Stable establishment of cotyledon identity during embryogenesis in Arabidopsis by ANGUSTIFOLIA3 and HANABA TARANU

Mari Kanei, Gorou Horiguchi and Hirokazu Tsukaya

SUMMARY

In seed plants, the shoot apical and root apical meristems form at the apical and basal poles of the embryonic axis, and leaves form at the flanks of the shoot apical meristem. ANGUSTIFOLIA3/GRF INTERACTING FACTOR1 (AN3/GIF1) encodes a putative transcriptional co-activator involved in various aspects of shoot development, including the maintenance of shoot apical meristems, cell proliferation and expansion in leaf primordia, and adaxial/abaxial patterning of leaves. Here, we report a novel function of AN3 involved in developmental fate establishment. We characterised an an3-like mutant that was found to be an allele of hanaba taranu (han), named han-30, and examined its genetic interactions with an3. an3 han double mutants exhibited severe defects in cotyledon development such that ectopic roots were formed at the apical region of the embryo, as confirmed by pWOX5::GFP expression. Additionally, gif2 enhanced the ectopic root phenotype of an3 han. Although the auxin accumulation pattern of the embryo was correct in an3 han-30, based on DR5rev::GFP expression at the globular stage, expression of the PLETHORA1 (PLT1), a master regulator of root development, expanded from the basal embryonic region to the apical region during the same developmental stage. Furthermore, the plt1 mutation suppressed ectopic root formation in an3 han. These data suggest that establishing cotyledon identity requires both AN3 and HAN to repress ectopic root formation by repressing PLT1 expression.

KEY WORDS: Leaf development, Embryogenesis, Apical-basal patterning, ANGUSTIFOLIA3, HANABA TARANU, Arabidopsis thaliana

INTRODUCTION

All organs produced during plant development are classified into root, stem and aerial lateral organs (including leaf and floral organs). The sources of these organs are the shoot apical meristem (SAM) and root apical meristem (RAM), which are established during embryogenesis. In the post-globular stage of eudicot embryo development, cotyledons are formed at the apical end of the embryo, while SAM develops between the two cotyledons and RAM develops at the basal end of the embryo.

Organ identity is established by selecting a specific genetic program and repressing undesirable programs, typically through the action of transcription factors. Shoot and root identities are dependent on the establishment of SAM and RAM, and these meristems can be induced by overexpressing even a single transcription factor gene in Arabidopsis thaliana (hereafter, Arabidopsis). For example, ectopic SAM is induced in RAM by overexpressing WUSCHEL (WUS), a transcription factor gene that is essential for stem cell maintenance in shoots (Gallois et al., 2004). Likewise, the class III homeodomain leucine zipper (HD-ZIP III) transcription factors and AP2-domain transcription factors PLETHORA1 (PLT1) and PLT2 function as master regulators of embryonic apical fate and basal fate, respectively. When a microRNA-resistant HD-ZIP III gene is expressed in RAM, a second shoot pole is established instead of the embryonic root pole (Smith and Long, 2010). However, induction of PLT genes leads to ectopic root formation in the shoot apex (Aida et al., 2004; Galinha et al., 2007).

Preventing inappropriate fates is crucial during the developmental process. For normal embryonic root development, HD-ZIP III genes must be repressed (Grigg et al., 2009). Similarly, repression of PLT at the apical region by TOPLESS (TPL) is necessary for normal shoot development (Long et al., 2002; Long et al., 2006; Smith and Long, 2010). Establishment of the shoot-root axis is achieved by an antagonistic relationship between HD-ZIP III and PLT (Smith and Long, 2010). Repression of SAM-specific gene expression is important for normal leaf development. AS1 and AS2 suppress class I KNOTTED-LIKE HOMEBOX (KNOX) genes during leaf development. This process induces determinate growth on the leaf primordium and differentiates the leaf primordium from SAM (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Lin et al., 2003; Guo et al., 2008). Recent studies have proposed a mechanism for preventing the SAM gene expression program from being activated in the leaf primordia by YABBY genes (Kumaran et al., 2002; Sarojam et al., 2010).

Despite this progress, the mechanisms that establish fundamental leaf identities are poorly understood. There are only two studies that show homoeotic transformation of cotyledons into roots: one by the expression of either a dominant-negative version of Rab5 GTPase Ara7 or RNA interference of a Rab5-GEF gene, AvVps9a (Dhonukshe et al., 2008); and one by expressing constitutively active RopGEF7 (Chen et al., 2011). In the former case, auxin response maxima at the tips of developing cotyledons were larger in transgenic plants than those in wild type (Dhonukshe et al., 2008).

