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## Trans-acting siRNA-mediated repression of ETTIN and ARF4 regulates heteroblasty in *Arabidopsis*

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### Abstract

Mutations in the *ARGONAUTE* gene *ZIPPY*(*ZIP*)/*AGO7* in *Arabidopsis* accelerate the juvenile-to-adult transition. A screen for mutations that suppress this precocious phenotype yielded alleles of two auxin-related transcription factors known to be upregulated in *zip*: *ETTIN* (*ETT*)/*ARF3* and *ARF4*. Mutations in *ETT/ARF3* and *ARF4* delay the expression of adult traits, demonstrating that these genes have non-redundant roles in shoot maturation. *ZIP* is not generally required for the production of trans-acting (ta) siRNAs, but is required for the production and/or stability of *tasiR-ARF*, a ta-siRNA that targets both *ETT/ARF3* and *ARF4*. *tasiR-ARF* is absent in *zip-2*, and overexpression of a *tasiR-ARF*-insensitive form of *ETT* mimics the *zip* phenotype. We conclude that the precocious phenotype of *zip* is attributable to the absence of *tasiR-ARF*-mediated repression of *ETT* and *ARF4*. The abundance of *tasiR-ARF*, *ETT/ARF3* and *ARF4* RNA does not change during vegetative development. This result suggests that *tasiR-ARF* regulation establishes the threshold at which leaves respond to a temporal signal, rather than being a component of this signal.

### Keywords

siRNA; RNAi; Heterochrony; Arabidopsis

## INTRODUCTION

Shoot growth is characterized by the repeated production of lateral organs from the shoot apical meristem. The nature of these organs varies over time, undergoing both gradual and discrete changes in morphology as the plant passes through the juvenile, adult and reproductive phases of development (Kerstetter and Poethig, 1998). This phenomenon is known as ‘heteroblasty’ or ‘phase change’, and is brought about by the intersection of pathways controlling developmental timing and leaf morphogenesis. In *Arabidopsis*, heteroblasty can be observed in the gradual transition from leaves with long petioles and round, smooth, flat blades to leaves with short petioles and elliptical, serrate, epinastic blades (Telfer et al., 1997). The number of hydathodes per leaf also increases steadily throughout vegetative growth (Hunter et al., 2003; Tsukaya and Uchimiya, 1997), whereas trichome distribution defines discrete phases, with leaves gaining abaxial trichomes at the juvenile-to-adult transition, and losing adaxial trichomes at the adult-to-reproductive transition (Telfer et al., 1997).

Several genes responsible for the temporal control of this process have recently been identified. These include the *ARGONAUTE* family member *ZIPPY* (*ZIP*) (Hunter et al., 2003), the RNA-

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While this paper was under review, evidence that *tasiR-ARF* is responsible for the morphological phenotype of *sgs3*, *rdm6* and *dcl4* was published by Adenot et al., Garcia et al. and Fahlgren et al. (Adenot et al., 2006; Garcia et al., 2006; Fahlgren et al., 2006).

dependent RNA polymerase *RDR6*, the plant-specific gene *SGS3* (Peragine et al., 2004), the Dicer-like gene *DCL4* (Gascioli et al., 2005; Xie et al., 2005; Yoshikawa et al., 2005), the exportin-5 homolog *HASTY (HST)* (Bollman et al., 2003; Park et al., 2005; Telfer and Poethig, 1998), and the zinc finger gene, *SERRATE (SE)* (Clarke et al., 1999; Prigge and Wagner, 2001). Mutations in these genes cause the precocious expression of traits associated with later stages of development; for example, mutations in *ZIP*, *RDR6*, *SGS3* and *DCL4* cause premature leaf elongation, downward curling of the leaf margin, serration, and abaxial trichome production (Gascioli et al., 2005; Hunter et al., 2003; Peragine et al., 2004; Yoshikawa et al., 2005). All of these genes have roles in the biogenesis of miRNAs or endogenous siRNAs (Allen et al., 2005; Dunoyer et al., 2005; Park et al., 2005; Peragine et al., 2004; Vazquez et al., 2004; Yoshikawa et al., 2005), which strongly suggests that their mutant phenotype can be attributed to the aberrant expression of genes normally repressed by these small RNAs. Expression profiling of *zip*, *rdr6* and *sgs3* revealed several genes whose transcripts accumulate in these mutants (Peragine et al., 2004), but which of these upregulated genes, if any, is responsible for their precocious phenotype is still unknown.

We addressed this question using a genetic approach. Assuming that the precocious phenotype of *zip* is indeed a result of upregulated gene expression, we looked for loss-of-function mutations that suppress this phenotype. Remarkably, this screen produced mutations in the two most-highly upregulated genes in *zip*, *rdr6* and *sgs3*: *ETTIN (ETT/ARF3)* and *AUXIN RESPONSE FACTOR 4 (ARF4)*. *ETT* and *ARF4* are closely related members of the auxin response factor family of transcription factors (Remington et al., 2004; Ulmasov et al., 1999). The first *ett* mutants were isolated as plants with severely malformed gynoecia: apical-basal defects caused the expansion of the style and stipe at the expense of the ovary, and adaxialization led to the peripheral expansion of central tissues, including the stigma and the transmitting tract (Sessions and Zambryski, 1995). The finding that *ETT* is expressed abaxially in the developing gynoecium (Sessions et al., 1997) supported its role in establishing abaxial/peripheral tissues. Both *ETT* and *ARF4* have been shown to be expressed during vegetative development: *ETT* mRNA is present in the shoot apical meristem, leaf primordia, and the margins, vascular bundles and stipules of mature leaves, whereas *ARF4* mRNA is expressed in the abaxial domain of leaf primordia and the phloem of mature leaves (Pekker et al., 2005). Plants doubly mutant for *ett* and *arf4* resemble *kan1;kan2* double mutants, suggesting that these genes are involved in the specification of abaxial identity (Alvarez et al., 2006; Pekker et al., 2005). However, neither gene has been shown to have an independent vegetative phenotype.

