreases the number of LR founder cells with an auxin response maximum for LR initiation. In addition, we provide evidence that the *fwr* LR phenotype was caused by a missense mutation in the *GNOM* gene. Our results indicate that LR initiation is one of the most sensitive processes among the GNOM-dependent developmental processes, and strongly suggest that GNOM is required for the establishment of an auxin response maximum for LR initiation, probably through the regulation of local and global auxin distribution.

Results

The *fwr* mutation decreases the frequency of LR primordium initiation sites in *Arabidopsis thaliana*

fwr was isolated from ethyl methanesulfonate (EMS)-mutagenized *A. thaliana* M_2 seedlings (**Fig. 1A**) as a single recessive mutant line that had fewer LRs. In 10-day-old seedlings, the primary root length of the *fwr* mutant was slightly shorter than that of the wild type, but the number of emerged LRs was dramatically reduced in *fwr*, compared with that of the wild type (**Fig. 1A–C**). Similarly, the total number of emerged LRs

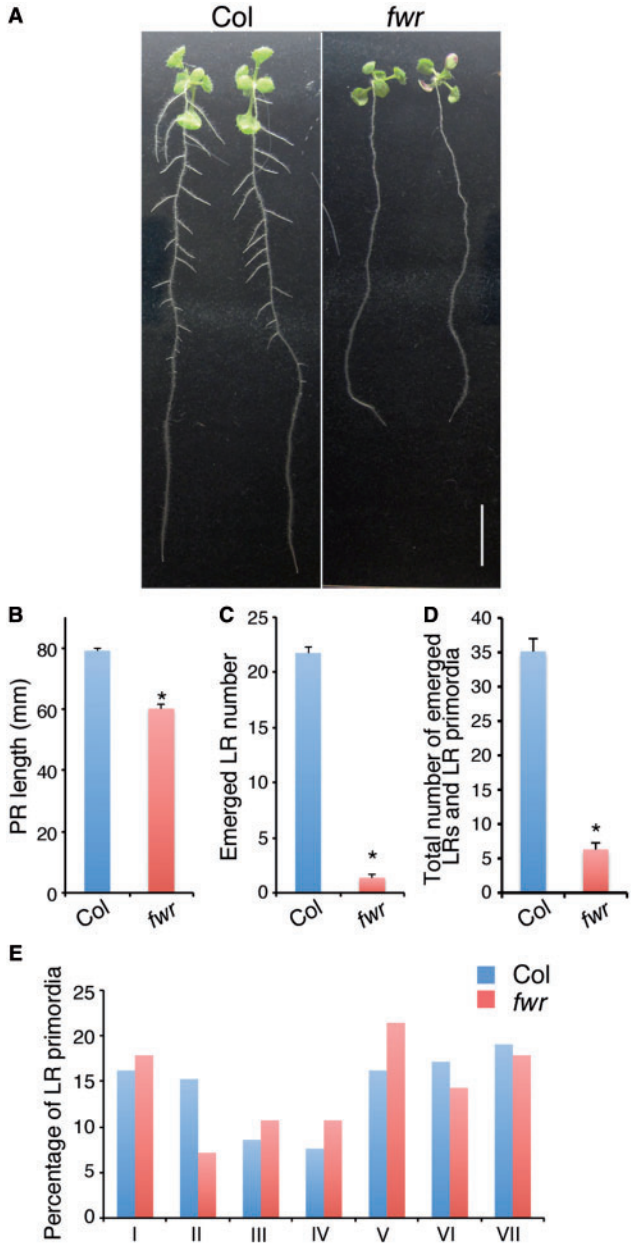

Fig. 1 Phenotype of the *fewer roots* (*fwr*) mutant plants. (A) Ten-day-old wild-type (Col) and *fwr* mutant seedlings. Scale bar = 10 mm. (B–D) Primary root (PR) length (B), emerged lateral root (LR) number (C), total number of emerged LRs and LR primordia of 10-day-old wild-type (Col) and *fwr* mutant seedlings (D). The error bars represent the SEM ($n = 25$). Asterisks indicate a statistical difference (*$P < 0.01$, Student's *t*-test). (E) Percentage of LR primordia at given developmental stages in 8-day-old wild-type (Col) and *fwr* mutant seedlings. Stage classification of LR primordia is based on Malamy and Benfey (1997). Total numbers of LR primordia are 105 in 14 Col seedlings and 28 in 31 *fwr* mutant seedlings, respectively.

plus non-emerged LR primordia of the 10-day-old seedlings was also reduced in the *fwr* primary roots, compared with that in the wild-type primary roots (**Fig. 1D**). In 15-day-old seedlings, LRs were formed in the *fwr* mutant but the number of emerged

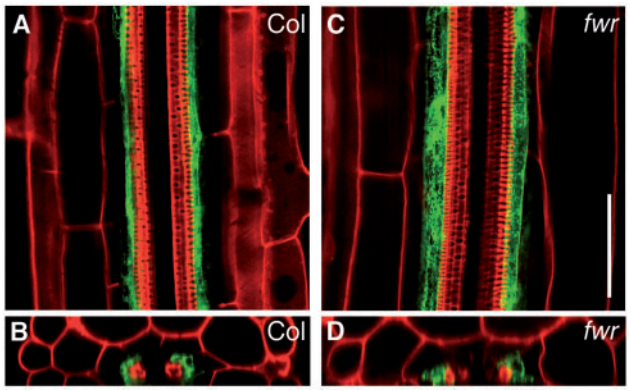

Fig. 2 Cell identity of the xylem pole pericycle in the *fwr* mutant seedling. (A–D) Expression of the J0121 enhancer trap line that expresses GFP at the xylem pole pericycle of Col (A, B) and *fwr* (C, D) seedlings. Scale bar = 50 μm.

LRs was still reduced in *fwr*, compared with that of the wild type [wild type, 63.4 ± 7.6 (mean ± SD); *fwr*, 20.1 ± 5.4 (mean ± SD), $13 < n < 17$]. These results indicate that the *fwr* mutation decreases the number of LRs while allowing limited formation of LRs. When LR primordia were formed in the *fwr*, no obvious stage-specific developmental arrests were observed except that the transition from Stage I to Stage II was slightly affected (**Fig. 1E**). In addition, the organization of the *fwr* LR primordia appeared to be the same as that of the wild type (**Supplementary Fig. S1**). These observations indicate that the *fwr* mutation specifically reduces the frequency of LR initiation but does not drastically affect LR primordium development and LR emergence.

