Auxin Response Factors Mediate Arabidopsis Organ Asymmetry via Modulation of KANADI Activity

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Members of the KANADI gene family in Arabidopsis thaliana regulate abaxial identity and laminar growth of lateral organs. Promoter APETALA3-mediated ectopic expression of KANADI restricts petal expansion and was used in a genetic screen for factors involved in KANADI-mediated signaling. Through this screen, mutations in ETTIN (ETT; also known as Auxin Response Factor3 [ARF3]) were isolated as second site suppressors and found to ameliorate ectopic KANADI activity throughout the plant as well. Mutant phenotypes of ett are restricted to flowers; however, double mutants with a closely related gene ARF4 exhibit transformation of abaxial tissues into adaxial ones in all aerial parts, resembling mutations in KANADI. Accordingly, the common RNA expression domain of both ARFs was found to be on the abaxial side of all lateral organs. Truncated, negatively acting gene products of strong ett alleles map to an ARF-specific, N-terminal domain of ETT. Such gene products strongly enhance abaxial tissue loss only when ARF activities are compromised. As KANADI is not required for either ETT or ARF4 transcription, and their overexpression cannot rescue kanadi mutants, cooperative activity is implied. ARF proteins are pivotal in mediating auxin responses; thus, we present a model linking transient local auxin gradients and gradual partitioning of lateral organs along the abaxial/adaxial axis.

INTRODUCTION

Formation of lateral organs, such as leaves and floral organs at the flanks of shoot and floral apices, involves the specification of a group of initial cells patterned distinctively relative to the meristem. Organ primordium cells adjacent to the meristem will develop adaxial characteristics and give rise to the upper layers, while cells away from the meristem will have abaxial characteristics and give rise to the lower organ layers. The physical continuum of the primordial adaxial domain with the meristem is also manifested in the expression and role of the PHABULOSA (PHB)-like class III HD-ZIP proteins in apical meristem establishment and maintenance as well as adaxial organ identity (McConnell and Barton, 1998; Emery et al., 2003; Prigge et al., 2005). Elaboration of the lateral organs into flat, laterally expanding organs requires concerted exclusion of the PHB-like activities from the abaxial domain and of the abaxial factors from the adaxial domain. The abaxial repression of the PHB-like adaxial factors is mediated by activities of the GARP-type transcription factors, KANADIIs, and by the activities of a group of microRNAs (miR165/6; Eshed et al., 2001, 2004; McConnell et al., 2001; Emery et al., 2003; Juarez et al., 2004; Kidner and Martienssen, 2004; Mallory et al., 2004; McHale and Koning, 2004). Whether the activities of the KANADI proteins and miR165/6 are connected is presently unknown. RNA in situ analyses suggest that initial restriction of the adaxial factors takes place between the onset of primordium initiation (P0) and outgrowth (P1) reflected by abaxial exclusion of PHB and REV (Eshed et al., 2001; Kerstetter et al., 2001; McConnell et al., 2001). Moreover, the codominant nature of the phb-1d and phv-1d mutants suggests a quantitative process that involves a gradual interplay of gene activities during organ primordium partitioning.

A surgical approach to mapping patterning events in lateral primordia has been performed by excision or laser separation of emerging leaf primordia from the shoot apex (Sussex, 1954; Snow and Snow, 1959; Reinhardt et al., 2005). Such surgical separation results in polarity-disrupted, radial leaves that fail to expand laterally. The most dramatic effects on subsequent morphology were achieved by separation of leaf primordia either at the P0 or P1 stage (depending on the report). Later separation of P2 primordia does not result in alterations of abaxial/adaxial patterning. These studies suggest that a central component of asymmetric pattern establishment derives from a morphogenic gradient either entering or leaving young primordia, providing graded positional information. That immediately affected leaves are formed following separation supports the idea that partitioning of the primordia occurs in a graded manner over the 48- to 72-h interval between P0 and P1 of tomato (Solanum lycopersicon) leaf primordia (Reinhardt et al., 2005).

Numerous lines of evidence support a central role of positional information in pattern formation in plants. However, only a few studied developmental modules have identified direct involvement of extracellular or mobile signals directing pattern formation (Nakajima et al., 2001; Lenhard and Laux, 2003). The small, soluble, organic molecule auxin appears to be used repeatedly in...
patterning and facilitation of axes throughout the plant life cycle. Auxin pools are present at high levels in many plant organs, but most of the hormone is in conjugated forms with no or limited morphogenetic function (Aloni et al., 2003). A small fraction of this pool, free auxin, is rapidly mobilized between cells and tissues by a group of influx and efflux carriers. As a consequence, steep local gradients of endogenous free auxin concentrations are created that can provide morphogenetic cues. Indeed, localized free auxin gradients were demonstrated to pattern the early apical basal embryo axis (Friml et al., 2003), root meristem zonation (Sabatini et al., 1999), and shoot and root lateral primordium initiation (Benkova et al., 2003; Reinhardt et al., 2003; Billou et al., 2005).

Repeated use of the same morphogenetic signaling molecule in early stages of embryo and organ primordium patterning complicates studies of the role of auxin during later stages of development and obscures distinctions between direct functions and developmental epistasis. A primary cellular response to elevated free auxin levels is the fast breakdown of the auxin/indole-3-acetic acid (AUX/IAA) proteins. These short-lived nuclear molecules appear to function as transcriptional repressors via dimerization with Auxin Response Factor (ARF) proteins that bind to promoters of auxin response genes containing auxin-responsive promoter elements (AuxREs). Such repression of primary/early auxin response genes is thought to occur following AUX/IAA dimerization with ARFs via protein–protein interaction domains (termed domains III and IV) present both in AUX/IAA and ARFs and through transcriptional repression via the AUX/IAA repression domain (domain I; recently reviewed in Woodward and Bartel, 2005). The ETTIN (ETT; also known as ARF3) and ARF17 proteins provide a somewhat enigmatic deviation from the canonical structure of ARFs, as they lack the conserved domains III and IV for interaction with AUX/IAA proteins (Hagen and Guilfoyle, 2002). Nevertheless, a microRNA-resistant version of ARF17 was recently shown to modulate the expression of early auxin response genes (Mallory et al., 2005). Furthermore, ETT is capable of binding AuxRE DNA motifs in vitro (Ulmasov et al., 1999) and differentially suppresses a synthetic AuxRE-based reporter in protoplasts in an auxin-dependent manner (Tiwari et al., 2003).