Here, we describe the effect of *ett* and *arf4* on leaf morphology, and their interaction with the *ZIP*-mediated timing pathway. We show that upregulation of *ETT* and *ARF4* is largely responsible for the vegetative morphology of *zip*. We further show that the *zip* phenotype can be attributed to a defect in the production or stability of a transacting (ta) siRNA from the *TAS3* locus (*tasiR-ARF*), which is known to direct the cleavage of the mRNA of these genes (Allen et al., 2005; Williams et al., 2005). The abundance of *tasiR-ARF*, as well as the *ETT* and *ARF4* mRNAs, does not change significantly during vegetative development. This result is consistent with the mutant phenotype of *zip* (Hunter et al., 2003), and suggests that *tasiR-ARF* regulation of *ETT* and *ARF4* transcript levels influences heteroblasty by determining the sensitivity of leaf primordia to a temporal signal, rather than by serving as a component of a developmental clock.

## MATERIALS AND METHODS

### Plant materials and phenotypic analysis

All mutations were in the Columbia background, unless otherwise indicated. The *arf4-2* T-DNA insertion mutation SALK\_070506 (Alonso et al., 2003) was obtained from Jason Reed

(Chapel Hill, NC). *ett-7* was a gift from Patricia Zambryski (Berkeley, CA), and the *zip* (*Ler*) T-DNA insertion line (CSH3629) was obtained from the Cold Spring Harbor Laboratory. Plant growth, leaf shape analysis and trichome counts were carried out as described by Peragine et al. (Peragine et al., 2004) and camera lucida tracings were performed as described by Kerstetter et al. (Kerstetter et al., 2001). Leaf length was measured as the distance between the leaf tip and the intersection point of lines drawn tangent to the leaf base. The width of the leaf blade was measured at the midpoint of this line.

### Mutant isolation and identification

*zip-2* seeds were mutagenized in 0.4% EMS for 6-9 hours prior to planting. Individually harvested M2 families ( $n=2400$ ) were screened for mutations that suppressed the leaf shape phenotype of *zip-2*. *ett-15* was mapped using the F2 plants from a cross to the *zip* (*Ler*) T-DNA insertion line CSH3629, which we obtained from the Cold Spring Harbor Laboratory. Five additional suppressors were classified as *ett* or *arf4* mutations by their failure to complement *ett-15* or *arf4-2*.

### RNA analysis

Isolation of low molecular weight RNA, northern analysis, and RLM-5'RACE were carried out as described previously (Yoshikawa et al., 2005). *tasiR-ARF* was identified on blots of low molecular weight RNA using the locked nucleic-acid oligonucleotide probe: 5'-T(+G)GGG(+T)CTT(+A)CAA(+G)GTCA(+A)GAA-3'.

For RT-PCR, total RNA was isolated from leaves with Trizol reagent (Invitrogen) and PCR amplification was carried out with the following primers for the analysis of ARF3 and ARF4 expression:

ARF3F, 5'-TGGTCCCAAGAGAAGCAGG-3';

ARF3R, 5'-TCCACCATCCGAACAAGTG-3';

ARF4F, 5'-GCCGCTGAAGATTGTTTTGCTC-3';

ARF4R, 5'-AGTAGATGCCTCCTTGGTTGACC-3';

EIF4AF, 5'-GCGCATCCTCCAAGCTGGTGTCC-3'; and

EIF4AR, 5'-GGTGGAAGAAGCTGGAATATGTCAT-3'.

The following primers were used for the analysis of splicing defects in *arf4* alleles:

Ex1F, 5'-GATGCTATGGTTTCATATTCGTCTCC-3';

Ex2R, 5'-TGTAGACCTCATCGGTGTCCTTATTAG-3';

Ex8F, 5'-AACTCTAAATGGAGGTGCTTGTTG-3';

Ex9R, 5'-GCCTTGGAGATGACTGAATGC-3';

Ex2F, 5'-CCAGTTGCTTGCTAATAAGGACAC-3';

Ex3R; 5'-CTTGACCTCTTCCCCTCCC-3';

Ex5F; 5'-CTCGTCTCTGGTGATGCGG-3';

Ex6R; 5'-TCAGGAAGTCCATTTCTTGGC-3'.

### **ETT overexpression constructs**

The *ETT* open reading frame (ORF) was amplified using primers adding *Bsp*HI and *Bst*EII sites to the 5' and 3' end of this transcript, respectively (ARF3fBsp, 5'-ATCATGAGCGGTGGTTTAATCGATCTGAACG-3'; ARF3rBst, 5'-TGGTTACCCTAGAGAGCAATGTCTAGCAAC-3'). Addition of the 5' *Bsp*HI site added a Ser residue after the initial Met, but this did not affect the ability of the construct to rescue *ett-15*. The resulting product was inserted into the T-easy vector (Promega) and used as a template for site-directed mutagenesis using the following oligonucleotides, as described previously (Wang and Malcolm, 1999).

mAf, 5'-CCAGAGGGTCCTGCAGGGACAGGAGATTTTTCCGGG-3';

mAr, 5'-CCCGGAAAAATCTCCTGTCCCTGCAGGACCCTCTGG-3';

mBf, 5'-CCATAAGGTCCTGCAGGGACAGGAGACAGTTCCCGCC-3';

mBr, 5'-GGCGGGAAGTGTCTCCTGTCCCTGCAGGACCTTATGG-3'.

The mutated *ETTIN* ORFs were inserted behind the CaMV 35S promoter in *Nco*I/*Bst*EII digested pCambia3301, and transformed into Col and *ett-15* plants by the floral dip method (Clough and Bent, 1998).

## **RESULTS**

### ***ett* and *arf4* suppress the *zip* phenotype**

A screen for EMS-induced mutations that suppress the curled, elongated leaf phenotype of the *zip-2* deletion yielded six phenotypically similar mutations in two complementation groups. Mutations in the first complementation group mapped near *ETT*, and failed to complement a strong allele (*ett-7*) of this gene. One of these mutations (*ett-15*) is a C to T change that generates a stop codon in the C-terminal region of *ETT*, and the second (*ett-16*) is a C to T change that generates a stop codon in the N-terminal region of this gene (Fig. 1A). Mutations in the second complementation group complemented *ett-7* but failed to complement a T-DNA insertion (*arf4-2*) (Pekker et al., 2005) in the closely related gene *ARF4*. One of these mutations (*arf4-3*) is located in the middle of the first intron and two others (*arf4-4* and *arf4-5*) are located at splice donor sites (Fig. 1A). All three of these mutations produce transcripts with unspliced introns (Fig. 1B) that introduce stop codons in the ARF4 ORF. *arf4-6* is a missense mutation that converts an arginine to a lysine (Fig. 1A,B). Most of the research presented here was conducted with *ett-7*, a point mutation that generates a stop codon at the extreme N-terminal end of *ETT*, and *arf4-2*, a T-DNA insertion near the C-terminal end of *ARF4* (Fig. 1A). These mutations were chosen because their phenotype is slightly stronger than the suppressors isolated in our screens.