To examine further whether the *fwr* mutation affects the identity of xylem pole pericycle from which LRs are initiated, we investigated the expression of the xylem pole pericycle-specific marker J0121 in the *fwr* mutant roots. J0121 was expressed in the xylem pole pericycle of the *fwr* mutant roots to the same levels as in the wild type (**Fig. 2**), suggesting that the identity of xylem pole pericycle is not affected in the *fwr* mutants.

In the *fwr* mutants, both root hair formation and root gravitropic response appeared to be unaffected, and leaf and flower development and embryogenesis also appeared not to be severely impaired (data not shown), although the rosette leaf shape was slightly narrow and curled downwards compared with that of the wild type (**Supplementary Fig. S2**). This indicates that the *fwr* mutation mainly affects post-embryonic root development, specifically LR formation.

The *fwr* mutation inhibits the establishment of an auxin response maximum in LR initiation sites

To investigate whether the *fwr* mutation affects the auxin response maximum in the xylem pole pericycle, which is the site of LR initiation, we examined the expression of a reporter *GUS* (β-glucuronidase) gene construct under the control of the *DR5* promoter, *DR5::GUS*, to monitor the auxin response maximum in both *fwr* and the wild type (Ulmasov et al. 1997, Benková

et al. 2003). The 5-day-old wild-type seedlings had several LR initiation sites and LR primordia marked by a strong *DR5::GUS* activity (**Fig. 3C, D**; **Supplementary Fig. S3**). In contrast, the 5-day-old *fwr* mutant seedlings had almost no LR initiation sites, and distinct *DR5::GUS* activity was not observed in the xylem pole pericycle (**Fig. 3E, F**; **Supplementary Fig. S3**). These observations suggest that the *fwr* mutation led to decreased frequency of LR initiation with the auxin response maximum at the xylem pole pericycle, thereby decreasing the number of LRs. In order to determine upon which developmental step, during the LR initiation, the *fwr* mutation exerts its inhibitory effect, we examined the expression of the *LBD16/ASL18* gene, which is the direct target of ARF7 and ARF19 for LR initiation (Okushima et al. 2007). It is reported that *LBD16/ASL18* expression occurs in the LR founder cells with an auxin response maximum (Goh et al. 2012a). In the 5-day-old seedlings, strong *pLBD16::GUS* activity was observed in the LR initiation sites at the xylem pole pericycle in the wild-type background (**Fig. 3G**; **Supplementary Fig. S4**), whereas almost no LR initiation sites with distinct *pLBD16::GUS* activity could be observed in the *fwr* seedlings, although weak GUS activity was observed along the root stele which was also shown in the wild type (**Fig. 3H**; **Supplementary Fig. S4**). This indicates that the *LBD16/ASL18* gene was not adequately activated in the xylem pole pericycle of the 5-day-old *fwr* mutant seedlings. On the other hand, when LR primordia were formed in the 10-day-old *fwr* mutant seedlings, *pLBD16::GUS* was normally expressed in the LR initiation sites and the developing LR primordia as observed in the wild type (**Supplementary Fig. S1**), although the number of LR primordia was reduced in the *fwr* mutant (data not shown). Taken together, these observations indicate that the *fwr* mutation inhibits the establishment of an auxin response maximum in LR initiation sites.

In the root tip, similar *DR5::GUS* expression patterns were detected in both *fwr* and the wild type except that GUS activity in the LR cap region tended to be absent or lower in *fwr* (16 cases /20 samples) than in the wild type (3 cases /20 samples) (**Fig. 3A, B**). This suggests that the *fwr* mutation may reduce auxin transport or signaling in the root tip region. Taken together, the *fwr* mutation affects the auxin response pattern in the root, resulting in the reduced LR phenotype.

The *fwr* mutant LR phenotype can be restored by low concentrations of auxins

Based on the *fwr* LR phenotype, we hypothesized that the *fwr* mutation might affect auxin responses. In order to investigate whether the *fwr* mutation changes the auxin responsiveness in the roots, we examined the effect of exogenous auxin on primary root growth and LR formation. When 4-day-old seedlings grown on auxin-free media were transferred onto auxin [IAA or naphthylphthalamic acid (NAA)]-containing media and incubated for an additional 3 d, the effect of exogenously applied auxin on primary root growth was found to be similar in both the *fwr* mutant and the wild type (**Fig. 4A, B**; **Supplementary Fig. S5**). In the wild type, LR formation was induced depending on the concentration of exogenous auxin, and the LR density (the number of LRs per portion of the primary root where LRs are present) increased with auxin treatment (**Fig. 4A, C, D**;