Surprisingly, only few overt developmental phenotypes have been reported from single mutants in individual ARF genes, suggesting extensive functional overlap among various family members (Okushima et al., 2005). The only ARF protein associated with organ asymmetry disruptions is ETT. The phenotype of ett plants shows altered floral organ numbers and exhibits disruptions in proximal/distal and abaxial/adaxial axes during gynoecium development (Sessions and Zambryski, 1995; Sessions, 1997; Sessions et al., 1997).

In a screen for factors mediating the role of KANADI in establishing abaxial organ identity, we isolated second site suppressors of KANADI1 misexpression. We describe here two functionally redundant genes, ETT and ARF4, which act to promote abaxial identity in conjunction with KANADI or its downstream targets. Activities of additional ARFs may be required for asymmetry establishment as manifested by an apparent competitive inhibition generated by truncated ETT gene products. We propose that lateral organ abaxial/adaxial domain separation is gradually refined via differential activation of ARFs through interpretations of auxin gradients.

RESULTS

Ectopic KANADI Activity Is Suppressed in an ett Mutant Background

Ectopic expression of either KANADI1 (KAN1) or KAN2 throughout leaf primordia resulted in the transformation of adaxial cell types into abaxial ones and the abolition of lateral expansion (Eshed et al., 2001, 2004). To identify factors linking KAN activities with abaxial identity and initiation of laminar expansion, a facile system for screening was established by restricting KAN expression to the flowers. When KAN1 was expressed throughout petals using the promoter of the floral B class gene APETALA3 (AP3;KAN1), various phenotypes were observed, ranging from plants with missing petals and rudimentary radialized stamens to plants with normal, fertile stamens with radialized petals (Figures 1A to 1C). Seeds from fertile AP3;KAN1 plants were mutagenized with ethyl methanesulfonate, and M2 plants were screened for second site suppressors of the radialized petal phenotype. Putative suppressors were tested for transgene activity by backcrossing, and genuine suppressors were identified. In all, four complementation groups of ectopic KAN suppressors were isolated, and the first one identified was analyzed.

Compared with AP3;KAN1 plants, plants homozygous for the suppressor mutation had expanded but narrower wild-type–like petals as well as a malformed gynoecium with reduced valves and basally expanded style tissue (Figure 1D). When examined in an otherwise wild-type background, these mutants had normal leaves and flowers resembling those of ett mutants described by Sessions and Zambryski (1995). An allelism test with ett-1 confirmed that the suppressor is a new allele (ett-11) of ETT.

The expression pattern of ETT and its mutant phenotype analyses suggested a role for ETT in patterning abaxial-adaxial and apical-basal axes of the Arabidopsis thaliana gynoecium (Sessions et al., 1997). However, ETT is also expressed in all other organs of the flower where such a developmental role was not phenotypically described. Therefore, we examined the morphology of epidermal cells of ett-11 and ett-1 organs. In the wild type, the abaxial epidermal cells of petals are relatively cubic and the adaxial ones are conical, whereas the radial abaxialized AP3;KAN1 petal cells are cubic all around (Figures 1E to 1G). Conversely, petal epidermal cells of both sides of both ett-1 and ett-11 are conical or partially conical, suggesting a partial loss of abaxial identity in these organs (Figure 1H for ett-11; data not shown for ett-1). No new polarity defects were detected in other ett-11 and ett-1 organs, suggesting that suppression of misexpressed KAN can be restricted to the AP3 domain. That possibility was tested by construction of ectopic expression of KAN using the flower meristem and A class gene promoter AP1 and the carpel-specific promoter CRABS CLAW. In these cases as well, suppression of ectopic KAN action was obtained in an ett-1 mutant background (see Supplemental Figure 1 online).

The relationship between ETT and KAN was further examined by construction of the triple mutant kan1-2 kan2-1 ett-1. The
Figure 1. ETT Mediates Ectopic KAN Activity.

Arabidopsis wild-type petals are laterally expanded and composed of epidermal cells that exhibit distinct abaxial/adaxial morphology (A). Ectopic expression of KAN1 using the floral-specific AP3 promoter results in radial petals (B) and (C). AP3::KAN1 ett-11 plants have laterally expanded petals despite ectopic KAN activity (D). In the wild type, abaxial petal cells are relatively cubic with wavy cuticular ridges, while adaxial ones are conical with undulated cuticular ridges (E) and (F). Distal epidermal petal cells of AP3::KAN1 are cubic all around (G), while the distal abaxial epidermal petal cells of ett-11 are intermediate between abaxial and adaxial wild-type petal cells (H). (I) shows a comparison of abaxial 7th leaf surface of wild-type (a) and kan1-2 kan2-1/+- (b), ett-1 kan1-2 kan2-1/+- (c), and kan1-2 kan2-1 double mutants (d). Novel phenotype of small blade outgrowths appears on the abaxial leaf surface of the ett-1 kan1-2 kan2-1/+- leaves, which resemble kan1-2 kan2-1 double mutants, ett-1 mutants are indistinguishable from the wild type (J) before flowering. Ectopic expression of KAN2 through the ANT promoter results in narrow cotyledons, radial leaves, and growth arrest (K), while in ett-1 ANT::KAN2 plants (L), all lateral organs form and expand, albeit with altered morphology; p, petal; rp, radial petal; ab, abaxial; ad, adaxial. Bars = 10 μm in (E) to (H).

 triple mutant plants were indistinguishable from kan1-2 kan2-1. Therefore, KAN is epistatic to ETT. However, leaves of kan1-2 kan2-1/+- plants were curled upwards, while leaves of ett-1 kan1-2 kan2-1/+- plants had abaxial blade outgrowths similar to those found in kan1-2 kan2-1 (Eshed et al., 2001), although at lesser density (Figure 1I). The results suggest that ETT and KAN operate in a common genetic pathway in the flower as well as in the leaves. Therefore, we tested whether ETT can suppress KAN action in leaf primordia. When KAN is expressed through leaf primordia using the ANT promoter, nearly radialized and abaxialized cotyledons and leaves are formed (Figure 1K; Eshed et al., 2001). However, in ANT::KAN2 ett-1 plants, all lateral organs expanded, although their morphology was altered (cf. Figures 1K to 1L). Thus, ectopic KAN effects were suppressed in the ett-1 mutant background throughout the plant, indicating that ETT is an integral component of the KAN abaxial promoting pathway.