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We have previously identified several factors contributing to leaf development. ANUGSTFOLIA3 (AN3)/GRF-INTERACTING FACTOR1 (GIF1) encodes a putative transcriptional co-activator (Kim and Kende, 2004; Horiguchi et al., 2005). an3 mutations lead to narrow leaves, owing to a significant reduction in cell number (Relichova, 1976; Kim and Kende, 2004; Horiguchi et al., 2005). AN3/GIF1 interacts with several members of GROWTH REGULATING FACTOR (GRF) and promotes cell proliferation in leaf primordia (Kim and Kende, 2004; Horiguchi et al., 2005). GIF and GRF families consist of three and nine members, respectively (Kim et al., 2003; Kim and Kende, 2004; Liu et al., 2009; Rodriguez et al., 2010). GRF and GIF members have redundant functions in determining lateral organ size (Kim et al., 2003; Kim and Kende, 2004; Lee et al., 2009). In the GIF family, AN3/GIF1 plays a predominant role (Kim and Kende, 2004; Lee et al., 2009). Triple gif mutants have a more severe cell-proliferation defect in leaves and reduced floral organ number (Lee et al., 2009). Moreover, the an3 mutation in combination with miR396-mediated silencing of most GIF family members causes the loss of SAM formation (Rodriguez et al., 2010).

Further characterisation of an3 mutants revealed additional phenotypes. Compensation is an event that increases cell size in response to a reduction in leaf cell number due to a genetic defect (Tsuge et al., 1996). To observe petal epidermis cells, we examined the first three fully opened flowers on the primary meristem (n=10) by the dried-gel method, as described previously (Horiguchi et al., 2006). To determine cell size, whole leaf size and petal width, we used IMAGE J software (http://rsb.info.nih.gov/ij/). The average leaf area and cell size were estimated as previously described (Horiguchi et al., 2005). Seeds were removed from fruits and cleared with chloral hydrate solution (chloral hydrate: water: glycerol: 8:2:1) on a microscope slide. Whole leaves, flowers, seeds and seedlings were observed using a stereoscopic microscope (MZ16F; Leica Microsystems, Tokyo, Japan). Leaf palisade cells, root cells, gel casts of petal epidermal cells and cleared embryos were observed under a Nomarski differential interference contrast microscope (DM4500B; Leica Microsystems). To observe reporter gene activity in embryos, the embryos were separated from seed coats and observed using a laser scanning confocal microscope (MetaS10; Carl Zeiss, Tokyo, Japan). Representative images of homozygous an3-4 han-30 embryos were chosen from self-pollinated AN3/an3-4 han-30/han-30 or an3-4/an3-4 HAN/han-30 plants.

Microscopic observations

To measure the number and size of palisade mesophyll cells, we used the first leaves of plants 21 days after sowing (n=8). Leaves were fixed with FAA and cleared using chloral hydrate solution, as described previously (Tsuge et al., 1996). To observe petal epidermis cells, we examined the first three fully opened flowers on the primary meristem (n=10) by the dried-gel method, as described previously (Horiguchi et al., 2006). To determine cell size, whole leaf size and petal width, we used IMAGE J software (http://rsb.info.nih.gov/ij/). The average leaf area and cell size were estimated as previously described (Horiguchi et al., 2005). Seeds were removed from fruits and cleared with chloral hydrate solution (chloral hydrate: water: glycerol: 8:2:1) on a microscope slide. Whole leaves, flowers, seeds and seedlings were observed using a stereoscopic microscope (MZ16F; Leica Microsystems, Tokyo, Japan). Leaf palisade cells, root cells, gel casts of petal epidermal cells and cleared embryos were observed under a Nomarski differential interference contrast microscope (DM4500B; Leica Microsystems). To observe reporter gene activity in embryos, the embryos were separated from seed coats and observed using a laser scanning confocal microscope (MetaS10; Carl Zeiss, Tokyo, Japan). Representative images of homozygous an3-4 han-30 embryos were chosen from self-pollinated AN3/an3-4 han-30/han-30 or an3-4/an3-4 HAN/han-30 plants.

Map-based cloning

The chromosomal position of the #2047 mutation was mapped using F2 progenies derived from a cross between an3 #2047 mutants and the Landsberg erecta accession. Genomic DNA was extracted from F2 progenies that exhibited abnormal seed shapes. Polymorphic DNA makers were designed according to data available from TAIR (http://www.arabidopsis.org).

In situ hybridisation

The PLT1 and PHABULOSA (PHB) riboprobes were generated as described previously (Aida et al., 2004; Smith and Long, 2010). Digoxygenin-labelled sense and antisense RNA probes were synthesised with the DIG RNA labelling Kit (Roche Diagnostics, Tokyo, Japan) using T3 or T7 RNA polymerase (Roche Diagnostics). Embryos were fixed in 4% paraformaldehyde and 4% dimethyl sulfoxide in phosphate-buffered saline (PBS). Samples were dehydrated in an ethanol series, replaced with xylene, and embedded in Paraplast Plus (Sigma-Aldrich, Tokyo, Japan). Sections (8 μm) were placed on glass slides. The sections were fixed to glass slides by overnight incubation at 45°C. Pre-treatment was performed as described previously (Kouchi and Hata, 1993), except that protease K (1 μg/ml; Sigma-Aldrich) treatment was performed for 30 minutes at 37°C. Hybridisation was performed according to Nakayama et al. (Nakayama et al., 2010). For immunological detection, detection buffer containing NBT/BCIP (Roche Diagnostics) was added to each slide, incubated at 4°C overnight, and subsequently incubated at room temperature for at least 3 hours.