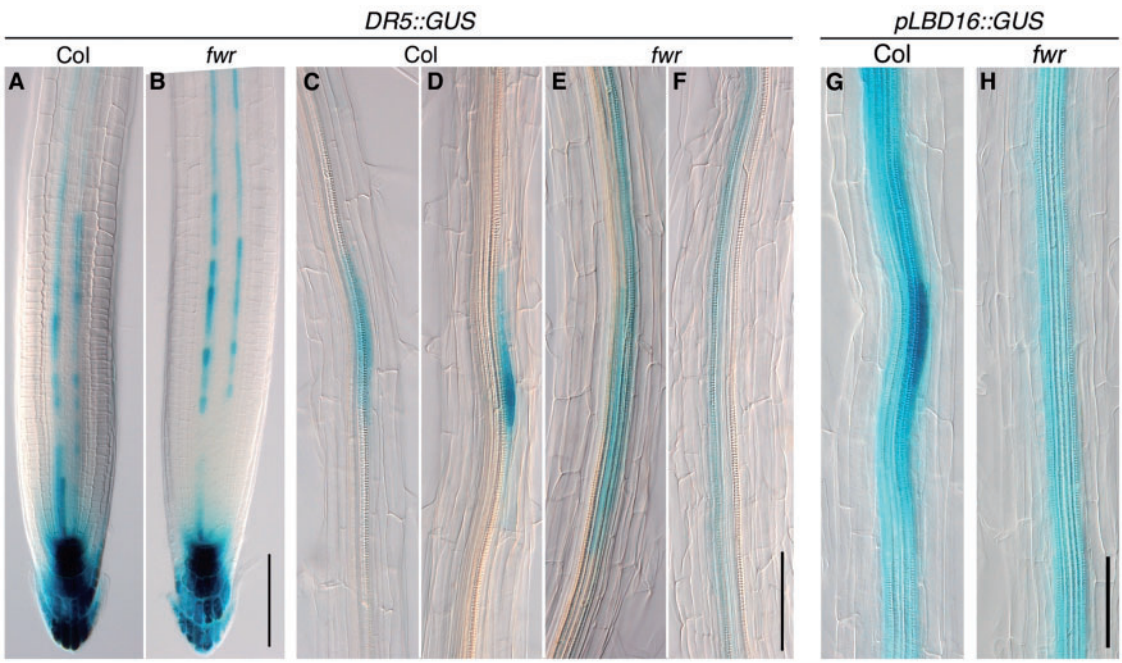

Fig. 3 The *fwr* mutation inhibits the establishment of an auxin response maximum for LR initiation. (A, B) Expression of *DR5::GUS* in the root tip of Col (A) and *fwr* (B) seedlings [5 days after germination (DAG)]. (C–F) Expression of *DR5::GUS* in the mature root region of Col (C, D) and *fwr* (E, F) seedlings (5 DAG). (G, H) Expression pattern of *pLBD16::GUS* in the Col (G) and *fwr* (H) roots. Scale bars = 100 μm.

Supplementary Fig. S5). In the *fwr* mutant, the auxin-induced LR formation was observed with either 0.03 μM IAA or 0.03 μM NAA and the LR density was restored to wild-type levels (**Fig. 4C, D**; **Supplementary Fig. S5**). Interestingly, higher concentrations of auxin (either 0.1 μM IAA, 1.0 μM IAA or 1.0 μM NAA) increased the LR density in the *fwr* mutant significantly more than in the wild type (**Fig. 4C, D**; **Supplementary Fig. S5**). These results indicate that the *fwr* mutant remains responsive to low concentrations of exogenous auxin to form LRs but shows enhanced sensitivity to higher concentrations of exogenous auxin, strongly suggesting that the *fwr* mutation may attenuate auxin distribution for LR formation.

fwr is a new weak *gnom* allele that specifically inhibits LR initiation

To isolate the *FWR* gene, detailed mapping using the F_2 generation of *fwr* (accession Columbia) and accession Landsberg *erecta* was performed. The *FWR* gene was identified within an approximately 120 kb genomic region of a bacterial artificial chromosome (BAC) clone (F7A19) of chromosome 1 (**Supplementary Fig. S6**). After sequencing the genes