The interaction between *zip-2* and *ett-7*, *ett-15* and *arf4-2* was studied in F2 families generated by the self-pollination of plants heterozygous for both mutations. The genotype of the plants in these families was determined by PCR, using allele-specific primers. The first two leaves of double mutants are both flatter and rounder than the first two leaves of *zip-2* (Fig. 2; Fig. 3A). In *zip-2*, the length:width (L:W) ratio of these leaves was 1.7, in *zip-2;ett-15* and *zip-2;ett-7* double mutants it was approximately 1.3, and in *zip-2;arf4-2* it was approximately 1.4; this difference in leaf shape was quite obvious and highly significant ( $n=14$ ;  $P<0.001$ ). The effect of *ett* and *arf4* mutations on the *zip-2* phenotype in subsequent leaves is more subtle, and is most evident at the leaf tip, which is slightly more rounded in double mutants than in

*zip-2* (Fig. 3A). *zip-2;ett-15* and *zip-2;ett-16* leaves had unusually short and thick petioles, but this phenotype was not observed in *zip-2;ett-7* (Fig. 3A). *ett-7* and *arf4-2* also affect leaf shape in the absence of *zip-2* (Fig. 3B). In wild-type plants, the L:W ratio of the lamina increased from a value of 1.3 for leaves 1 and 2, to a value of  $\approx 2.7$  for leaf 7 and remained constant for several leaves before declining. *ett-7* and *arf4-2* produced a significant ( $n=10$ ;  $P<0.01$ ) decrease in the L:W ratio of leaves 1-8, but did not affect the morphology of the last several leaves of the shoot. Consistent with this effect, *ett* and *arf4* delayed abaxial trichome production in both the presence and the absence of *zip-2* (Fig. 3C,D). On average, *zip-2* plants had  $2.8\pm 0.3$  leaves without abaxial trichomes (compared with  $3.5\pm 0.3$  in Col). This number was increased to  $6.6\pm 0.5$  in *zip-2;ett-15*, to  $5.6\pm 0.7$  in *zip-2;ett-7*, and to approximately 4 in *zip-2;arf4-2*, *zip-2;arf4-3*, *zip-2;arf4-4*, *zip-2;arf4-5* and *zip-2;arf4-6*. These mutations produced a slightly greater delay in abaxial trichome production as single mutants, but in most cases this effect was so small that it was not statistically significant (Fig. 3C; data not shown). Interestingly, the trichome phenotype of *+ett-7* was intermediate between that of homozygous mutant and homozygous wild-type plants (Fig. 3D). Although *ett* mutations that truncate within the DNA-binding domain appear to have dominant-negative activity (Pekker et al., 2005), the stop codon in *ett-7* is located at the extreme N terminus of this region, and should therefore eliminate most of this conserved domain. Whether the semi-dominant phenotype of *ett-7* results from haploinsufficiency or dominant-negative activity, this result implies that abaxial trichome production is sensitive to the dose of *ETT*. In summary, these results indicate that *ETT* and *ARF4* promote the expression of at least two juvenile traits - 'circular/elliptical' leaf morphology and the absence of abaxial trichomes- and are required for the effect of *zip-2* on these traits.

In addition to affecting leaf shape and abaxial trichome production, *zip* mutations increase hydathode number (Hunter et al., 2003). To determine whether *ett-7* also affects this trait, we introduced the hydathode marker E340 (Hunter et al., 2003) into an *ett-7* background. *ett-7* had no effect on the number of hydathodes on leaves 1 through 7, indicating either that upregulation of *ETT* is not responsible for this aspect of the *zip* phenotype, or that hydathode production is regulated redundantly by *ETT* and *ARF4*.

*zip* plants are often semi-sterile because stamen elongation is delayed relative to the elongation of the pistil, and because the septum is usually split near the tip of the carpels (Hunter et al., 2003). *ett-7* and *ett-15* also cause splitting of the septum, with *ett-7* having a much more severe phenotype than *ett-15*, whereas *arf4-2* has no observable carpel phenotype. *zip-2;ett-15* and *zip-2;arf4-2* double mutants had a lower frequency of septum splitting than did *zip-2*, suggesting that the effect of *zip-2* on septum development is attributable to the increased expression of *ETT* and *ARF4* in this mutant (Fig. 3C). *ett-15* had a less significant effect than did *arf4*, probably because *ett-15* causes septum splitting in the absence of *zip-2*. The fact that septum splitting is associated with both increased expression (in *zip-2*) and decreased expression (in *ett-7* and *ett-15*) of *ETT* may reflect the marginal origin of the septum (Liu et al., 2000): marginal outgrowth typically only occurs at the boundary of two distinct cell types, and loss of either cell fate can disrupt this process (Bowman, 2000). Indeed, this effect is consistent with the role of *ETT* and *ARF4* in the promotion of abaxial identity (Pekker et al., 2005).

### ***ETT* has a non-redundant function in leaf polarity**

In contrast to *kan1* mutants, which have up-curved or unusually flat leaves (Fig. 4A) (Kerstetter et al., 2001), *ett* and *arf* have little or no effect on leaf expansion as single mutants and are therefore thought to have redundant functions in leaf polarity (Pekker et al., 2005). To further explore this theory, we examined the morphology of the mesophyll cells in leaf 6 of wild type, *kan1-12*, *ett-7*, *ett-15* and *arf4-2* (Fig. 4B). Adaxial (palisade) mesophyll cells of wild-type

plants are round and tightly packed, whereas abaxial (spongy) mesophyll cells are highly convoluted and have abundant intercellular spaces. *kan1-12* has a minor effect on adaxial cell size, but simplifies the morphology of abaxial mesophyll cells and reduces the amount of intercellular space in this tissue, causing it to resemble adaxial mesophyll (Kerstetter et al., 2001). *arf4-2* and *ett-15* did not have a major effect on mesophyll morphology, but *ett-7* produced a noticeable reduction in the irregularity of spongy mesophyll cells. This observation correlates with the observation that *ett*, but not *arf4*, can correct the abaxialized phenotype of *ANT::KAN2* plants (Pekker et al., 2005). The observation that *ett-7* has a more dramatic effect on mesophyll cell shape than *ett-15* is consistent with the strength of their floral phenotypes. These results demonstrate that *ETT* promotes the differentiation of abaxial tissue in the leaf blade, and that this process is more sensitive to the loss of *ETT* than to the loss of *ARF4*.