RESULTS

Isolation of mutants displaying an an3-like leaf phenotype

To search for novel factors related to AN3 function, we first isolated mutants with an an3-like leaf shape (Horiguchi et al., 2006). The first leaves of wild-type plants developed nearly round leaf blades, whereas those of an3 developed spoon-like leaves (Fig. 1A,B,E,F). Based on these phenotypic differences, we isolated only one recessive mutant with an an3-like leaves, #2047 (Fig. 1C,G). The leaf index (the ratio of leaf blade length to width) of the first leaves in #2047 was 1.38 (n=8, s.d.=0.075), which was greater than the wild-type value of 1.28 (n=8, s.d.=0.065). The leaf index of an3-4 was 1.73 (n=8, s.d.=0.060).

To examine leaf phenotype similarities between #2047 and an3 at a cellular level, we measured the number of palisade cells in the subepidermal layer (per leaf) and the size of palisade cells

MATERIALS AND METHODS

Plant materials and growth conditions

The wild-type accession used in this study was Col-0. The isolation of #2047 mutant has been reported previously (Horiguchi et al., 2006). han-1, originally isolated in the Wassilewskijia (Ws) background (Zhao et al., 2004), was back-crossed to Col-0 three times. The gif2 mutant (SAIL_32K_A03) has been reported previously (Lee et al., 2009). The T-DNA insertion mutants for PLT1 and GATA19 are SALK_116254 and SALK_138626, respectively (Alonso et al., 2003). The transgenic lines DR5rev::GFP (Friml et al., 2003) and pWOX5::GFP (Bilion et al., 2005) were crossed with an3 and #2047. Seeds were sown on Murashige and Skoog (MS) inorganic salts (Murashige and Skoog, 1962) supplemented with Gamborg’s B5 vitamins (Gamborg et al., 1968) and 2% (w/v) sucrose, and then solidified using 0.5% (w/v) gellan gum. Ten days after sowing, seedlings were transferred to rock wool (Nitobobo, Tokyo, Japan) and watered daily with 0.5 g/l Hypoxen solution (Hypoxen Japan, Osaka, Japan). For the quantitative analysis of leaves, seedlings were sown on rock wool. Plants were grown at 22°C under white fluorescent lamps (~50 μmol m−2 sec−1) with a 16-hour light/8-hour dark cycle.
using first leaves in an3-4 (a null allele of an3) (Horiguchi et al., 2005) and #2047 mutants. In an3, the reduced cell number induces enhanced cell expansion through non-cell-autonomous signalling (Horiguchi et al., 2005; Kawade et al., 2010). The cell numbers in an3-4 and #2047 were reduced to 29.5% and 49.9%, respectively, compared with wild type (Fig. 1K). In addition, palisade cell size increased in both mutants, by 75.7% and 32.9%, respectively, compared with wild type (Fig. 1H-J,L), demonstrating compensation in #2047. As a result, leaf blade area was reduced to only 54.3% and 70.9% in an3-4 and #2047, respectively, compared with wild type (Fig. 1M). These results suggest that #2047 has similar phenotypes to an3-4 mutants at the cellular and organ level.

Additionally, variation in cotyledon number was observed in #2047 seedlings with the three-cotyledon phenotype being most frequent (11.5%) (Fig. 2D,E; Table 1), which was rarely observed in an3 alleles and wild type (Fig. 2A-C). #2047 seedlings with fused cotyledons were also observed (data not shown).

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**Fig. 1. Comparison of leaf phenotypes between #2047 and an3-4 mutants, and wild type.**

(A-D) Shoots of wild type (A), an3-4 (B), #2047 (C) and an3-4 #2047 (D) grown for 21 days after sowing (DAS) (A-C) and 31 DAS (D). (E-G) First leaves of wild type (E), an3-4 (F) and #2047 (G) photographed at 21 DAS. (H-J) Paradermal view of palisade cells in the subepidermal layer in wild type (H), an3-4 (I) and #2047 (J). (K-M) The number of palisade cells in the subepidermal layer (K), the cell area of palisade cells (L) and leaf area (M). The first leaves from 21 DAS plants were analysed. Data are expressed as the mean ± s.d. Scale bars: 1 cm in A-D; 5 mm in E-G; 100 μm in H-J.