ETT and ARF4 Act Redundantly to Establish Abaxial Cell Fate

While ETT mediates ectopic KAN activity in all lateral organs examined, the manifestation of the ett mutant phenotype is restricted to flowers. A possible explanation is that other ARF genes compensate for the reduction in ETT function. Cladistics analyses of the Arabidopsis ARF family place ARF4 as the most closely related gene to ETT (Liscum and Reed, 2002; Remington et al., 2004). Hence, we examined an arf4-1 Ds insertion line presumably resulting in a null allele (Parinov et al., 1999) and an arf4-2 mutant resulting from T-DNA insertion in exon 10 (salk_070506). Homozygous arf4-1 and arf4-2 plants were indistinguishable from the wild type under the growth conditions examined. Moreover, ectopic KAN activity was as potent in the arf4-1 mutant background as in the wild type. We constructed ett arf4 double mutants. Remarkably, ett-1 arf4-1 and ett-1 arf4-2 plants resembled ett-1 kan2-1, having narrow, dark-green leaves that develop ectopic blade outgrowths on their abaxial sides only (Figures 2A to 2C). Formation of these outgrowths was greatly enhanced in plants grown in short days but remained restricted to the abaxial side of the leaves. Ectopic stipules were formed at the abaxial base of the leaves, an alteration also found in adaxialized kan1-2 kan2-1 and phb-1d/+ plants (Figure 2D; Eshed et al., 2001). Unlike kan1-2 kan2-1 plants, the abaxial outgrowths in ett-1 arf4-1 leaves occur later in plant development (from the third leaf on versus first leaf in kann1-2 kann2-1) and emerge relatively later after leaf initiation (Figure 2E).

The entire abaxial surface of ett-1 arf4-1 leaves is composed of very small cells and exhibits delayed maturation of guard cells (Figures 2E and 2F). As the final size of ett-1 arf4-1 leaves is only slightly smaller than the wild type, we examined whether extra cell divisions account for this discrepancy. Cell divisions were monitored by cyclin activity, as determined by the CYCLINB1;1::β-glucuronidase (GUS) reporter (Donnelly et al., 1999). As shown in Figures 2G and 2H, a prolonged period of CYCB1 activity was found in mutant leaves, primarily on the abaxial side, suggesting the impairment of a mechanism of cell maturation possibly regulated by auxin.

Leaf main vascular bundles of ett-1 arf4-1 were normally organized, while single vascular bundles connected the blade outgrowths with main leaf bundles (Figure 2I). The most severe malformation found in ett-1 single mutant plants was the formation of a short-valved gynoecium capped by an enlarged style expanded basally (Figures 2J and 2K). Similar alterations were found in plants with partially compromised KAN activity such as kann1-2 kann2-1/+- (Figure 2L). Like kann1-2 kann2-1 plants, ett-1 arf4-1 double mutants had short internodes and altered phyllotaxis of flowers. All floral organs of ett-1 arf4-1 were misshapen: sepals were short with abaxial protrusions, petals were narrow, and stamens were often misshapen and sterile. Organ numbers in each whorl varied between flowers: similar to ett-1, ett-1 arf4-1 flowers usually had one extra sepal and petal and a reduced number of stamens (four to five usually). The gynoecium of ett-1 arf4-1 plants was nearly devoid of valve tissue, the placenta was shifted upwards, and ovules could be found on the distal end, positioned adaxially and abaxially (Figures 2M and 2N). Taken together, all aspects of KAN loss of function were present in ett-1 arf4-1 double mutants, although phenotypes were weaker and...
ETT and ARF4 are indistinguishable from the wild type at the vegetative stage (A); however ett-1 arf4-1 double mutants exhibit severe morphological alterations (B): leaves are narrow and curled upwards and have ectopic blade outgrowths on their abaxial side only. Similar narrow leaves with abaxial outgrowth formed by kan1-2 kan2-1 mutants are shown (C). At the base of ett-1 arf4-1 leaves, ectopic stipules (asterisks) can be found (D). The abaxial blade outgrowths of these leaves emerge in expanding leaf primordia and are nearly radial with unexpanded cells all around (E). Abaxial epidermal cells are extremely small, similar in size to guard cells (F). The temporal pattern of cell divisions is monitored by CYC1B:GUS reporter activity in the wild type (G). In ett-1 arf4-1 leaves of comparable age, it displays prolonged activity (H). A single vascular bundle connects the ett-1 arf4-1 blade outgrowth to the leaf main bundles (I). Unlike the wild type (J), the ett-1 gynoecium is composed of a short valve and basally expanded style (K). Similar alterations are found in plants with partially compromised activity of the redundant KAN genes as in kan1-2 kan2-1/+ (L). All floral organs of ett-1 arf4-1 are misshapen (M). The gynoecium of ett-1 arf4-1 (N) is short and nearly devoid of valve tissue, and ovules are distally positioned both adaxially and abaxially. bl, blade; blo, blade outgrowths; gc, guard cell; vb, vascular bundle; v, valve; st, style. Bars = 50 μm in (D) and (E), 10 μm in (F), and 200 μm in (N).

3SS:ETT plants are mildly different from the wild type, with leaves slightly curled downward and the flower organs tending to bend outwards at maturity. This phenotype is reminiscent of sgs-2 plants that were recently reported to have elevated levels of ETT and ARF4 transcripts (Peragine et al., 2004). However, no clear polarity or patterning alterations were observed among several dozen independent T1 plants. A line with a single insertion (#2) fully complemented ett-1 and was crossed into the kan1-2kan2-1 background (as summarized in Figure 3A). No rescue of the kan1-2 kan2-1 phenotype was observed, yet kan1-2 kan2-1/+ plants had enhanced up-curling of the young leaves in the transgene background, but without any signs of local blade outgrowths. The 3SS:ETT#2 ett-1 arf4-1 plants were indistinguishable from the wild type before flowering but had slightly longer gynophores (Figures 3B and 3C) similar to that observed in the weak ett-2 allele (Sessions and Zambryski, 1995). None of several dozen 3SS:ARF4 plants were phenotypically different from the wild type, but partial rescue of the ett-1 phenotype in an ectopic ARF4 background was common. Moreover, 3SS:ARF4 arf4-1 ett-1 plants were similar to weak ett mutant alleles, verifying full arf4 and partial ett complementation (Figure 3D). As with ectopic ETT expression in the kan1-2 kan2-1 background, 3SS:ARF4 failed to rescue the kan1-2 kan2-1 phenotype.