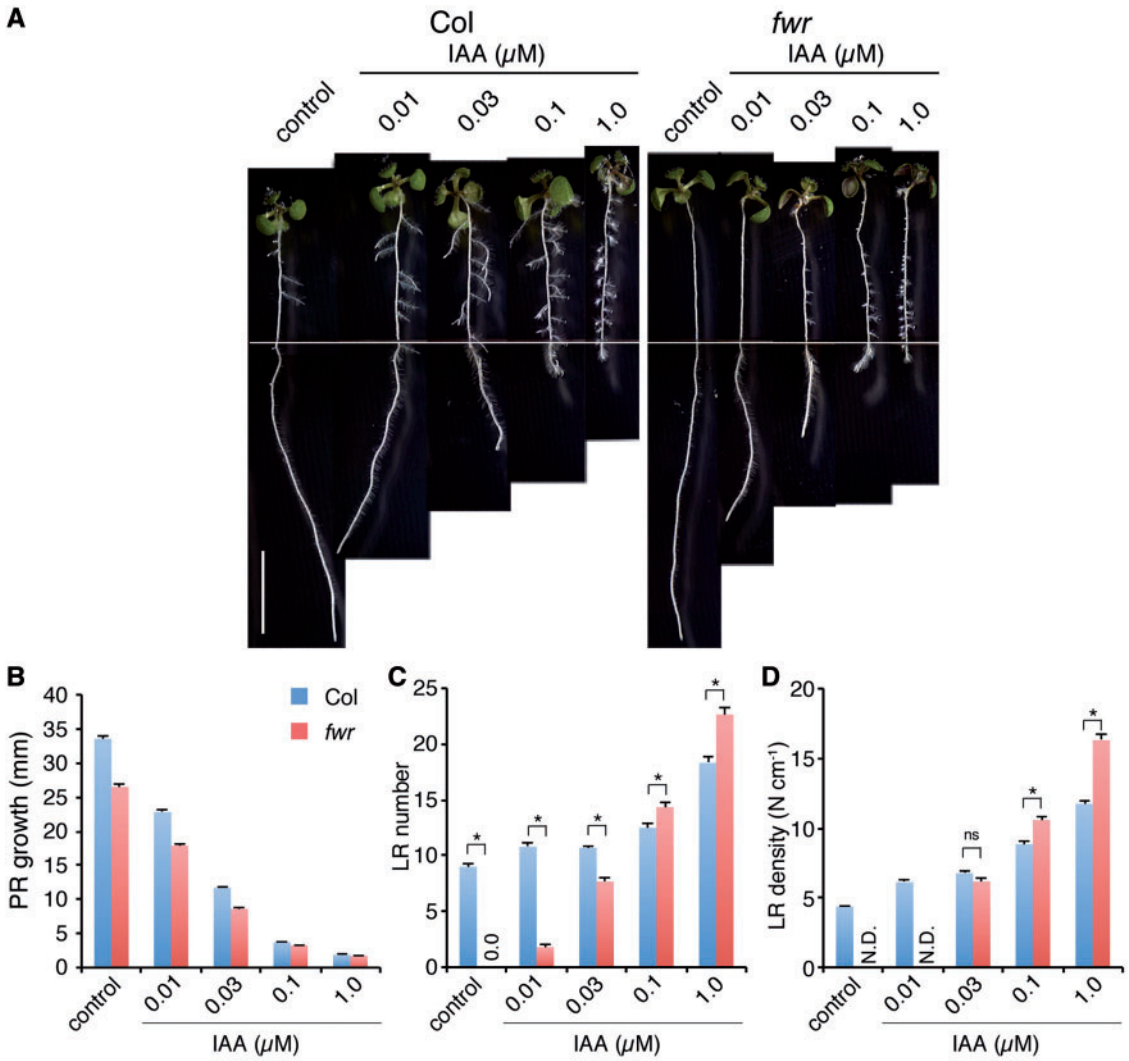

Fig. 4 Auxin inhibition of primary root growth and auxin induction of LR formation in Col and *fwr* mutant seedlings. (A) Four-day-old Col and *fwr* mutant seedlings were transferred onto IAA-free (control) or IAA-containing media, and incubated for an additional 3 d. Scale bar = 10 mm. Representative seedlings are shown for each treatment. (B) Root elongation of seedlings on IAA-containing media for 72 h. The error bars represent the SEM ($n = 25$). (C) LR number of seedlings grown on IAA-containing media for 72 h. The values for *fwr* mutants on hormone-free media (control) are 0.0 ± 0.0 (mean ± SEM). The error bars represent the SEM. The asterisks indicate a statistical difference between Col and the *fwr* mutant (*$P < 0.01$ by Student's *t*-test). ns, not significant. $n = 25$. (D) LR density of seedlings on IAA-containing media for 72 h. The values of *fwr* mutants on hormone-free (control) and 0.01 μM IAA media is not determined due to a limited number of LR primordia (N.D.). The error bars represent the SEM. The asterisks indicate a statistical difference between Col and the *fwr* mutant (*$P < 0.01$ by Student's *t*-test). ns, not significant. $n = 25$. Experiments were repeated twice, and similar results were obtained in each experiment.

annotated in this region, we found that the *fwr* mutant genome has a single base pair substitution in the *At1g13980* gene, which encodes GNOM/EMB30, an Arf-GEF that functions in vesicle trafficking and is required for embryogenesis (Mayer et al. 1991, Mayer et al. 1993, Shevell et al. 1994, Busch et al. 1996). This mutation caused a missense mutation (C to T) in the open reading frame that changes the 1,005th serine to phenyalanine in the HDS2 (Homology Downstream of SEC7 2) domain (**Fig. 5A**; **Supplementary Fig. S6**). Interestingly, this serine residue is conserved among several GNOM-related proteins, AtGNL1 and AtGNL2 in Arabidopsis, and CeGBF1 and HsGBF1 in animals (Mouratou et al. 2005) (**Fig. 5B**), suggesting the functional importance of this residue. Expression analysis by semi-quantitative reverse transcription–PCR (RT–PCR) showed that mutant *GNOM* mRNAs were expressed in the *fwr* mutant seedlings, suggesting that the mutant GNOM protein is expressed in the *fwr* mutant (**Fig. 5D**). To confirm genetically that the *fwr* is a mutant allele of the *GNOM* gene, the allelism test using a T-DNA insertion line of *GNOM*, SALK_103014, $gnom^{SALK\_103014}$ was performed. The $gnom^{SALK\_103014}$ line showed the aberrant seedling lethal phenotype in which shoot and root apical meristems were not produced, similar to that of *gnom/emb30* (**Fig. 5C**; Mayer et al. 1991, Mayer et al. 1993). All heterozygous plants of the *fwr* allele and the $gnom^{SALK\_103014}$ $gnom^{SALK\_103014}$ allele had a shorter primary root without any LRs, which is a more severe phenotype than *fwr* but a milder phenotype than $gnom^{SALK\_103014}$, indicating that *fwr* is genetically allelic to $gnom^{SALK\_103014}$ (**Fig. 5C**). In addition, we also performed a molecular complementation test by introducing the *genomicGNOM-GFP* construct into the *fwr* mutant. The *fwr* mutant plants expressing the GNOM–green fluorescent protein (GFP) fusion protein under the control of the native *GNOM* promoter (*genomicGNOM-GFP/fwr*) produced wild-type levels of LRs (**Supplementary Fig. S7**). The primary root growth was also restored in the *genomicGNOM-GFP/fwr* seedlings (**Supplementary Fig. S7**). Furthermore, we confirmed that the subcellular localization of GNOM–GFP fluorescence was observed at the specific compartments closely associated and partially overlapping with FM4-64-labeled endosomes in these transgenic root cells, consistent with previous reports on the subcellular localization of GNOM (**Supplementary Fig. S7**; Geldner et al. 2003). These results indicate that GNOM–GFP functionally rescued the *fwr* phenotype for both LR formation and primary root growth. To examine whether the *fwr* mutation affects the subcellular localization of GNOM–GFP, we also produced transgenic plants expressing the $gnom^{fwr}$–GFP fusion protein under the control of the native *GNOM* promoter. Interestingly, the subcellular localization of $gnom^{fwr}$–GFP fluorescence was mainly observed at the plasma membrane, which is different from that of GNOM–GFP (**Supplementary Fig. S7**). This suggests that the *fwr* mutation might affect the subcellular localization of GNOM. However, the $gnom^{fwr}$–GFP fusion protein was functional as well as the GNOM-GFP in the $gnom^{SALK\_103014}$ mutant background, suggesting that the