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**Fig. 2. Genetic interactions between an3 and #2047.**

(A-I) The 9 DAS seedlings of wild type (A), an3-4 (B), an3-2 (C), #2047 (D), #2047 with a tricot phenotype (E) and an3-4 #2047 (F,G), as well as 12 DAS an3-2 #2047 seedlings (H) and 5 DAS an3-4 han-1 seedlings (I). The arrow in G indicates a trichome produced on the protrusion of an3-4 #2047. (J) Gross morphology of wild type, an3-4, han-30 and an3-4 han-30 during the reproductive stage. All plants shown (except for the rightmost plant) are at 32 DAS. The rightmost one is an3-4 han-30 at 44 DAS. (K) Flowers of wild type, an3-4, han-30 and an3-4 han-30. (L-N) Petal width (L), the number of cells in the transverse direction in petal epidermis (M) and the cell area of petal epidermis (N). Data are expressed as the mean ± s.d. Scale bars: 1 mm in A-F,H,I,K; 100 μm in G; 1 cm in J.
Control of cotyledon identity

We analysed the genetic interaction between mutation play a similar role in leaf development. To explore this

an3-4 han-1

Double mutants exhibited more

#2047

The 10-23th flowers

Reproductive phenotypes of an3 han-30

The an3 han double mutant seedlings were not viable when sown on rock wool, but could be rescued when they are grown in vitro and then transferred to rock wool. Using this method, an3 han-30 developed many leaves and produced flowers (Fig. 1D). The pollen produced by an3 han-30 flowers was fertile (data not shown) and the flowers of an3 han-30 did not set seeds (Fig. 2J,K). Petal and stamen numbers decreased in han-30, as reported in other han

mutants (Table 2; Zhao et al., 2004). Organ number did not differ between an3 and wild type (Table 2). The misregulation of sepal and petal numbers in han-30 was influenced by the an3 mutation; a significant increase in sepal number and a decrease in petal number were observed in the double mutant (Table 2). By contrast, the lower number of stamens in han-30 was partially restored in an3 han-30 (Table 2). The an3 mutation did affect regulation of floral organ number in the han-30 mutant, but the effect seemed to be different in each whorl.

We also examined the interaction between an3 and han-30 in terms of post-embryonic lateral organ development. An appropriate comparison of leaves in different mutants was difficult because an3 han-30 had many more leaves than wild-type plants at the same developmental stage, probably owing to the occurrence of multiple shoots, and the size and number of cells in a leaf progressively changes according to node position (Usami et al., 2009). We instead examined petals, which are modified leaf organs. The widths of an3-4, han-30 and an3-4 han-30 petals were reduced to 71.6%, 85.2% and 52.0% compared with wild-type petals, respectively (Fig. 2L). Correspondingly, the number of adaxial epidermal cells along the transverse axis of an3-4, han-30 and an3-4 han-30 petals was reduced to 69.0%, 81.0% and 43.9% compared with wild-type petals, respectively (Fig. 2M). The petal cell number in an3-4 han-30 was smaller than expected if we assume additive effects of the two mutations. The 43.9% reduction in cell number was less than the expected value of 55.9% (0.69 × 0.81). These results indicate that a synergistic interaction between AN3 and HAN regulates cell proliferation in petals. Compensation occurred in the petals of all mutants examined, as seen in the leaves (Fig. 2M,N).

The an3 han seedling generates ectopic roots in the apical region

Next, we characterised developmental defects in late embryogenesis and post-germinative development in an3 han-30. The an3-4 han-30 seeds were irregularly cone-shaped (Fig. 3A,B), which was due to a defect in cotyledon development and subsequent failure of cotyledon bending (Fig. 3C,D). an3-4 seeds were small and had pale orange seed coats (supplementary material Fig. S2A,C), whereas han-30 seeds were not significantly different from wild-type seeds (supplementary material Fig. S2B,D).

Surprisingly, some of the double mutant seedlings that germinated in vitro developed root-like protrusions in the apical region, in addition to the malformed leaf-like protrusions (Fig. 3F-

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<th>Stamens</th>
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The 10-23th flowers (n=50) on the primary meristem were scored. Fused organs were counted separately.
suggesting that with the strong phenotype (supplementary material Table S1), triple homozygous mutants were found only in seedlings. AN3/AN3 genotypes are indistinguishable. Nevertheless, were homozygous for han-30 (Fig. 3J) or strong (Fig. 3M) phenotype. All seedlings these abnormal seedlings with a moderate (like 3J,K). We carried out genotyping of 12 individuals from each of seedlings (Fig. 4). We identified the primary defect of the double mutants have root identity, and a subset of leaf-like protrusions might have a mixed root/leaf identity.