Ectopic expression of ETT and ARF4 complemented their corresponding single mutant phenotypes but did not cause morphological alterations in an otherwise wild-type background. The activity of transcriptional regulators can be enhanced via fusion with potent activation or repression domains. These include factors such as the VP16 activation domain, which interacts with multiple targets within the RNA polymerase II initiation complex as well as with adaptor proteins (Sadowski et al., 1988), or mSin3 interaction domain (Sid), which represents a dominant repression domain whose activity can be transferred to a heterologous DNA binding region (Guan et al., 2002). To look for further evidence of the involvement of ETT and ARF4 in the KAN-mediated pathway, we fused both proteins to the repression domain Sid or the activation domain VP16. Expression of the Sid fusions with the 3SS promoter did not complement the mutant phenotypes and was not pursued further. No differences were observed between the native and VP16-fused versions of ETT, as both could rescue the ett-1 mutant phenotype, the leaf phenotype of ett-1 arf4, and only partially rescue the ett-1 arf4-1 flower phenotype (Figure 3E). However, 3SS:ARF4-VP16 could fully complement ett-1 (2/2) and ett-1 arf4-1 (no arf4-1 ett-1 or ett-like plants in 90 progeny T1s of arf4-1 ett-1/+ independent trans- formants of which two were confirmed to be double mutants by progeny testing; Figure 3F). Yet, the only alteration from wild-type appearance in 3SS:ARF4-VP16 plants was a slight, transient up-curling of young leaves. As with 3SS:ARF4, no new phenotypes were observed in either wild-type or kan1 kan2 backgrounds. Previous studies reported that in carrot (Daucus carota) protoplast assays, ARF4 repressed the auxin response reporter construct in the presence of auxin (Tiwarı et al., 2003). In other cases where VP16 was fused to a known repressor, it could not reverse the protein function and only reduced its activity (Ohta et al., 2001; Tiwarı et al., 2004). Thus, ARF4 might also be involved in transcriptional activation complexes and together with VP16 could substitute for ETT as well.
In summary, while ETT and ARF4 activities are partly interchangeable, neither could complement loss of KAN activities.

KAN Is Not Required for ETT and ARF4 Transcription

The failure of ectopic ETT and ARF4 to rescue kan mutants does not preclude them from being KAN targets. To establish if KAN action functions through regulation of ETT and ARF4 transcription, their RNA expression patterns were compared between wild-type and kan1-2 kan2-1 mutants. In wild-type tissue, weak levels of ETT transcript were detected throughout the shoot apical meristem (SAM), but much higher levels were found in leaf anlagen (P-1 and P0). In older primordia (P1 to P6), expression was gradually confined to lateral leaf margins, vascular bundles, and stipules (Figures 4A and 4B). While early expression in the vasculature corresponded with provascular tissues, later expression was confined to procambium (note the proximity of ETT signal to tracheary elements compared with ARF4 expression in the vascular bundle). In kan1-2 kan2-1 seedlings, initial ETT expression appeared normal; however, shortly after leaf primordia emerged, stronger than wild type expression was detected apparently prior to expansion of the ectopic abaxial outgrowths, consistent with ETT marking early primordia (Figure 4C). Subsequently, strong ETT expression was observed in the abaxial outgrowths, similar to the enhanced late expression of the abaxial and primordial gene FIL in these structures (Eshed et al., 2004).

ARF4 mRNA was not detected in the SAM but was restricted to the abaxial domains of the young developing leaf primordia mostly in the leaf base (P0 to P4). As leaves expanded, ARF4 expression was restricted to a narrow band of cells connecting the vascular bundle with the leaf lateral margins and soon afterwards became confined to the phloem (Figures 4D and 4E). A similar expression pattern of ARF4 is detected in kan1-2 kan2-1 vegetative apices with a slight expansion to more distal leaf domains (Figure 4F). Even though ARF4 can compensate for the loss of ETT in leaves, no alteration of transcript distribution was found in ett-1 plants, implying that the polarity defects in arf4-1 ett-1 leaves can be attributed to abaxial expression in developing primordia (Figure 4G).

During flower initiation, ETT transcription was detected throughout the inflorescence meristem and marked the sites of provascular differentiation. Much higher ETT mRNA levels were found throughout flower meristem anlagen and gradually became confined to cryptic bract primordia. In older flowers, primordial expression became restricted to abaxial domains of sepals, stamens, and carpels (Figures 4H and 4I). This expression pattern is similar to that previously reported by Sessions et al. (1997), apart from the expression in inflorescence meristem and sepal primordia, which could be ecotype-derived differences. ARF4 mRNA was first detected in low levels throughout both inflorescence and flower meristems and later resolved to cryptic bract primordia and throughout all initiating floral organ primordia (Figures 4J to 4L). At later stages of organ development, expression of ARF4 was restricted to the phloem (Figure 4M).

Thus, the expression domain of ETT and ARF4 colocalized to the abaxial domain of all lateral organs. Their transcription does not require KAN activity, indicating regulation by other mechanisms, probably associated with organ initiation processes.