### **zip upregulates *ETT* by blocking *tasiR-ARF* production**

The *ETT* and *ARF4* transcripts are targets of a ta-siRNA from the *TAS3* locus, *tasiR-ARF*, and accumulate in mutants that block the production of this ta-siRNA (Allen et al., 2005; Peragine et al., 2004; Williams et al., 2005). Both of these transcripts are also upregulated in *zip*, and the isolation of loss-of-function mutations of *ETT* and *ARF4* as suppressors of *zip* suggests that this increase plays a key role in the *zip* phenotype. The basis for this increase is unclear, however, because *zip* does not affect the accumulation of any of the siRNAs that have been examined, including ta-siRNAs from the *TAS1* and *TAS2* loci (Allen et al., 2005; Peragine et al., 2004; Vazquez et al., 2004; Williams et al., 2005; Yoshikawa et al., 2005). Whether *ZIP* is required for the biogenesis of *tasiR-ARF* is unknown.

To determine whether *ZIP* regulates *ETT* and *ARF4* expression via the ta-siRNA pathway, we examined the effect of *zip-2* on the expression of *tasiR-ARF* and *mir390*, a miRNA that is involved in the biogenesis of *tasiR-ARF* (Allen et al., 2005). *sgs3-11* and *rdr6-11* had no effect on the level of *mir390*, but the level of this miRNA was slightly increased in *zip-1*. All three mutations dramatically reduced the level of *tasiR-ARF* and produced a corresponding loss of *tasiR-ARF*-mediated cleavage of *ETT*, as revealed by RLM-RACE (Fig. 5A). As expected (Yoshikawa et al., 2005), *siR1511* was absent in *sgs3-11* and *rdr6-11*, but present in *zip-2*. These results suggest that *ZIP* is required for the biogenesis of siRNAs from *TAS3*, and also indicate that *ZIP* either directly or indirectly represses the transcription or biogenesis of the miRNA (*mir390*) that contributes to the generation of these siRNAs. The observation that *sgs3* and *rdr6* do not affect *mir390* suggests that this effect is not mediated by a ta-siRNA, as *SGS3* and *RDR6* appear to be generally required for ta-siRNA biogenesis.

Based on its mutant phenotype, we originally suggested that *zip* acts in a pathway that sets the threshold for the juvenile-to-adult transition, rather than being a component of the developmental ‘clock’ that initiates this transition (Hunter et al., 2003). This hypothesis was based on the observation that *zip* affects the onset of the juvenile-to-adult transition (e.g. abaxial trichome production, hydathode number) without affecting the number or the character of transition leaves. This is in contrast to mutations, such as *hst*, which accelerate both the onset of phase change and the rate at which this process occurs (Telfer and Poethig, 1998). To test this hypothesis, we examined the level of *tasiR-ARF*, *ETT* and *ARF4* at different times in shoot development. Northern analysis revealed that the *tasiR-ARF* and *ARF3* transcripts are present at a relatively constant level during the first three weeks of growth in plants grown in continuous light (Fig. 5B). To obtain a more accurate picture of the expression of *ETT* and *ARF4*, we performed semi-quantitative RT-PCR on 3-mm and 6-mm long leaf primordia of leaves 1 through 8 from *zip* and wild-type plants grown in either short days (Fig. 5C) or continuous light (Fig. 5D). Both transcripts were more abundant in *zip* than in wild type, and this difference was accentuated in short days. However, in both genotypes, there was no apparent difference in the level of *ETT* or *ARF4* mRNA in successive leaves. These results suggest that *tasiR-*

*ARF* constitutively represses *ETT* and *ARF4*, and they support the hypothesis that *ZIP* sets the threshold at which leaves respond to a temporal signal (via its effect on the level of *ETT* and *ARF4*), rather than by regulating the production of this signal.

### Overexpression of *ETT* causes *zip* and *as2*-like phenotypes

To determine whether *ETT* upregulation is the primary cause of the *zip* phenotype, we attempted to phenocopy *zip* by overexpressing *ETT* in wild-type plants. For this purpose, we produced transgenic plants expressing a wild-type *ETT* cDNA, or cDNAs in which both *tasiR-ARF* target sites (Allen et al., 2005; Williams et al., 2005) were mutated (Fig. 6A), under the regulation of the 35S promoter. The mutations that were introduced in the *tasiR-ARF* target sites do not affect the amino acid sequence of the protein, but are expected to eliminate or diminish the ability of *tasiR-ARF* to repress *ETT*. *35S::ETT* produced a lower frequency of morphologically aberrant phenotypes than did *35S::ETTmAB*, and also had a less significant effect on abaxial trichome production. Twenty-seven percent of *35S::ETTmAB* plants produced abaxial trichomes at an abnormally early position (leaf 1-4), as compared with 8% of plants expressing *35S::ETT* (Fig. 6B); furthermore, only *35S::ETTmAB* produced plants with abaxial trichomes on leaf 1 or 2. Many plants transformed with either *35S::ETT* or *35S::ETTmAB* had abnormally late trichomes, presumably reflecting a co-suppression of *ETT* by these overexpressed transgenes. RT-PCR analysis demonstrated that the severity of the mutant phenotype in *35S::ETTmAB* transformants was correlated with the level of *ETT* expression, and also revealed that many plants that appeared phenotypically normal had equivalent, or higher, levels of *ETT* than did *zip* plants (Fig. 6C). This latter result suggests that *ARF4*, or another as yet unknown target of *ZIP*, also contributes to the precocious phenotype of *zip* mutations.

About 16% of the *35S::ETTmAB* primary transformants resembled *zip* in that they had elongated, epinastic leaves and abaxial trichomes on leaf 3 or 4 (Fig. 6D). These plants also resembled *zip* in having flowers with short stamens and split replums (Fig. 6F). Plants with the highest levels of *ETT* had a more severe phenotype that was strikingly similar to that of mutations in *asymmetric leaves 2 (as2)* (Iwakawa et al., 2002; Ori et al., 2000; Semiarti et al., 2001) and *blade-on-petiole1* (Ha et al., 2003) (Fig. 6D-F). These plants were very small, with tightly curled, deeply lobed leaves that produced abaxial trichomes starting with leaf 1 or 2. Many leaves also had small leaflets extending from the petiole; this phenotype was apparent on leaf 3 or 4 and became more severe on successive leaves. Petal elongation was delayed in severely affected plants, and fully mature flowers had outwardly curved, irregularly positioned petals (Fig. 6F). These flowers had short stamens that often failed to produce pollen, but had relatively normal carpels with a low frequency of septum splitting.