To identify the primary defect of the double mutants, we used the pWOX5::GFP reporter as a quiescent centre (QC) marker (Bilou et al., 2005). A GFP signal was observed only in the QC of primary roots of wild type, an3-4 and han-30 (Fig. 4D; supplementary material Fig. S2J,L). In an3-4 han-30, a GFP signal was observed at both the tip of the primary roots and the tip of root-like protrusions (Fig. 4E,F). In addition, a GFP signal was detectable as a spot in the intermediate type protrusions (Fig. 4G). We found that eight out of 30 an3-4 han-30 seedlings had leaf-like protrusions with GFP signals. Leaf-like protrusions that had no GFP signal were also identified (six out of 30) (Fig. 4H). These results suggest that the root-like protrusions of the double mutants have root identity, and a subset of leaf-like protrusions might have a mixed root/leaf identity.

**Development of an3 han-30 embryos**

To identify the primary defect of the an3-4 han-30 double mutant, we analysed embryo development in AN3/an3-4 han-30/han-30 plants and compared them with wild type, an3-4 and han-30 single-mutant embryos. Most embryos from AN3/an3-4 han-30/han-30 plants did not exhibit aberrant cell division during early embryogenesis, but some embryos showed inappropriate cell division directions starting at the eight-cell stage (data not shown). Aberrant cell division was seen in only 9% of 16-cell stage embryos from AN3/an3-4 han-30/han-30 plants (Table 4), similar to the frequency in the han-30 single mutant (7.1%, Table 4), suggesting that there was no enhancement of aberrant cell division at the 16-cell stage by an3-4 on han-30. A lens-shaped cell that is the progenitor of QC divided normally in all genotypes.

As G1F family members have redundant functions in leaf development (Lee et al., 2009), we examined the effect of the gif2 mutation on the an3 han ectopic root phenotype. gif2 never produced ectopic roots on the aerial parts (Fig. 3L). Strikingly, a subset of seedlings of the F2 progeny between an3 han and gif2 showed even stronger transformation of cotyledons into roots (Fig. 3M) compared with that in an3 han seedlings (Fig. 3J,K). We carried out genotyping of 12 individuals from each of these abnormal seedlings with a moderate (like an3-4 han-30 shown in Fig. 3I) or strong (Fig. 3M) phenotype. All seedlings were homozygous for han-30 (supplementary material Table S1). Because an3-4 is a deletion allele and its chromosomal breakpoint is unknown (Horiguchi et al., 2005), an3-4/AN3 and AN3/AN3 genotypes are indistinguishable. Nevertheless, gif2 an3 han triple homozygous mutants were found only in seedlings with the strong phenotype (supplementary material Table S1), suggesting that gif2 enhanced the an3 han ectopic root phenotype. We also investigated whether GATA19 has a redundant role with HAN, and found that the gata19 mutation did not significantly affect the an3 han phenotype (data not shown).

To determine whether intermediate and leaf-like protrusions also had some root-like features, we used the pWOX5::GFP reporter as a quiescent centre (QC) marker (Bilou et al., 2005). A GFP signal was observed only in the QC of primary roots of wild type, an3-4 and han-30 (Fig. 4D; supplementary material Fig. S2J,L). In an3-4 han-30, a GFP signal was observed at both the tip of the primary roots and the tip of root-like protrusions (Fig. 4E,F). In addition, a GFP signal was detectable as a spot in the intermediate type protrusions (Fig. 4G). We found that eight out of 30 an3-4 han-30 seedlings had leaf-like protrusions with GFP signals. Leaf-like protrusions that had no GFP signal were also identified (six out of 30) (Fig. 4H). These results suggest that the root-like protrusions of the double mutants have root identity, and a subset of leaf-like protrusions might have a mixed root/leaf identity.
Owing to the delayed primordia formation, some primordia in the apical region (Fig. 5B; supplementary material mutants showed retardation during development of organ heart stage, wherein between mutants and wild-type embryos was observed at the (Fig. 5; supplementary material Fig. S2Q,R). A clear difference Control of cotyledon identity (an3-4 han-30 structures in Fig. 4. Root-specific features observed in ectopic root-like (Fig. 4L; 87.5%, n=10), in oval-shaped embryos from an3-4/an3-4 HAN/han-30 (Fig. 4L; 87.5%, n=16) and in some an3-4 embryos (supplementary material Fig. S2K; 31.8%, n=22), but was always restricted to the basal part of the embryo. Similarly, no pWOX5::GFP expression was observed in the apical region in the torpedo stage in any of the mutants examined (Fig. 4I,M; supplementary material Fig. S2L,O). In mature embryos, in which organ primordia containing root-like protrusions were morphologically evident, ectopic pWOX5::GFP expression was detected in the apical part of the an3-4 han-30 embryo (Fig. 4N) but not in wild type, an3-4 or han-30 (Fig. 4K; supplementary material Fig. S2M,P) embryos. This result indicates that ectopic root identity in an3-4 han-30 is obtained during embryogenesis, but ectopic induction of pWOX5::GFP is not a primary cause of ectopic root formation.