Strong ett Alleles Result from Truncated Gene Products That Exert Negative Effects

Earlier studies demonstrated that ett gynoecium malformations vary significantly among different alleles and ecotypes (Sessions and Zambryski, 1995). In addition, new ett alleles with T-DNA...
insertions close to the translation start site (18 bp downstream of ATG in ett-13 and 8 bp upstream of ATG in ett-22; Figure 5A) were isolated and examined. Surprisingly, plants homozygous for these alleles are indistinguishable from the wild type apart from having a gynoecium with a short gynophore and split style (Figure 5B). Despite their weak phenotype, ett-13 plants could suppress the ectopic KAN action in petals and enhance the kan1-2 kan2-1/++ phenotype as efficiently as ett-1 plants (see Supplemental Figure 2 online). As the new weak alleles are in the Columbia (Col) background, we compared them with a different T-DNA allele, ett-114, generated in the same background to discount ecotype as a basis for the observed differences (Figure 5C). The Col allele ett-114 results from a T-DNA insertion very close to the point of T-DNA insertion in ett-1 and, as predicted, results in a similar strong phenotype, including an increase in perianth organ numbers and severely malformed gynoecium. Likewise, the weak phenotype of ett-13 remained unchanged after backcrossing four times into the Landsberg erecta (Ler)

Figure 4. In Situ RNA Distribution of ARF4 and ETT in Vegetative and Reproductive Tissues.

Twelve-day-old seedlings of wild type ([A], [B], [D], and [E]), kan1-2 kan2-1 ([C] and [F]), and ett-1 ([G]) probed with antisense digoxigenin-labeled RNA for ETT ([A] to [C]) and ARF4 ([D] to [G]). m, meristem; s, stipules; blo, leaf blade outgrowth; vb, vascular bundle; ph, phloem; im, inflorescence meristem; fn, flower meristem; cb, cryptic bract; sp, sepal; st, stamen; g, gynoecium. (A) ETT transcript is detected at low levels throughout the SAM and at much higher levels in leaf anlagen. Expression marks the sites of provascular differentiation both in the shoot and leaves. (B) In the transverse section, a gradual restriction from throughout leaf primordia to the adaxial marginal domain of leaves (arrowheads), stipules, and vascular bundles is evident. (C) In kan1-2 kan2-1 background, ETT expression initiates normally, but further expression is restricted to the abaxial leaf side where high levels of ETT mRNA demarcate the initiation sites of abaxial blade outgrowths. (D) and (E) ARF4 is expressed abaxially in the proximal part of wild-type leaf primordia starting at P0 to P4, and its expression is gradually restricted to a narrow band of cells connecting the vascular bundle with the leaf lateral margins (arrow) and the phloem. (F) and (G) An expression pattern of ARF4 similar to the wild type is detected in kan1-2 kan2-1 vegetative apices (F) with slight expansion to more distal leaf domains (G). No alteration relative to wild-type ARF4 transcript distribution was found in ett-1 apices. (H) and (I) ETT mRNA is detected throughout the inflorescence meristem and the sites of provascular differentiation, but much higher mRNA levels are found in initiating flower meristems, which is later confined to the cryptic bract and abaxial domain of sepals, stamens, and gynoecium. (J) to (L) Low ARF4 expression is detected in inflorescence and flower meristems. Later, it resolves to cryptic bract primordia and throughout all initiating floral organ primordia. (M) At later stages, expression of ARF4 is restricted to the phloem as seen in a transverse section of a stage 10 flower pedicel.
background. These tests indicated that the lack of observed 
phenotype in ett-13 and ett-22 was not due to the Col back-
ground. Furthermore, the strength of the different ett alleles also 
corresponded with the strength of their double mutants with arf4. 
ett-1 arf4-1 plants (Col background) were similar to ett-1 arf4-2 
plants (Ler background). Yet, ett-13 arf4-2 (Col background) 
plants had only slight blade abaxial outgrowths and the normal 
number of perianth organs and retained valve tissue in a fertile 
gynoecium (see Supplemental Figure 2 online).

To examine the phenotype of the double mutant between ett 
presumed RNA null allele and arf4-1 in the same ecotype back-
ground, the Ler backcrossed ett-13 was combined with arf4-1. 
The resulting ett-13 arf4-1 plants had a much weaker phenotype 
than ett-1 arf4-1 plants, exhibiting slightly up-curved leaves with 
few abaxial outgrowths (Figure 5D). The flowers of ett-13 arf4-1 
had nearly normal organ numbers (apart from the second whorl, 
where an extra petal was occasionally formed) and narrow petals 
with relatively normal epidermal cell morphology. Occasionally, 
neared radial petals were formed, and a stamen lacked a locule. 
The gynoecium had prolonged gynophore with very reduced or 
complete loss of valve tissue, a style expanded basally, and 
distal placenta (Figures 5E and 5F). In summary, mutant 
phenotypes were slightly stronger in the Ler background, but 
presumptive ett null alleles are much weaker than alleles with 
disruptions toward the more C-terminal end of the transcript.

No ETT transcripts were detected in ett-13 seedlings by RNA 
in situ analysis (Figure 5G), and only minor amounts of ETT 
transcript were detected by RT-PCR (see Supplemental Figure 2 
online). Yet, in ett-1 plants, truncated ett-1 RNA distribution was 
similar to the wild type, although the difference in levels between 
primordia (high in the wild type) and meristem (low in the wild 
type) was less pronounced in this background (Figure 5H). We 
suggest that ett-13 and ett-22 represent close to null alleles with 
significantly reduced levels of ETT transcript, while ett-1 and 
ett-114 RNAs would encode truncated polypeptides having 
~120 and 109 amino acids, respectively, identical to the wild-
type ETT N terminus. The relative severity of the phenotypes of 
the latter alleles may indicate that the truncated protein of ett-1 
and ett-114 now acts negatively in the ETT pathway. To test for 
this possibility, truncated versions of an ETT cDNA encoding a 
150- and 333-amino acid protein (ETT-N150 and ETT-N333) 
were introduced into the wild-type and ett-12 backgrounds 
(Figure 5A). The 35S:ETT-N150 and 35S:ETT-N333 plants 
were indistinguishable from wild-type plants. By contrast, in 
ett-13 background, these constructs displayed variable strengths 
of phenotypes. In the strong 35S:ETT-N150 (4/45) and 35S:ETT-N333 (5/59) plants, the flower organ number (see 
Supplemental Figure 2 online) and gynoecium defects of ett-13 
were greatly enhanced, such that 35S:ETT-N333 ett-13 plants 
resembled the strong ett-1 mutants (Figures 5I and 5J). In these 
lines, endogenous ETT transcript levels were similar to those 
found in ett-13 untransformed plants, suggesting that cosup-
pression was not the cause of the strong phenotypes. These 
results indicate that the truncated ETT gene product can 
interfere with normal development in planta.