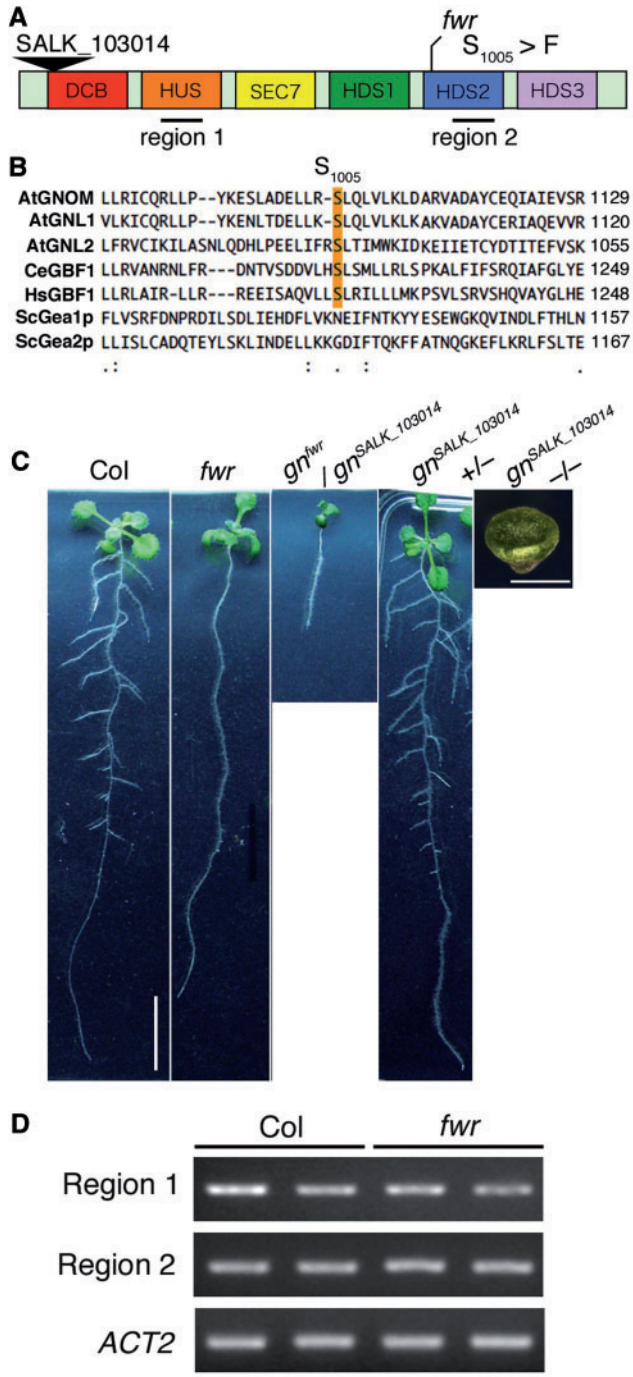

Fig. 5 The *FWR* gene encodes GNOM protein. (A) Protein structure of GNOM (GN) and mutation point of *fwr*. GN has several characteristic domains: DCB, dimerization/cyclophilin-binding domain; HUS, homology upstream of Sec7 domain; Sec7, Sec7 domain; HDS, homology downstream of Sec7 domain. *fwr* has a single nucleotide mutation in HDS2 that caused $Ser_{1,005}$Phe alteration. The black triangle represents the insertion site of T-DNA in the SALK_103014 line. (B) Amino acid sequence alignment of the flanking region of the *fwr* mutation of Gea/GNOM/GBF family members. Budding yeast Gea1p and Gea2p (ScGea1p and ScGea2p), *Caenorhabditis elegans* GBF1 (CeGBF1), human GBF1 (HsGBF1) and Arabidopsis GNL1 and GNL2. Sequences were aligned using ClutstalW. The mutated residue

(continued)

addition of GFP to the C-terminus of the gnom^{fwr} mutant protein might rescue the function (data not shown).

It is reported that several partial loss-of-function *gnom* mutant alleles affect most of the growth and developmental processes (Koizumi et al. 2000, Geldner at al. 2004, Richter et al. 2010). *gnom*^{R5}, one of the weak *gnom* alleles, has a pleiotropic phenotype including dwarf shoots, agravitropic roots, arrested root meristematic activity and defects in LR primordium initiation and development (Geldner et al. 2004). However, no *gnom* mutant allele has been reported specifically to impair LR initiation. It is noted that the embryogenesis, shoot development and fertility were not drastically affected by the *fwr* mutation. These results indicate that *fwr* is a new partial loss-of-function mutant allele of the *GNOM* gene, which mainly inhibits GNOM's function in LR initiation.

The *fwr* mutation confers hypersensitivity to BFA-mediated root growth inhibition

To examine whether the *fwr* mutation affects the Arf-GEF activity of GNOM, we examined the sensitivity of the *fwr* mutant to brefeldin A (BFA) that inhibits the Arf-GEF activity in vesicle transport. When 4-day-old seedlings grown on BFA-free media were transferred onto BFA-containing media and incubated for an additional 3 d, the primary root growth of the *fwr* mutant was more inhibited at either 1 or 5 μM BFA, compared with that of the wild type, indicating that the *fwr* mutation confers enhanced sensitivity to BFA-mediated root growth inhibition (**Fig. 6**). This result indicates that the *fwr* mutation reduces the Arf-GEF activity of GNOM, thereby confirming that *fwr* is a weak *gnom* allele.

Localization of PIN1–GFP in the *fwr* mutant roots

GNOM is known to regulate the recycling of PIN1, an auxin efflux carrier, thereby affecting the subcellular localization of PIN1 at the basal plasma membrane, which is important for several auxin-dependent growth and developmental processes (Steinmann et al. 1999, Geldner et al. 2001, Geldner et al. 2003). To determine whether the *fwr* mutation affects the localization of PIN1 in the root, we genetically crossed the PIN1–GFP line (Friml et al. 2003) into the *fwr* mutant and observed PIN1–GFP fluorescence in the *fwr* roots. As shown in **Fig. 7**, PIN1–GFP was localized at the basal membrane of the root stele cells in *fwr* as observed in the wild type (**Fig. 7A**). In the Stage I LR primordia and later stages, no distinct difference in PIN1–GFP localization was observed between the wild type and *fwr* (**Fig. 7B, C**), which suggest that at least PIN1-mediated auxin transport for LR formation is dramatically unaffected by the *fwr* mutation.

Fig. 5 Continued
(Ser$_{1,005}$Phe) in *fwr* is highlighted in orange. (C) Allelism test between the *fwr* mutant and the *GNOM* knockout T-DNA mutant (SALK_103014). Scale bars indicate 10 mm (Col) and 2 mm (*gnom*^{SALK_103014}), respectively. (D) Accumulation of *GNOM* mRNA in Col and *fwr* mutant seedling roots. The black lines in (A) indicate the amplified regions by RT–PCR. The expression of the *ACT2* gene was used as a control.

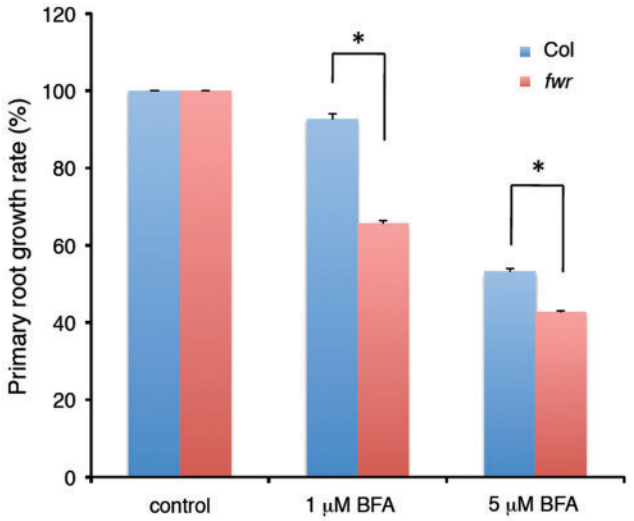

Fig. 6 BFA inhibition of primary root growth in Col and *fwr* mutant seedlings. Four-day-old seedlings grown on MS medium were transferred to medium containing different concentrations of BFA. Root growth after 3 d with each concentration of BFA was measured. For each genotype, the inhibition of root growth relative to the growth on unsupplemented medium (mock) was shown. Root increment (elongation) of mock-treated seedlings during 3 d was 25.1 ± 0.5 (mean ± SEM) mm for wild-type Col, and 22.1 ± 0.9 mm for *fwr*. The error bars represent the SEM. The asterisks indicate a statistical difference between Col and the *fwr* mutant (*$P < 0.0001$ by Student's *t* test). ns, not significant. $n = 17$. Experiments were repeated twice, and similar results were obtained in each experiment.