To determine when the ectopic root identity was established in the apical region of an3 han embryos, we again applied the pWOX5::GFP reporter. Even in the heart stage, during which morphological defects were observed in han-30 and an3-4 han-30, pWOX5::GFP was expressed only at the basal end of the embryo (Fig. 4I,L; supplementary material Fig. S2K,N). The area of WOX5 expression was slightly expanded in han-30 (supplementary material Fig. S2N; 60.0%, n=10), in oval-shaped embryos from an3-4/an3-4 HAN/han-30 (Fig. 4L; 87.5%, n=16) and in some an3-4 embryos (supplementary material Fig. S2K; 31.8%, n=22), but was always restricted to the basal part of the embryo. Similarly, no pWOX5::GFP expression was observed in the apical region in the torpedo stage in any of the mutants examined (Fig. 4I,M; supplementary material Fig. S2L,O). In mature embryos, in which organ primordia containing root-like protrusions were morphologically evident, ectopic pWOX5::GFP expression was detected in the apical part of the an3-4 han-30 embryo (Fig. 4N) but not in wild type, an3-4 or han-30 (Fig. 4K; supplementary material Fig. S2M,P) embryos. This result indicates that ectopic root identity in an3-4 han-30 is obtained during embryogenesis, but ectopic induction of pWOX5::GFP is not a primary cause of ectopic root formation.

To clarify whether the an3 han double mutant embryo has shoot identity in the apical region, we examined the expression pattern of the class III HD-ZIP gene PHB (Fig. 6). PHB mRNA was expressed in provascular cells and adaxial sides of cotyledon primordia and prospective SAM in all samples examined, as reported previously (McConnell et al., 2001). No difference in expression pattern was observed among wild-type, an3-4 and han-30 embryos (Fig. 6A-C). PHB mRNA was also detected in the apical region of a han-30 oval-shaped embryo (data not shown). This expression in the apical region was detected in all embryos from AN3/an3-4 han-30/han-30 that we examined (Fig. 6D-F, n=33), indicating that PHB mRNA accumulated in the apical region of the an3-4 han-30 double mutant. This result suggests that the an3 han-30 embryos retain shoot and cotyledon identities in their apical tissues.

**The auxin accumulation pattern is not disrupted in an3 han-30**

There are several reasons why ectopic roots form in the double mutant embryo. One possible explanation is that the defect in auxin accumulation leads to a disturbance of axial patterning. We used the DR5rev::GFP reporter to monitor the auxin response maxima in the embryos. During the globular stage in all genotypes
examined, the GFP signal was visualised in the basal region, and no ectopic signal was observed in the apical region (Fig. 7, Table 5), suggesting that the apical-basal patterning was initiated relatively normally in an3-4 han-30 at the globular stage as long as auxin response pattern concerns. At the heart stage, a more broadly distributed GFP signal was seen in the basal region of the han-30 and an3-4 han-30 embryos than in the wild-type and an3-4 embryos, but was never detected in the apical region of any oval-shaped embryos of han-30 or the putative an3-4 han-30 (Fig. 7, Table 5). As cotyledon primordia develop during the late heart stage, the GFP signal was detected at the tip of the cotyledon primordia in wild type, an3-4 and han-30 (Fig. 7A-C). At this stage, an3-4 han-30 embryos had a broad GFP signal in the apical region (Fig. 7D).

**Misexpression of PLT1 and its effect in an3-4 han-30**

To determine the earliest molecular phenotypes in an3-4 han-30 embryos, we examined the expression pattern of PLT1 by in situ hybridisation. At the globular stage, PLT1 was expressed in the provascular cells and in the lens-shaped QC progenitor cells of all genotypes examined (Fig. 8A,D,G,J), as reported by Aida et al. (Aida et al., 2004). We did not observe any differences in PLT1 expression in the an3-4 embryos (Fig. 8D-F). However, in han-30, a slightly broader pattern of PLT1 expression was observed compared with wild-type samples (Fig. 8A,G), but it normalised during the heart stage (Fig. 8H,I compared with 8B,C). In most of the globular-stage putative an3-4 han-30 embryos, the PLT1 expression domain extended into the upper tiers (66.7%, n=15), whereas PLTI was never expressed there throughout embryogenesis in wild-type samples (Fig. 8A,J). This ectopic PLT1 expression continued during subsequent developmental stages (Fig. 8B,K), then was gradually restricted to each primordium in the apical region (Fig. 8L,N). PLTI was not expressed in developing cotyledons in wild type (Fig. 8M). In summary, ectopic PLT1 expression occurred earlier than the changes in apical auxin response, the induction of pWOX5::GUS misexpression and the patterning defect during embryogenesis.