## DISCUSSION

Heteroblasty is regulated by the interaction between a developmental timing mechanism and morphogenetic pathways that respond to this mechanism. The identification of *ETT* and *ARF4* as targets of the heterochronic gene *ZIP* provides the first insight into how these two processes are connected in *Arabidopsis*. *zip* was originally identified as a mutation that altered the threshold for the juvenile-to-adult transition, and encodes *ARGONAUTE7*, a member of a family of genes with known roles in the processing of small RNAs (Hunter et al., 2003). Here, we show that the increase in *ETT* and *ARF4* expression in *zip* (Peragine et al., 2004) is a consequence of a reduction in the level of a ta-siRNA from the *TAS3* locus, *tasiR-ARF*, and demonstrate that these transcription factors are responsible for the precocious phenotype of *zip*. Our results also indicate that *tasiR-ARF* may act as a temporally constitutive repressor of *ETT* and *ARF4*, and that the level of *tasiR-ARF* determines the sensitivity of leaf primordia to the signal(s) responsible for the temporal variation in leaf morphology.

### ***ETT* and *ARF4* promote adult leaf traits**

*ETT* and *ARF4* have been studied primarily for their role in organ polarity. *ett* mutations affect both the polarity and number of floral organs (Sessions et al., 1997; Sessions and Zambryski, 1995), and *ett;arf4* double mutants have a vegetative phenotype that is characteristic of adaxialized mutants, such as *kan1;kan2* or *phb-D* (Alvarez et al., 2006; Eshed et al., 2004; McConnell and Barton, 1998; Pekker et al., 2005). The discoveries that loss-of-function mutations in *ETT* and *ARF4* partially suppress the phenotype of *zip*, and that overexpression of *ETT* in wild-type plants phenocopies *zip*, demonstrate that overexpression of *ETT* and *ARF4* is largely responsible for the precocious phenotype of *zip*, and points to a relationship between leaf polarity and heteroblasty.

Many, if not most, heteroblastic aspects of leaf morphology are polarized along either an adaxial-abaxial or proximodistal axis. For example, the increase in leaf epinasty that occurs during rosette development reflects an increase in the growth rate of adaxial tissue relative to the growth rate of abaxial tissue. Similarly, the differential distribution of trichomes on rosette and inflorescence leaves is a consequence of an inherent difference in the developmental potential of the adaxial and abaxial epidermis (Telfer et al., 1997). It is not surprising, therefore, that genes involved in the specification of leaf polarity have a role in heteroblasty. But, while the ability of *ett* and *arf4* to suppress epinasty is readily explained by their adaxialized phenotype, their effect on abaxial trichome production does not have this explanation. Trichomes are absent or less abundant on the abaxial than on the adaxial surface of rosette leaves, and mutations that cause adaxialization, such as *kan1-12* (Kerstetter et al., 2001) and *phb-D* (McConnell and Barton, 1998), typically enhance trichome production on the abaxial leaf surface. The delayed production of abaxial trichomes in *ett* and *arf4* is therefore inconsistent with an adaxialized phenotype. This phenotype may reflect a separable function of these genes, as *ett-7* and *ett-15* have almost equivalent effects on trichome production despite the much stronger effect of *ett-7* on leaf and gynoecium polarity. Similarly, *arf4-2* delays abaxial trichome production and affects leaf shape, but has no independent effect on leaf or carpel polarity. Whether the effect of *ett* and *arf4* on leaf shape is linked to their function in leaf polarity or some other developmental pathway is unclear. Whatever the case, the phenotype of these mutations individually and in combination with *zip* suggests that *ETT* and *ARF4* sit at a branch point in the mechanism of heteroblasty - linking a variety of morphogenetic pathways to one or more temporal regulatory signals (Fig. 7A).

How is the activity of *ETT* and *ARF4* regulated? Analyses of RNA levels in wild-type rosette leaves suggest that these genes are constitutively transcribed during vegetative development. Regulation by *tasiR-ARF* is clearly important, but because the abundance of *tasiR-ARF* does not change dramatically during vegetative development, this ta-siRNA cannot be a source of temporal information. If their activity is temporally regulated, this regulation must occur post-transcriptionally, and by a mechanism that does not involve *tasiR-ARF*. *ETT* has several upstream ORFs in its 5' UTR that negatively affect the translation of the coding region and appear to affect its activity in flowers (Nishimura et al., 2004). The fact that the *ETT* transcript is not localized in the leaf (Pekker et al., 2005), despite its clear role in abaxial-adaxial polarity, suggests that *ETT* is subject to additional levels of regulation during vegetative development as well. In addition to regulation at the level of translation, it is likely that *ETT* and *ARF4* activities are regulated by their interaction with other auxin-related proteins. The floral phenotype of *ett* can be partially phenocopied by the application of polar auxin transport inhibitors, and *ETT* is thought to establish boundaries between the stipe, ovary and style in response to auxin levels (Nemhauser et al., 2000). In the absence of auxin, ARF proteins that activate transcription are inhibited by binding to transcriptional repressors of the AUX/IAA family via domains III and IV. Auxin promotes the degradation of AUX/IAA proteins, freeing the ARF protein to promote transcription (Tiwari et al., 2003). However, both *ETT* and *ARF4*



have both been shown to act as transcriptional repressors, and *ETT* lacks domains III and IV and should therefore be incapable of interacting with AUX/IAA proteins (Tiwari et al., 2003; Ulmasov et al., 1999). It will be important to determine whether the activity of ARF4 is regulated by its interaction with AUX/IAA proteins and if ARF4 has a role in the activity of *ETT*.