The *fwr* mutation increases the endogenous IAA level in roots

PIN1 is necessary for polar auxin transport from the shoots toward the roots (Okada et al. 1991). As this process is important for LR formation (Reed et al. 1998, Casimiro et al. 2001), it is possible that the *fwr* mutation impairs PIN1-dependent polar auxin transport from the shoots toward the roots, thereby inhibiting LR formation. To examine whether the *fwr* mutation in GNOM decrease the levels of endogenous IAA in the roots, we measured endogenous IAA levels in the roots of 5-day-old wild-type and *fwr* seedlings by LC-ESI-MS/MS (liquid chromatography-electrospray ionization-tandem mass spectroscopy) analysis (see the Materials and Methods). Interestingly, the level of endogenous IAA in the roots of *fwr* seedlings is much higher than that in wild-type seedlings (**Fig. 8**). These results indicate that that GNOM/FWR negatively control the endogenous IAA levels in roots and that the *fwr* mutation inhibits LR initiation without decreasing the total levels of endogenous IAA in the roots.

Discussion

In this study, we have identified the *fwr* mutant in Arabidopsis that is specifically defective in LR initiation and we demonstrated that *fwr* is a weak mutant allele of *gnom*, which revealed

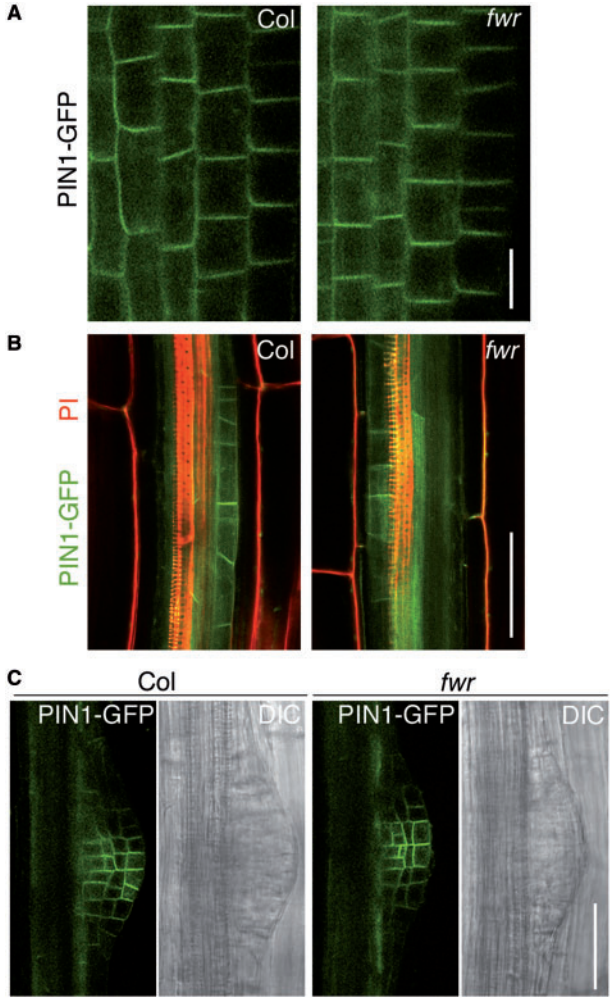

Fig. 7 Subcellular localization of PIN1–GFP in Col and *fwr* mutant seedling. (A–C) Subcellular localization of PIN1–GFP in the stele cells of the root tip (A), Stage I LR primordium (B) and Stage IV LR primordium (C). Scale bar in (A) = 10 μm. Scale bar in (B, C) = 50 μm.

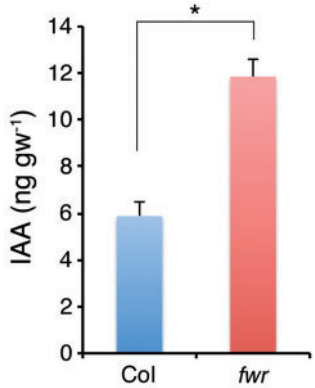

Fig. 8 IAA levels in Col and *fwr* mutant seedlings. IAA levels (ng g^{-1} FW) in the roots of the 5-day-old Col and *fwr* mutant seedlings are shown ($n = 3$). Error bars indicate the SEM. The asterisk indicates a statistical difference ($P < 0.001$ by a two-sided *t*-test). Experiments were repeated twice, and similar results were obtained in each experiment.

a more critical role for GNOM in LR initiation. Our results indicated that LR initiation is one of the most sensitive developmental processes among those regulated by GNOM, and strongly suggest that GNOM is required for the establishment of an auxin response maximum for LR initiation, probably through the regulation of local and global auxin distribution.

GNOM is required for the establishment of an auxin response maximum for LR initiation