We generated an an3-4 han-30 plt1 triple mutant to evaluate the effect of PLT1 misexpression in an3 han. The plt1 mutation suppressed the ectopic root phenotype seen in an3 han (Fig. 8O,P). This suppression occurred in eight of 15 plt1 heterozygous seedlings and in all of 60 plt1 homozygous seedlings. The multiple shoot formation is also mostly suppressed. The narrow-leaf phenotype and sterility of an3 han-30 did not recover by the plt1 mutation (data not shown).

**AN3 expression pattern in the embryo and the absence of a direct physical interaction between AN3 and HAN**

HAN expression is observed broadly in early embryos until the globular stage and is gradually restricted to provascular tissues during subsequent stages of embryogenesis (Zhao et al., 2004; Nawy et al., 2010). We observed AN3 expression during embryogenesis using pAN3::GUS to understand how HAN and AN3 act to regulate embryogenesis. AN3 was expressed in the basal half of globular-stage embryos but was absent in epidermal cells (supplementary material Fig. S3A). AN3 was broadly expressed during the heart stage but not in the tips of the cotyledon primordia or in epidermal cells (supplementary material Fig. S3B). At later stages, AN3 expression was observed in the basal region of cotyledon primordia, SAM, RAM and some provascular cells (supplementary material Fig. S3C,D). These expression patterns overlapped with those of HAN (Zhao et al., 2004; Nawy et al., 2010).

We next examined whether HAN and AN3 interact with each other using yeast two-hybrid assays (supplementary material Fig. S4). Neither wild-type HAN nor a mutant version of HAN (han-30) interacted with AN3. We also tested the interaction between HAN and GRF5, but no interaction was found.

**DISCUSSION**

In this study, we found that an3/gif1 and han have phenotypic similarities in several aspects of leaf and floral organ development. Mutations in either gene inhibited cell proliferation in leaves, induced compensation and produced spoon-shaped leaves and a variable number of floral organs. han seedlings often have an altered number of cotyledons. This phenotype is also observed in gif1 gif2 gif3 triple mutants that are defective in the interacting partners of AN3/GIF1 (Kim et al., 2003). More importantly, an3 and han mutations genetically interact with each other and cause ectopic root formation in the apical region of the embryo where cotyledons normally develop. This phenotype is further enhanced by the gif2 mutation. PLT1 is ectopically expressed in the apical region of the an3 han embryo, and plt1 suppresses the ectopic root phenotype. This suggests that the HAN and GIF family, represented by AN3, are cooperatively involved in regulating PLT1 expression, which is required for establishing leaf identity during embryogenesis.

Here, we discuss the causes of ectopic root formation in an3 han. First, during embryogenesis, a disturbance in the auxin response pattern could cause disruption of the apical-basal axis,
leading to ectopic root formation. Polar auxin transport affects the expression of some genes, along with the apical-basal axis and other contributing developmental events (Blilou et al., 2005). However, our data do not support this possibility. Maximum DR5 activity was observed only in the basal region of the an3-4 han-30 embryo in the globular stage, indicating that the relatively normal auxin response and apical-basal axis that requires an appropriate distribution of auxin are established once in an3-4 han-30. By contrast, Dhonukshe et al. (Dhonukshe et al., 2008) reported that increased auxin response in cotyledons stimulates the initiation of roots at cotyledon tips. In wild-type heart-stage embryos, polar auxin transport generates strong auxin maxima at the root pole and also creates weaker maxima at the cotyledon tips. Broad auxin accumulation in primordia of an3-4 han-30 embryos was observed during the late heart stage. However, this accumulation occurred after patterning defects, such as retardation of organ primordia development and ectopic PLT1 expression, which do not require auxin accumulation (Aida et al., 2004). Therefore, we consider this auxin response to be a secondary effect of altered embryonic development or the result of primordia formation. The final possible explanation for the formation of ectopic roots is the ectopic expression of a master regulator of root development. Ectopic expression of PLT1 is sufficient to induce ectopic root formation (Aida et al., 2004; Galinha et al., 2007). Consistent with this finding, PLT1 mRNA accumulated ectopically in the apical region of the an3-4 han-30 embryo. PLTs are auxin-inducible genes (Aida et al., 2004), but DR5rev::GFP expression was normal when ectopic expression of PLT1 was observed in early heart-stage embryos of an3-4 han-30. This result indicates that the ectopic expression of PLT1 in an3-4 han-30 was not induced by auxin. Additionally, the mutation in the PLT1 gene fully suppressed the ectopic root phenotype seen in an3 han. Therefore, ectopic expression of PLT1 is sufficient to account for ectopic root formation in an3-4 han-30 embryos.