### Is AS2 a target of the *zip* pathway?

*ETT* and *AS2* appear to play opposing roles in leaf polarity, with *as2* mutants having a weak abaxialized phenotype (Lin et al., 2003; Xu et al., 2003) and *ett* mutants having a weak adaxialized phenotype. The possibility that these genes may have closely related functions in leaf polarity is suggested by our observation that plants with high levels of ectopic *ETT* expression strongly resemble *as2* mutant plants. This conclusion receives additional support from the report that *rdr6* (which upregulates *ETT*) enhances the *as2* phenotype (Li et al., 2005), and the observation that overexpressing *AS2* produces a phenotype very similar to that of *ett;arf4* double mutants (Lin et al., 2003; Xu et al., 2003). All of these observations are consistent with a model in which *ETT* and *AS2* act to mutually repress the activity of each other. This repression does not appear to take place at a transcriptional level, however. Although *rdr6* upregulates *ETT* and enhances the *as2* phenotype, it does not alter *AS2* or *AS1* expression, nor do *as1* and *as2* alter the level of the *RDR6* transcript (Li et al., 2005). This would suggest that *AS2* is not a transcriptional target of *ETT* or *ARF4*, and that the interaction of these genes either takes place post-transcriptionally or at a convergent point in their pathways. Alternatively, the phenotype of *35S::ETTmAB* may reflect a role for *ETT* in the repression of *BLADE-ON-PETIOLE1 (BOP1)*, as mutations in *BOP1* resemble *as2* (Ha et al., 2003).

### The role of threshold genes in defining the juvenile-to-adult transition

Two classes of mutations have been identified in screens for mutants with defects in the juvenile-to adult-transition: those that affect the time at which adult traits are first produced without altering the length of the transition zone or the total number of leaves produced by the shoot, and mutations such as *hasty* (Telfer and Poethig, 1998) and *squint* (Berardini et al., 2001), which accelerate the appearance of adult traits, nearly completely eliminate transition leaves, and reduce leaf number. *zip* is an example of the first class of mutations, and we hypothesized that *ZIP* acts to establish a threshold for entry into the adult phase of development (Hunter et al., 2003). Loss of *ZIP* lowers this threshold, allowing the shoot to undergo phase change prematurely (Fig. 7B). The evidence that *ZIP*- and genes (*RDR6*, *SGS3* and *DCL4*) that have mutant phenotypes identical to *zip* - is required for the production of *tasiR-ARF* suggests that this threshold is set by ta-siRNA-mediated repression of *ETT* and *ARF4*. Loss of *tasiR-ARF* regulation in *zip*, *rdr6* and *sgs3* mutants, or as a result of introducing target site mutations in *ETT* (*35S::ETTmAB*), lowers or bypasses the threshold for entry into the adult phase. Although we did not determine the effect of repressing *ETT* and *ARF4* by overexpressing *tasiR-ARF*, loss-of-function mutations of these genes produce the expected prolonged juvenile phenotype, supporting the conclusion that the expression level of these genes plays a crucial role in this transition. This conclusion receives further support from the observation that *ett-7* has a semi-dominant (i.e. dose-dependent) effect on abaxial trichome production.

The independence of the threshold and clock mechanisms can be seen even in plants with the most severe phenotypes, e.g. *ett;arf4* double mutants (Pekker et al., 2005) and severely affected *35S::ETTmAB* plants, which still show the gradual changes in leaf morphology indicative of an intact developmental clock. This result is consistent with the phenotype of *zip*, *sgs3*, *rdr6* and *dcl4*, and demonstrates that *ETT* and *ARF4* transcript levels are important for leaf identity and morphogenesis, but that subsequent input from a temporal signal is needed to complete the regulation of heteroblasty and phase change.

## The function of the RNAi pathway in plants

In plants, RNAi has been studied primarily for its role in virus resistance (Baulcombe, 2004). The evidence that genes required for this process (*SGS3*, *RDR6* and *DCL4*) also affect vegetative phase change (Gascioli et al., 2005; Peragine et al., 2004; Xie et al., 2005; Yoshikawa et al., 2005) and salt sensitivity (Borsani et al., 2005) reveal that this regulatory mechanism is involved in a wider array of processes than has previously been recognized. However, mutations in these genes cause relatively subtle phenotypes - particularly in comparison to the highly pleiotropic phenotype of mutations in genes involved in miRNA biogenesis (Schauer et al., 2002). This may be a result of the redundancy in the pathways that produce siRNAs; however, we think this is unlikely because plants doubly mutant for *DCL3* and *DCL4* - the dicers that generate the vast majority of endogenous siRNAs - resemble *sgs3*, *rdr6* and *dcl4* (Gascioli et al., 2005). Furthermore, although these plants show severe stochastic effects in advanced generations - probably as a result of the reactivation of transcriptionally silenced genes (Gascioli et al., 2005) - these epigenetic effects cannot be the basis for rapid, reproducible developmental changes and physiological responses. This suggests that RNAi is either employed sparingly to regulate endogenous gene expression, or that only a few of its endogenous targets have important biological functions.

Given that other ARF genes are targets of miRNAs, and that the biogenesis of ta-siRNAs requires miRNA-directed cleavage, it is worth asking why *ETT* and *ARF4* are not direct targets of a miRNA. Is this simply an accident of evolution, or is there something special about ta-siRNA regulation that makes it particularly useful for some types of processes? There is evidence that miRNAs act in a spatially restricted fashion (Alvarez et al., 2006a; Parizotto et al., 2004; Schwab et al., 2006), whereas siRNAs are capable of moving long distances (Dunoyer et al., 2005; Himber et al., 2003; Schwach et al., 2005). Although this makes it tempting to think that ta-siRNAs may act as inducers of phase change (the vegetative equivalent of a 'florigen'), it is important to remember that ta-siRNAs repress adult development, and do not accumulate (or decline) over the course of development. The analogy may hold, however, in the power of siRNAs to act non-autonomously to coordinate developmental transitions that must take place simultaneously in a broad range of tissues. Mobile siRNAs play an important role in repressing transgene expression throughout a plant, and it is possible that they play a similar role in the regulation of endogenous gene expression. This makes them excellent candidates for establishing a uniform threshold for entry into the adult phase (Fig. 7B). Finding the additional targets of these ta-siRNAs, and understanding their interactions with the developmental clock, are important problems for future research.