**DISCUSSION**

The partitioning of organ primordia into discrete abaxial and 
adaxial domains involves gradual separation of the KAN-
expressing domain from the PHB-like–expressing domain. In 
this study, the ARF protein ETT was identified as an essential 
intermediary for the gradual establishment of abaxial identity

**Figure 5.** Strong ett Alleles Result from Truncated Gene Products with Negative Effects. 

(A) Structure of the ETT gene and position of lesions in the different ett alleles (exons shown as boxes, and introns are marked by lines). T-DNA insertion positions and the premature stop codon in ett-11 are indicated by arrows. Regions indicated by A and B correspond to TAS3 trans-
acting siRNA (ta-siRNA) target sites (Allen et al., 2005). The black lines denote the extent of the ETT-N150 and ETT-N333 constructs. 

(B) ett-13 gynoecia have an elongated gynophore topped by valves of unequal length as indicated by arrows. 

(C) ett-114 gynoecium has the typical strong ett phenotype with apical 
cell types shifted basally. 

(D) In ett-13 arf4-1 plants, leaves are slightly up-curved with hardly any 
abaxial outgrowths. 

(E) ett-13 arf4-1 flowers have normal numbers of organs (one petal 
removed), albeit narrower. 

(F) Occasionally, nearly radial petals or stamens missing locules are 
formed (asterisk). The gynoecium has an extended gynophore with very 
reduced or complete loss of valve tissue, style expanded basally, and 
limited distal placenta. 

(G) and (H) Close to null nature of ett-13 is evident by lack of RNA signal 
in the shoot (G), compared with the strong and more equal expression 
between meristem and primordia detected in ett-1 (H). 

(I) and (J) Misexpression of ETT-N150 (I) and ETT-N333 (J) lead to a 
significant enhancement of the ett-13 gynoecium defects, phenocopy-
ing the strong ett-1 allele. 

Bars = 2 mm in (B), (C), (I), and (J). DBD, DNA binding domain; MR, 
middle region; v, valve; s, stamen; st, style; gy, gynophore; p, petal; rp, 
radial petal.
initiated by KAN. It was further demonstrated that this activity partially overlaps with a second ARF protein—ARF4. ARF proteins participate in auxin signaling and transcriptional regulation of the auxin response targets (Ulmasov et al., 1997), thus establishing a novel and essential link between lateral organ abaxial/adaxial axis development and signaling of a central phytohormone.

**ETT and ARF4 Mediate the KAN Abaxial Pathway**

The monophyletic ARF proteins ETT and ARF4 overlapped in RNA expression in the abaxial domain of all lateral organ primordia. Consistent with this, the most pronounced defects observed in ett-1 arf4-1 double mutants occurred on the abaxial side of lateral organs. Taken together, ETT and ARF4 are redundantly required for specification of abaxial cell types, although their combined expression is found in the apical meristem and the adaxial domain of lateral organs as well (Figure 4). On examination of the role of these genes in the establishment of abaxial cell fate, it was notable that ARF4 abaxial expression in leaf primordia was first observed shortly after leaf primordium specification (P0). This may suggest that ARF4 is not part of the initial abaxial/adaxial partitioning program but rather participates in refinement and maintenance of the molecular distinction between the abaxial and adaxial domains. This is in support of a developmental scenario in which specification of the adaxial/abaxial organ boundary in lateral organ formation is a gradual and continual process.

The role of ETT in the KAN pathway for establishment of abaxial fate was uncovered via a screen for second site suppressors of ectopic KAN. Such a screen cannot distinguish between downstream targets, cofactors, or independent regulators that participate in genetically but not biochemically related processes. Several lines of evidence suggest that ETT and ARF4 activities operate in conjunction with, and not downstream of, KAN function. First, to our knowledge, the striking phenotypic similarity between the double mutants ett-1 arf4-1 and kan1-2 kan2-1 has not been observed in any loss-of-function mutants thus far. Second, the role of ETT and ARF4 is linked to KAN activity since kan1-2 kan2-1 is epistatic to either ett or arf4. Third, ett can enhance the intermediate leaf kan1-2 kan2-1/+ phenotype. Fourth, transcription of both ETT and ARF4 does not require KAN activity and is not immediately induced by it (Figure 4; I. Pekker and Y. Eshed, unpublished data). In addition, neither native nor activated versions of either ETT or ARF4 can rescue kan mutants. Therefore, a possible scenario is that both ETT and ARF4 serve as cofactors either directly with KAN or alternatively with its downstream factors to promote abaxial identity. While not all expression domains of the two ARFs overlap with KAN, it is the abaxial expression of ARF4 in the leaves of ett-1 that is most indicative of a role in promoting abaxial identity. This expression domain overlaps with KAN both in abaxial leaf primordia and in the phloem domain of vascular bundles. In the absence of identification of additional mutants that result in similar transformation of abaxial cell types, cooperative activities of ARFs and KANs are preferable.

The recent identification of trans-acting derived short interfering RNAs (siRNAs) that negatively regulate both ETT and ARF4 transcripts suggests that the full potential of these genes in induction of abaxial identity and in the rescue of the kan mutants has yet to be tested (e.g., using siRNA resistant versions; Allen et al., 2005). Notably, mutants impaired in microRNA processing, such as dicer-like1 or various alleles of argonaute1, exhibit complex polarity defects (Bao et al., 2004). In this case, impairment of microRNA-mediated regulation of both adaxial (PHB-like) and abaxial (ETT/ARF4) promoting factors provides a possible mechanism for these cryptic phenotypes. It is intriguing that both abaxial and adaxial negating factors are regulated through common mechanisms. It is tempting to speculate that the necessity for prolonged partitioning of organ primordia in a cell population incapable of migration requires multiple refinement steps to establish strict domain boundaries.