GNOM is known to be required for embryogenesis and post-embryogenic development (Mayer et al. 1991, Mayer et al. 1993, Geldner et al. 2004, Wolters et al. 2011). The use of *gnom*^{R5}, a weak mutant allele, has shown that GNOM plays a role in the establishment of an auxin gradient in the developing LR primordia (Geldner et al. 2004). The multiple mutations among the PIN members including PIN1, PIN3, PIN4 and PIN7 also showed the seedling-lethal phenotype in which the auxin-induced LR primordium development was also altered; aberrant pericycle cell divisions occurred, indicating that PIN-dependent auxin transport is important for proper LR primordium development (Benková et al. 2003). Although the *gnom*^{R5} mutation blocks the expression of the *ARABIDOPSIS CRINKY 4* (*ACR4*) gene in the xylem pole pericycle, that is known as a marker gene for the LR initiation site (De Smet et al. 2008), it is difficult to distinguish this from the possibility that the *gnom*^{R5} LR phenotypes might be due to the secondary effects of *gnom*^{R5} primary growth arrest because *gnom*^{R5} had pleiotropic phenotypes both in the shoots and in the roots, in which the primary root growth was inhibited to 20–30% of the wild-type level along with reduced root meristematic activity (Geldner et al. 2004). In contrast, the *fwr* mutant had a strong phenotype in LR initiation but not in LR primordium development, LR emergence or other growth and developmental processes. While the *gnom*^{R5} allele exhibited aberrant LR primordium development in response to exogenous NAA at 0.1 μM, that showed multilayered dividing pericycle cells with altered PIN1 localization (Geldner et al. 2004), the *fwr* mutant did not show similar aberrant LR primordium in response to exogenous auxin treatments at 0.1 μM NAA (data not shown). These observations indicate that LR initiation is more sensitive to the activity of GNOM than to other developmental processes. Measurements of endogenous auxin and physiological analysis with exogenous auxin suggested that the *fwr* mutation might have reduced the availability of endogenous auxin for LR initiation in spite of the increased levels of IAA in the roots. This strongly suggests that the *fwr* mutation affects local and global auxin distribution for the establishment of an auxin response maximum in the LR founder cells. It is possible that GNOM mediates auxin transport from the adjacent tissues towards the LR founder cells. It will be necessary to determine which tissues/cells and which components contribute to such local and global auxin transport for LR initiation.

Our results indicate that GNOM is necessary for LR initiation, most probably for the step for the establishment of an

auxin response maximum in the LR founder cells. It was reported that GNOM regulates LR primordium development probably through the regulation of PIN1 relocalization from Stage I to Stage II (Geldner et al. 2004). As the *gnom*^{R5} seedlings had no LR initiation events in the absence of exogenous auxin and showed aberrant formation of LR primordia in the presence of exogenous auxin, it was postulated that GNOM regulates PIN1 localization for the initial anticlinal cell divisions of the pericycle toward Stage I, and also in periclinal cell divisions from Stage I to Stage II during LR formation (Geldner et al. 2004). Kleine-Vehn et al. (2008) demonstrated that PIN1 is localized at both the apical and basal sides of the divided pericycle cells at Stage I and also at the lateral side of the inner layer cells at Stage II. However, the location of PIN1 in the LR founder cells before the first anticlinal, asymmetric cell divisions of the xylem pole pericycle is unknown, because of the difficulty in locating the protein before Stage I. The role played by PIN1 before Stage I, for the specification of LR founder cells, or the establishment of an auxin response maximum in the LR founder cells, remains unknown. The *pin1* loss-of-function mutation did not alter LR density (Laskowski et al. 2008), strongly suggesting the redundant functions of PIN members in LR primordium initiation and development. It is unknown whether GNOM regulates the localization of other PIN members, thereby mediating auxin transport from the adjacent tissues toward the LR founder cells. Previous results on the *gnom*^{R5} mutant (Geldner et al. 2004) and our results on the *fwr* mutant suggest that GNOM probably regulates PIN-mediated auxin transport to establish the auxin response maximum in the LR founder cells, thereby activating auxin signaling modules to induce LR initiation, although we do not exclude the possibility that the other proteins regulated by GNOM also regulate auxin distribution for LR initiation.

The gnom^{fwr} protein acts in a dose-dependent manner and is sufficient for embryogenesis and post-embryonic shoot development

In our allelism test using a T-DNA insertion line of *GNOM*, SALK_103014, *gnom*$^{SALK\_103014}$, all F_1 plants from the cross between *fwr* and *gnom*$^{SALK\_103014}$ showed a more severe phenotype than *fwr* whereas the F_1 plants from the cross between the wild type and *gnom*$^{SALK\_103014}$ showed the wild-type phenotype (**Fig. 5C**). Although the accumulation of gnom^{fwr} mutant protein in both the *fwr/fwr* homozygote and *fwr/gnom*$^{SALK\_103014}$ heterozygote was not determined in this study, the genetic analysis suggests that the *fwr* (*gnom*^{fwr}) mutant gene acts in a dose-dependent manner in the absence of the wild-type *GNOM* gene. It is possible that the level of gnom^{fwr} mutant protein in the *fwr/fwr* homozygote was sufficient to support shoot development and primary root growth but insufficient for normal levels of LR initiation. It is also possible that the *fwr* mutation in the HDS2 domain affects the activity or stability of the GNOM protein. Further analysis of the gnom^{fwr} mutant protein will reveal the mechanism of how GNOM functions in regulating LR initiation.

The *fwr* mutant is not embryonic/seedling lethal and it produces leaves, flowers and seeds as well as the wild type, indicating that the *gnom*^{fwr} mutation does not impair the molecular function of GNOM in embryogenesis and shoot development. This suggests that GNOM-dependent vesicle transport for root development, particularly LR initiation, is more sensitive to the alteration of GNOM activity. It is unknown how the HDS domains in the GNOM protein control the activity of the protein, but our results and previous results indicate that these domains may be involved in determining GNOM activity. The *mizukussei2* (*miz2*) is another *gnom* mutant allele that specifically inhibits root hydrotropism (Miyazawa et al. 2009). The *miz2* mutation caused an amino acid substitution located in the region between the HDS1 and HDS2 domains in the C-terminal half of GNOM. Interestingly, the *miz2* mutation does not cause any other developmental defects, indicating that it specifically affects GNOM's activity in the regulation of hydrotropism (Miyazawa et al. 2009). The *miz2* mutation was also reported to have no effect on the cellular localization of PIN1 (Miyazawa et al. 2009), suggesting that GNOM regulates hydrotropism without involving PIN1. The mutant phenotypes are totally different between *miz2* and *fwr*, suggesting that the HDS domains of the GNOM C-terminal region affect specifically its function in LR initiation and root hydrotropism.