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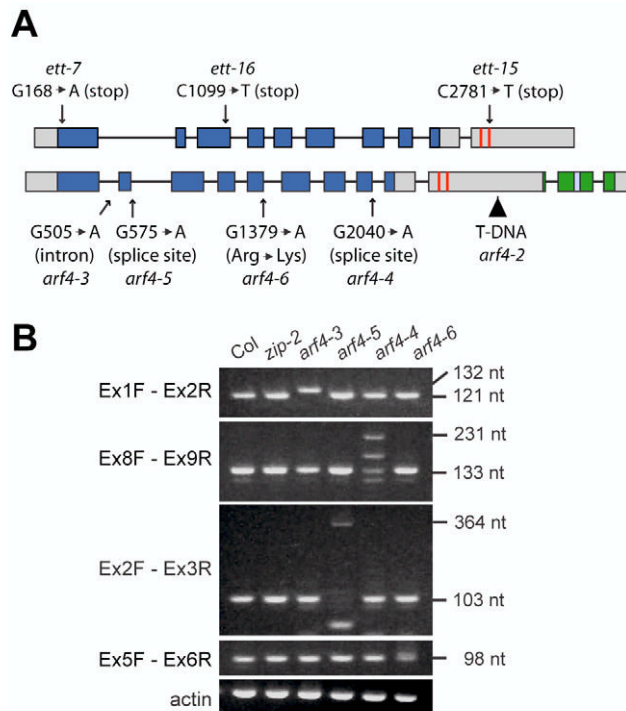
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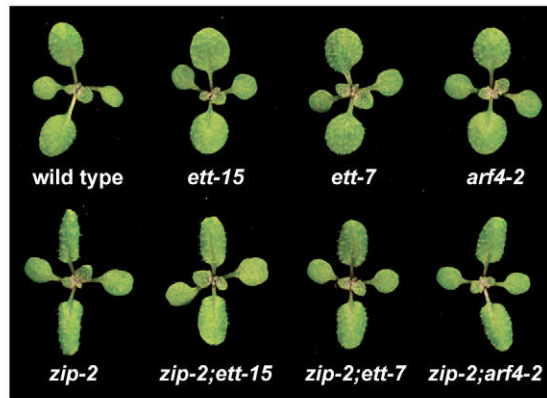
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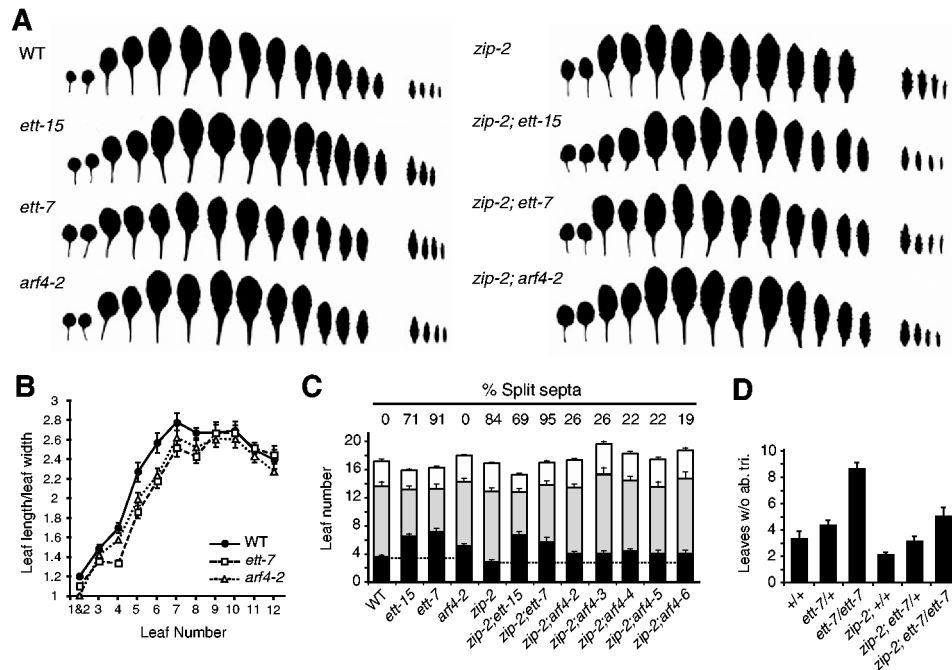
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**Fig. 1.** **The structure of wild-type and mutant alleles of *ETT* and *ARF4*.** (A) Genomic structure of *ETT* and *ARF4*, and the nature and position of mutant alleles of these genes. Grey box, exon; blue, conserved DNA binding domain; red, *tasi-ARF* target site; green, domains III and IV; triangle, T-DNA insertion. (B) RT-PCR analysis of *ARF4* mRNA in *arf4* mutants. PCR amplification was performed with exon primers that flanked the site of the mutation.

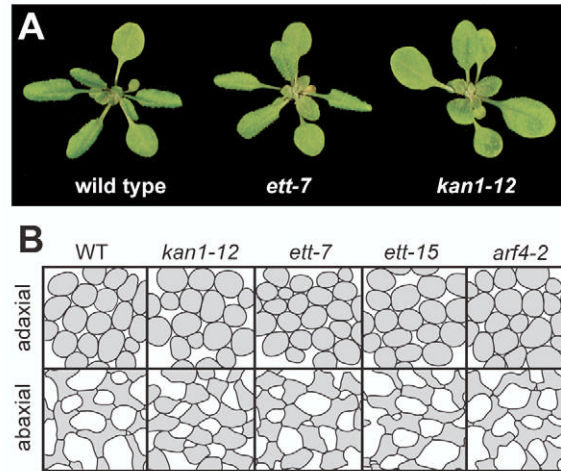


**Fig. 2.**  
***ett* and *arf4* mutations suppress the *zip* phenotype.** Photographs of 11-day-old plants showing the effect of *ett-15*, *ett-7* and *arf4-2* on leaf shape. In a wild-type background, these mutations cause a slight rounding and flattening of the first two leaves. In a *zip* background, they partially suppress the elongation of the lamina and the epinasty that results from the premature expression of adult traits in this mutant.