**ETT and ARF4 Functions Overlap with Those of Additional ARFs**

Despite the widespread use of auxin signals and the substantial biochemical evidence for the central role of ARF proteins in the process, only a handful of phenotypic alterations are associated with single mutants of the 22 ARFs encoded in the Arabidopsis genome (Okushima et al., 2005). The dramatic enhancement of ett mutants by arf4 implies that different ARF proteins can partially compensate for the loss of other members. Indeed, several studies uncovered overlapping activities of closely related ARF pairs (Li et al., 2004; Remington et al., 2004; Okushima et al., 2005). In the double mutant ett arf4, we could not uncover any role for ETT in the early adaxial domains of leaf primordia or in the shoot apical meristem even though it is expressed in these domains. However, in certain mutant backgrounds, ETT is essential for flower primordium initiation, a role that is reminiscent of ARF5 (MP) function (I. Pekker and Y. Eshed, unpublished data; Nishimura et al., 2004; Reinhardt et al., 2003). Thus, there is evidence that ETT is redundant at least in establishing the early floral meristem and possibly in its maintenance.

**Truncated ett Transcripts Suggest Complex Mechanisms in ARF Functions**

The striking phenotypic differences between the weak ett-13/ett-22 and strong ett-1/ett-114 alleles suggest that the resulting truncated protein interferes with the pathway in which the wild-type protein normally acts. Since the effects of the truncated protein are only evident in the absence of the wild-type copy, it is possible that the truncated form has a reduced affinity to targets and/or partners, turning a potentially dominant negative form into a dose-sensitive recessive one. Although ett-1 truncated transcripts are likely no longer under siRNA regulation, this does not contribute to the strong ett-1 phenotype, as ett-11 has similar alterations resulting from a nonsense mutation (the ETT siRNA binding site maps to the 3’ third of the transcript). However, a lack of siRNA regulation can underlie the higher than normal meristem expression observed in the ett-1 background (Figure 5).

The ett-1 arf4-1 double mutant phenotype is strikingly more severe than the double mutant combination ett-13 arf4-1 (Figures 2B, 2M, 5D, and 5E). Therefore, the wild-type function of
ETT is likely redundant with additional abaxial-promoting factors whose activities are interfered with by the ett-1 protein (in ett-1 homozygotes). The T-DNA insertions in ett-1 and ett-114 delimit the negatively acting domain to the 109 N-terminal amino acids. This part of the ETT protein contains two highly conserved motifs found throughout the ARF family of both Arabidopsis and rice (Oryza sativa; Sato et al., 2001). Using protein truncation and gel mobility shift assays with palindromic AuxRE, this domain was demonstrated to be a part of the DNA binding domain but incapable of binding DNA by itself. Likewise, it was demonstrated that ARF1 lacking domains III and IV can still form homodimers and heterodimers in the presence of the palindromic AuxREs (Ulmason et al., 1999). Taken together, this part of the protein might serve as a protein–protein interaction domain common to the whole ARF family. Therefore, the most obvious candidates for redundancy with ETT in the phylotaxis and organ asymmetry establishment pathways are other ARFs, consistent with ETT’s proposed role in auxin-mediated organ patterning (Nemhauser et al., 2000). In this model, the activities of other ARFs can be inhibited by dimerization with the ett-1 variants or by binding competition in essential protein complexes. Notably, a similar negative inhibition has been reported for other truncated ARFs. The strongest arf7 allele, nph-3, gives rise to a short truncated N-terminal protein that behaves genetically as a poisonous protein with stronger alterations than presumed null alleles (Harper et al., 2000). Thus, it will be important to obtain true nulls for the other ARF family members prior to conclusive assignment of unique versus common functions for the individual members. Unfortunately, no immediate ARF candidates can be found from cladistic analysis of the ARF family N terminus (L. Pekker and Y. Eshed, unpublished data). However, three ARF genes, ARF2, ETT, and ARF4, were recently demonstrated to be a common target of ta-siRNAs derived from the TAS3 gene (Allen et al., 2005). Regions of complementarity to the ta-siRNA in all three genes are conserved in all plant species examined, suggesting an evolutionarily conserved coregulation of these genes. As the arf2 mutation has no organ asymmetry phenotype (Okushima et al., 2005), generating an arf2/3/4 triple mutant can determine if ARF2 is also redundantly involved in abaxial/adaxial axis development of lateral organs.

**Auxin, Organ Initiation, and Establishment of Lateral Organ Asymmetry**

Recent seminal studies have linked localized gradients of auxin concentration with patterning in key axes of the plant body. These include establishment of the distal organizer in the root (Sabatini et al., 1999), the apical basal axis of the embryo (Friml et al., 2003; Billoiu et al., 2005), and lateral organ primordium initiation (Benkova et al., 2003; Reinhardt et al., 2003). In all of these instances, normal patterning disruptions were associated with alterations of local auxin concentrations. Strikingly, local application of auxin is sufficient to restore lateral organ formation in Arabidopsis mutants defective in auxin polar transport and in vegetative tomato apices treated with auxin transport inhibitors (Reinhardt et al., 2000, 2003). However, no disruptions in adaxial/abaxial organ asymmetry have been reported in association with altered auxin distribution, possibly because the establishment of organ asymmetry may be less sensitive to auxin perturbations than organ initiation.

A possible role for an auxin gradient in organ asymmetry patterning is suggested by the dynamic distribution of auxin transporters in the developing organ. At P0, leaf primordium cells are a sink for auxin as evidenced by cellular distribution of the auxin efflux carrier PIN1 (Benkova et al., 2003; Reinhardt et al., 2003). During the rapid expansion of the primordium (P1), an abaxial distribution of the auxin influx factor AUX1 is evident. In addition, alterations of PIN1 localization result in reversion of the primordia from being sinks to new sources of auxin (Reinhardt et al., 2003). Based on these observations and the roles of ETT

**Figure 6. Proposed Role for Auxin in Mediating Abaxial/Adaxial Partitioning of Organ Primordia.**

Initiating organ primordia cells coexpress abaxial and adaxial factors. The partitioning of lateral organs into the abaxial KAN-expressing domain and the adaxial PHB-like-expressing domain is gradual and evolves by mutual antagonism between the two types of factors and external morphogenetic input. With the rapid expansion of the growing primordium, auxin concentrations form a slight gradient via asymmetric auxin influx carrier distribution and due to conversion from being a sink to a new source of auxin synthesis (Reinhardt et al., 2003). This gradient of auxin is translated into differential action of specific subsets of ARFs (ETT, ARF4, and others), enabling KAN to override PHB-like activities at the abaxial domain. Subsequently, gradients of these ARFs help differentially translate auxin presence to maintain abaxial fate, leading to stable abaxial/adaxial partitioning.
and ARF4 in primordium patterning described in this work, we propose a model whereby ARF protein activities modulated by auxin concentrations act to facilitate lateral organ asymmetry (Figure 6). This model suggests that a central component of asymmetric pattern establishment derives from dynamic auxin gradients in emerging primordia. The resulting auxin gradient leads to differential AUX/IAA degradation and increased activity of specific subsets of ARFs. Joint action of the ARFs ETT and ARF4, together with factors such as KAN and miR165/6, differentially tip the mutual antagonistic activities of the primary asymmetry genes (PHB-like and KAN), gradually leading to stable asymmetric partitioning. Auxin will further act as a fine tuner of patterning via asymmetric ARF distribution. Direct manipulations of auxin activities in specific cells will help to further resolve auxin’s role as a facilitator or generator of pattern in this process.