In this study, we have provided evidence showing that GNOM functions in the specific step of LR initiation, which is controlling the establishment of an auxin response maximum to activate LR initiation. It is possible that GNOM regulates local and global auxin distribution, thereby affecting auxin availability for the establishment of an auxin response maximum in the LR founder cells. It remains unknown whether GNOM regulates this step through the control of cellular localization of several PIN members, or, as in the case of the *miz2* mutant, that vesicle transport of unknown components other than PIN members may be involved in LR initiation. In *A. thaliana*, several genes that are unrelated to auxin physiology, such as *ABERRANT LATERAL ROOT FORMATION4* (*ALF4*; Didonato et al. 2004) and *REDUCED LATERAL ROOT FORMATION* (*RLF*; Ikeyama et al. 2010), have been shown to be involved in LR initiation as well. It would be of interest to examine the relationship between GNOM and these regulators and to study how LR founder cells with an auxin response maximum are established in the xylem pole pericycle. Analysis with the use of the *fwr* mutant allele could prove to be helpful to unravel the genetic and molecular mechanisms of GNOM-mediated LR formation as well as other growth and developmental processes in higher plants.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana used in this study were Columbia (Col) and Landsberg *erecta* (L*er*) accessions. The *DR5::GUS* seeds were kindly provided by Tom Guilfoyle (University of Missouri, USA).

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The *PIN1-GFP* seeds were kindly provided by Jiří Friml (Ghent University, Belgium). The *pLBD16::GUS* line was described by Okushima et al. (2007). The T-DNA insertion line (*gnom*$^{SALK\_103014}$) in the *GNOM* (SALK_103014) seeds was obtained from the Arabidopsis Biological Resource Center (ABRC). The J0121 line was obtained from the Nottingham Arabidopsis Stock Centre (NASC). The *fwr* mutant was isolated from the EMS-mutagenized M_2 Col seeds obtained from LEHLE SEEDS (http://www.arabidopsis.com/). The *fwr* mutant was backcrossed more than three times before the analysis. Seeds were surface-sterilized and plated on Murashige and Skoog (MS) medium containing 1.0% sucrose solidified with 0.5% gellan gum as described previously (Fukaki et al. 2002). Plants were grown at 23°C under continuous light. The number of LRs and root lengths were determined using a dissecting microscope and ImageJ software (NIH).

Mapping of the *FWR* gene

DNA from individual wild-type F_2 plants from a cross between *fwr* (Col) and the wild type (*Ler*) was isolated and analyzed for co-segregation with various cleaved amplified polymorphic sequence (CAPS) and simple sequence length polymorphism (SSLP) markers (Konieczny and Ausubel 1993, Bell and Ecker 1994). Primers used for mapping are listed in **Supplementary Table S1**. Polymorphisms between Col and *Ler* ecotypes were found with the use of a dCAPS (F7A19C) marker and an SSLP (F7A19) marker on chromosome 1, respectively (**Supplementary Fig. S4**). Of the 2,920 chromosomes examined, four recombination events were found between the F7A19C marker and the *fwr* mutation. Of the 3,042 chromosomes examined, two recombination events were found between F7A19 and the *fwr* mutation. These markers mapped the *fwr* mutation to within about a 120 kb genomic region in the BAC clone F7A19. The *At1g13980* gene encoding GNOM in this region was amplified from the *fwr* mutant genomic DNA with several pairs of primers based on the genomic sequence containing the *At1g13980* gene. PCR products were purified and directly sequenced by standard methods using these PCR primers and additional internal primers.

Vector construction and plant transformation

Primers used for plasmid construction of *GNOM-GFP* are listed in **Supplementary Table S2**. The genomic *GNOM-GFP* line was constructed by the technique of fluorescent tagging of full-length proteins (Tian et al. 2004). The cDNA encoding GFP was inserted in-frame immediately preceding the stop codon of the *GNOM* gene-coding sequence. The complete sequence includes 2.4 kb of the 5′- and 0.6 kb of the 3′-flanking sequences of *GNOM*. The amplified sequence was subcloned into pENTR D-TOPO (Invitrogen) and then transferred to pGWB501 (Nakagawa et al. 2007) by Gateway technology (Invitrogen). The genomic *GNOM*^{fwr}*-GFP* was produced by PCR-based site-directed mutagenesis. The transformation of Arabidopsis plants was performed via floral dipping using *Agrobacterium tumefaciens* (strain C58MP90) (Clough and Bent 1998).

RNA extraction and RT–PCR analysis

Total RNA was isolated from 5-day-old root tissues using an RNeasy Plant mini kit (Qiagen). First-strand cDNA was synthesized from 1 μg of total RNA with a ReverTra Ace qPCR RT kit (TOYOBO). Transcripts were quantified by PCR using 1/30 (*GNOM*) or 1/100 (*ACT2*) of the resulting cDNA as template, respectively. Transcripts were amplified by 30 cycles of PCR with gene-specific primers. Primers used for RT–PCR are listed in **Supplementary Table S3**.

Microscopic analysis

GUS staining, fixation and whole-mount clearing preparation of roots were performed essentially as described previously (Malamy and Benfey 1997), and samples were observed with a Leica DM6000 microscope equipped with Nomarski optics (Leica Microsystems). For confocal microscopy, roots were counterstained with propidium iodide (10 μg ml^{-1}) and analyzed with an Olympus FV1000 confocal microscope. For labeling with FM4-64, the roots were soaked in MS liquid medium plus 4 μM FM4-64 on ice for 10 min. Samples were washed twice, incubated for 15 min at 23°C and then observed under a confocal laser scanning microscope.

Measurement of endogenous IAA

Fresh plant tissues (30–40 mg) were homogenized in 80% acetonitrile/1% acetic acid/H_2O (v/v, 0.5 ml) containing [$^{13}C_6$]IAA (Cambridge Isotope Laboratories) as an internal standard by using a Tissue Lyser (Qiagen. The extract was centrifuged at 14,000 × *g* for 10 min and the supernatant was transferred to a glass tube (5 ml). The pellet was further washed with 80% acetonitrile containing 1% acetic acid (v/v) and centrifuged. The supernatants were combined and evaporated by a Speed Vac (Thermo Scientific). The extract was dissolved in 1% acetic acid and loaded in a Waters Oasis WAX extraction cartridge (1 ml). After washing with 1% acetic acid (1 ml) and 80% acetonitrile (2 ml), IAA was eluted with 80% acetonitrile/1% acetic acid/H_2O (1 ml). After evaporation by a Speed Vac, the IAA fraction was re-dissolved in 1% acetic acid (20 μl) and injected into an LC-ESI-MS/MS apparatus 6410 (Agilent) equipped with a ZORBAX Eclipse XDB-C18 column (Agilent). Endogenous IAA levels were quantified as previously described (Yoshimoto et al. 2009).

Supplementary data

Supplementary data are available at PCP online.

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