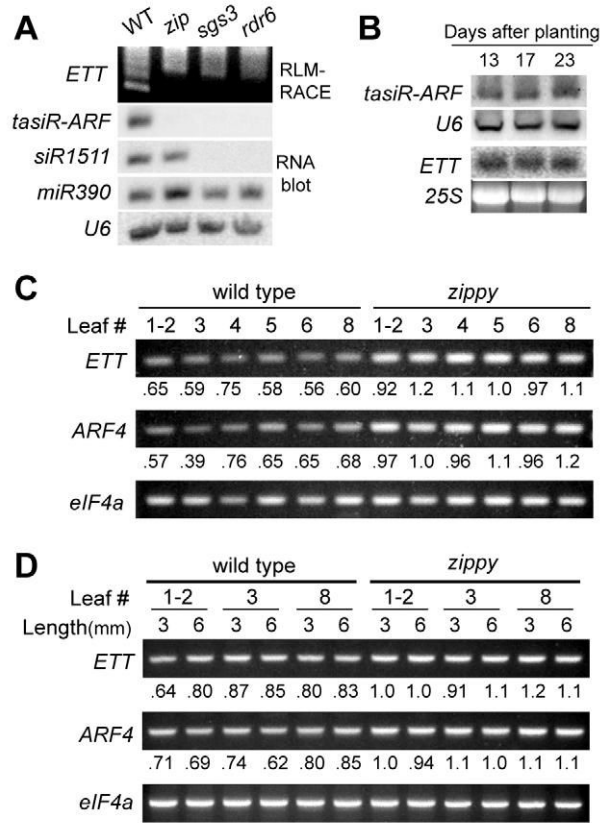


**Fig. 3.** *ett* and *arf4* affect leaf shape and trichome distribution. (A) Successive rosette and inflorescence leaves from wild-type and mutant plants, arranged from the base (left) to the tip (right) of the stem. *ett* and *arf4* suppress the narrow, elongated phenotype of the first two *zip* leaves. Later leaves of double mutants have rounder tips than *zip-2*, and the distal portion of the leaf blade is often wider than the proximal portion. *ett* and *arf4* do not affect the serration of the leaf blade. (B) The L:W ratio of the leaf blade of successive leaves of wild-type, *ett-7* and *arf4-2* plants ( $n=10$  plants of each genotype;  $\pm$ s.e.m.). In wild-type plants, this ratio increases gradually until leaf 8, after which it remains constant until flowering. *ett-7* and *arf4-2* cause leaves 4-7 to be slightly rounder than normal. (C) *ett-15*, *ett-7* and *arf4-2* increase the number of leaves without abaxial trichomes (black) in both a wild-type and a *zip* background. This is associated with a compensatory decrease in the number of adult leaves (grey;  $n \geq 18$ ,  $\pm$ s.e.m.). The number of cauline leaves is indicated by the white bar. The numbers above each bar represent the percentage of flowers with a split septum, based on an analysis of the first 10 flowers of five plants of each genotype. *ett-15* and *arf4* partially suppress the septum splitting observed in *zip*. Other *arf4* mutations also delay abaxial trichome production in a *zip-2* background. (D) The number of leaves without abaxial trichomes among plants segregating *ett-7* and *zip-2*. *ett-7* has a semidominant effect on abaxial trichome production.



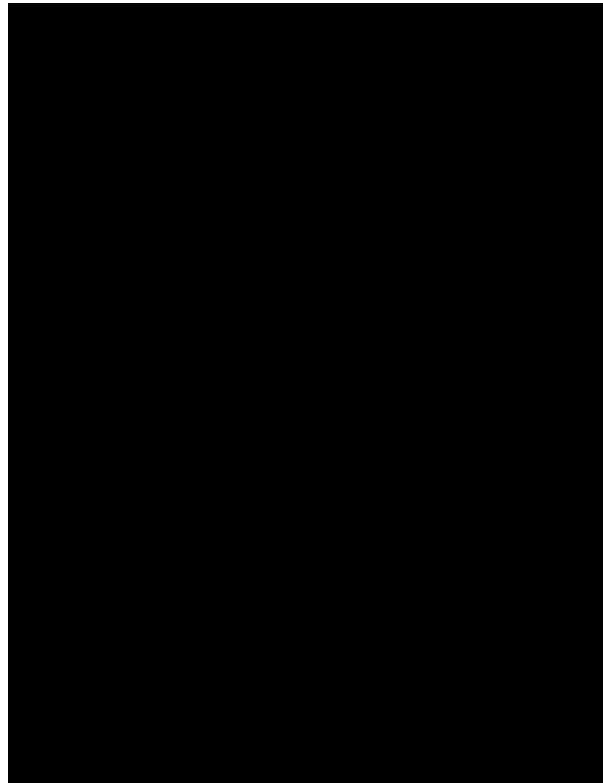


**Fig. 4.** *ett* has an adaxialized phenotype. (A) The morphology of 3-week-old wild-type, *ett-7* and *kan-12* plants. *kan-12* has flat leaves, whereas *ett-7* is not dramatically different from wild type. (B) Camera lucida drawings of adaxial and abaxial mesophyll cells of leaf 6 of wild-type and mutant plants. The adaxialized phenotype of *kan1-12* is shown for comparison. The phenotype of *ett-7* is slightly weaker than that of *kan1-12*. *ett-15* and *arf4-2* have a much less significant effect, if any, on the shape of abaxial mesophyll cells.



**Fig. 5.** *zip* reduces *tasiR-ARF* levels and increases *ETT* and *ARF4* expression. (A) Northern analysis of *zip-2*, *sgs3-11* and *rdr6-11* shows that all three reduce levels of *tasiR-ARF*, whereas only *sgs3* and *rdr6* reduce levels of the *TAS2* product *siR1511*. 5'RLM-RACE demonstrates that the reduction in *tasiR-ARF* correlates with a reduction in the cleavage of the *ETT* transcript. (B) Northern analysis of *tasiR-ARF* and *ETT* RNA at different times in rosette development in continuous light. (C) RT-PCR analysis of *ETT* and *ARF4* RNA from successive 3-mm leaves of wild-type and *zip* plants grown in short days. *zip-2* causes a consistent level of increase in both *ETT* and *ARF4* expression, but both genes are expressed uniformly in successive leaves. The ratio of the signal relative to the loading control *eIF4a* is shown. (D) RT-PCR analysis of *ETT* and *ARF4* RNA from leaves of wild-type and *zip-2* plants grown in continuous light.





**Fig. 7.**

**A model for temporal regulation of leaf morphology.** (A) Heteroblasty is regulated by the interaction between factors that change temporally during shoot development (developmental clocks) and by the pathways controlling leaf morphology. *ETT* and *ARF4* regulate a subset of the traits associated with vegetative phase change and heteroblasty. Their expression is repressed by the *tasiR-ARF*, the production of which requires *ZIP*, *SGS3*, *RDR6* and *DCLA*. Other heteroblastic traits, such as leaf serration and hydathode number, are regulated by another, as-yet-unknown, target of these four genes. (B) Expression of adult traits requires *ETT* and *ARF4*. *tasiR-ARF* creates a threshold for entry into the adult phase by constitutively limiting levels of *ETT* and *ARF4* transcripts. This threshold is lowered by mutations that block *tasiR-ARF* production (*zip*, *rdr6*, *sgs3*, *dcl4*). The developmental clock may progress to the adult phase by promoting *ETT* and *ARF4* translation or activity.