The complexity of ARF action and the difficulty in interpreting the consequences of auxin distribution changes in organ polarity might also stem from the interplay of other ARFs acting in conjunction with the adaxial pathway, maintaining a balanced auxin signaling when its levels are generally reduced. Preliminary evidence suggests that MONOPTEROS (ARF5) activity is required for activation of the PHB-like gene ATHB-8 in provascular tissues (Mattsson et al., 2003). In this respect, a tight association between auxin and both vascular development and organ asymmetry establishment is evident, along with the patterning requirement in both tissues for antagonistic KAN and the PHB-like functions. That both gene classes are involved has led to the proposal of shared developmental patterning modules in lateral organs and vasculature morphogenesis (Emery et al., 2003). The expression of ARF4 in the abaxial domain of the organ primordium and abaxial phloem extends this parallel.

METHODS

Plant Material, Growth, Mutagenesis, Crosses, and Transformation

All plants described apart from SALK T-DNA (Alonso et al., 2003) lines were in the Ler background. Plants were grown under 18-h cool white fluorescent light at 20°C. AP3::KAN1 seeds were mutagenized with 15 mM ethyl methanesulphonate for 12 h, and phenotypic suppressors were selected in the M2. The suppressors were backcrossed to Ler, and only those in which the transgene phenotype was restored were analyzed further. kan1-2 and kan1-2 kan2-1 were described earlier (Eshed et al., 1999, 2001). ett-1 (a kind gift from Alan Sessions) was originally in a Wassilewskija background but was backcrossed into Ler for eight generations before any further analysis; ett-11 has a premature stop codon at Trp 305, arf4-1 (a kind gift from Venkatesan Sundaresan) contains a Ds insertion at position –12 relative to the first Met codon. ett-13 is salk_040513 with a T-DNA inserted 18 bp downstream of the first Met codon; ett-22 is salk_052222 with a T-DNA inserted 8 bp upstream of the first Met codon; ett-114 is salk_060114 with a T-DNA insertion 740 bp downstream of the first Met codon; arf4-2 is salk_070506 with a T-DNA inserted in exon 10. Multiple mutant plants were generated by cross-fertilizing homozygous mutants and identifying desired mutant combinations among phenotypic categories in the F2 segregants. Genotypes were confirmed by monitoring Mendelian ratios and by progeny testing. Trans-activation lines were generated by transcriptional fusion of promoters in front of the chimeric LH4 and cDNAs subcloned behind an operator array (Moore et al., 1998) in the BJ36 vector. The ANT::LH4 line was a generous gift from Michael Lehnd (Schoof et al., 2000), and AP1::LH4 (Emery et al., 2003), CRC::LH4 (Greenboim-Wainberg et al., 2004), and 100P::KAN2 (Eshed et al., 2001) were described earlier. Promoter::LH4 lines were crossed to 100P::KAN2 to generate F1 plants where KAN2 was trans-activated with different promoters (Promoter::—KAN2). All transgenic plants were generated by the floral dipping method, and transformants were selected on soil on the basis of resistance to the herbicide BASTA.

Plasmids and cDNA Clones

For AP3::KAN1, a 500-bp fragment 5’ to the ATG of the AP3 gene (kindly provided by Tom Jack) was subcloned upstream to KAN1 cDNA in the BJ36 vector. ETT cDNA containing the open reading frame only (a kind gift from Jennifer Nemhauser) was subcloned into the ART7 vector using Xhol and XbaI sites. ARF4 cDNA was obtained from The Arabidopsis Information Resource (C00031), and the open reading frame was PCR amplified with Kprl and BgIII linkers and cloned into the ART7 vector. The VP16 activation domain (amino acids 413 to 490) was a generous gift from Detlef Weigel. It was amplified by PCR with BamHI and HindIII linkers. ETT and ARF4 cDNAs were PCR amplified with 5’ Xhol linker and 3’ BgIII linker, which eliminated the stop codon. VP16 was inserted as a carboxy-translational fusion with ETT and ARF4 and subcloned into the ART7 vector. The N terminus of Mad (Sid repression domain amino acids 1 to 35; a generous gift from Robert Eisenman) was inserted as a carboxy-translational fusion with ETT and ARF4 and subcloned into the ART7 vector. The 3SS::ETT-N150 construct, ETT cDNA was amplified by PCR with Xhol and BamHI linkers and cloned into the ART7 vector. The 3SS::ETT-N333 was generated by insertion of full-length ETT into the ART7 vector, followed by cleavage of internal and 3’ BamHI sites. The self-ligation results in a 333–amino acid coding protein. CYC18::GUS reporter was a kind gift from John Celenza. All constructs were subcloned into pMIBART binary vector and were introduced into Agrobacterium tumefaciens strain GV3101 by electroporation.

Microscopy

Tissue preparation, histological analyses, tissue clearing, GUS staining, and in situ hybridization were performed according to Eshed et al. (1999). Scanning electron microscopy was performed using an XL30 ESEM FEG microscope (FEI). ETT and OAR4 probes were generated by linearizing the above-described cDNA plasmids and synthesizing digoxigenin-labeled antisense RNA using T7 RNA polymerase.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. ETT44 Mediates Ectopic KANADI Activity in Multiple Organs.

Supplemental Figure 2. Phenotypic and Molecular Expression of the Presumed Null ett Allele.

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REFERENCES