Table 5. DR5rev::GFP expression pattern in embryos

<table>
<thead>
<tr>
<th>Parental genotype</th>
<th>Stage</th>
<th>Region of GFP signal</th>
<th>n (oval shaped)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Basal</td>
<td>Basal and apical*</td>
</tr>
<tr>
<td>Wild type</td>
<td>Globar</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>an3-4/an3-4</td>
<td>Globar</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>han-30/han-30</td>
<td>Globar</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>an3-4/an3-4 HAN/han-30</td>
<td>Globar</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>64</td>
<td>0</td>
</tr>
</tbody>
</table>

*Signals in the tip of the cotyledons were excluded.
The *han-30* mutant phenotype is similar to those of other *han* mutant alleles in terms of floral organ numbers (this study; Zhao et al., 2004), but there is a difference in the auxin response distribution pattern. The DR5rev::GFP signal shifted apically from the hypophysis during the globular stage in other *han* alleles, probably owing to misregulation of PIN1 and PIN7 (Nawy et al., 2010). However, in this study no significant changes were observed in either *han-30* or *an3-4 han-30* embryos. As *han-30* is probably a very weak allele that carries a missense mutation at a conserved region among *HAN*-like genes, it may not interfere with normal auxin distribution in developing embryos. *han-30* seems to have a similar or sufficient effect to the *han-1*-null allele in the *an3* background for ectopic root formation. We also found that the *gif2* mutation enhanced ectopic root formation in the *an3 han-30* background. Based on these findings, we propose that the *HAN* and *GIF* family, represented by *AN3*, coordinately repress *PLT1* expression in the apical region of embryos without auxin involvement, and repress ectopic root formation during embryogenesis. Genetic interaction analysis using a weak *han* allele and an *an3* mutant revealed that *HAN* could regulate *PLT1* expression, in addition to its previously reported role in regulating auxin distribution.

How *AN3* and *HAN* regulate *PLT1* expression is a key question to understand their roles in establishing cotyledon identity. *an3 han* double mutants induced severe defects in embryogenesis, which were not observed in either single mutant. It is interesting that the *AN3* and *HAN* expression domains partially overlapped during the globular stage (supplementary material Fig. S3A) (Zhao et al., 2004). However, *HAN* did not interact with *AN3* or an *AN3* interaction partner, GRF5, in yeast two-hybrid assays. These data suggest that *AN3/GRF5* and *HAN* function in parallel pathways and may have downstream targets that regulate *PLT1* expression through which cotyledon identity is stably established. *TPL* acts as a transcriptional co-repressor and negatively regulates *PLT* expression (Szemenyei et al., 2008; Smith and Long, 2010). As *AN3* is a putative transcriptional co-activator, *AN3* may positively regulate the expression of a negative regulator of *PLT1* expression. However, the role of *HAN* in transcriptional regulation is unknown. Some GATA members seem to be involved in both transcriptional activation and repression (Luo et al., 2010; Richter et al., 2010; Hudson et al., 2011). Further study is needed to understand the molecular mechanisms of *AN3*- and *HAN*-dependent repression of *PLT1* and whether *TPL* is involved in such mechanisms.

A second scenario is that *AN3* and/or *HAN* may positively regulate the genes required to stably establish cotyledon identity. We found that *PHB* was normally expressed at the adaxial side of abnormal organ primordia of the *an3-4 han-30* embryo. This suggests that *AN3* and *HAN* are not essential for deciding the fate of cotyledon development. As the *an3 gif2* triple mutant transformed the cotyledon into a root nearly completely, *AN3*, *GIF2* and *HAN* would play a role in stably establishing cotyledon identity in embryos. Their gradual loss of function might weaken cotyledon identity and then leak the root developmental program in the apical region of the mutant embryos. Thus, organ primordia in the apical region of the mutant embryos undergo both cotyledon and root developmental programs. This may explain why some protrusions of *an3 han* seedlings showed mixed root/cotyledon identity and delayed *WOX5* misexpression compared with *PLT1*. However, the mixed root/cotyledon identities seemed to be inconsistent with the finding concerning the mutual repression between root and shoot identity in apical meristems (Smith and Long, 2010). We believe that the ectopic root identity induced in *an3 han* is insufficient to suppress shoot identity completely or that *PHB* expression in cotyledons is not regulated by the same mechanism operating in the SAM. The molecular mechanisms that repress ectopic root formation via *AN3* and *HAN* should be addressed in future studies.

*AN3* is emerging as a key player in leaf development, as it promotes cell proliferation and adaxial identity, and coordinates cell proliferation and cell expansion (Kim and Kende, 2004; Horiguchi et al., 2005; Ferjani et al., 2007; Fujikura et al., 2007; Kawade et al., 2010; Horiguchi et al., 2011). In this study, we
demonstrate that both AN3 and HAN are essential to repress root fate in cotyledon primordia during embryogenesis. Thus, AN3 is an important factor that integrates multiple processes throughout leaf development.

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Competing interests statement
The authors declare no competing financial interests.

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