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The *Arabidopsis* Heterochronic Gene *ZIPPY* Is an *ARGONAUTE* Family Member

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Summary

Plants progress through a temporal sequence of juvenile, adult, and reproductive phases, each marked by the expression of phase-specific traits. Here we show that loss-of-function mutations in *ZIPPY (ZIP)* cause the premature expression of adult vegetative traits but do not accelerate the onset of reproductive competence or flowering time. *ZIP* encodes *ARGONAUTE7 (AGO7)*, one of ten members of the *ARGONAUTE* family in *Arabidopsis* [1]. In addition to playing developmental roles, some *ARGONAUTE* family members are required for RNAi-like phenomena, such as posttranscriptional gene silencing [2]. In contrast to *Arabidopsis ARGONAUTE1* [1], *ZIP* has no significant role in transgene silencing; its primary function is in the regulation of developmental timing.

Results and Discussion

In *Arabidopsis*, the juvenile phase of shoot development is marked by the production of small round leaves that have a smooth margin and lack abaxial trichomes [3]. Plants in the adult phase produce elongated leaves that curl downward, have a serrate margin and short petioles, and produce abaxial trichomes. The *ZIP* gene was identified in a screen for mutations that cause the precocious appearance of adult leaf traits. *zip* mutations cause the first two rosette leaves to become elongated and curl downward (Figure 1A) and also cause a forward shift in the expression of other phase-related traits, including leaf serration (Figure 1C) and the number of hydathodes per leaf ([4]; Figure 1D). Although these mutations affect all rosette leaves, their phenotype is most obvious in leaves 1 and 2 (Figure 1B).

Under constant light, *zip-1* and *zip-2* reduced the number of leaves without abaxial trichomes from 3.3 $\pm$ 0.2 to 2.1 $\pm$ 0.1 (Table 1) and produced abaxial trichomes almost 2 days earlier than Col (Figure 1E). Under short-day conditions, both Col and *zip-1* plants had delayed abaxial trichome production, but *zip-1* plants produced 4.5 $\pm$ 0.4 leaves without abaxial trichomes, whereas Col plants produced 7.9 $\pm$ 0.3 such leaves (Table 1). We conclude that *zip* causes a premature transition to the adult state but does not override environmental cues or cause leaves to unconditionally adopt an adult fate. Although *zip* accelerated the onset of abaxial-trichome production, it did not alter the gradual increase in their production; mutant and wild-type plants both produced about three transition leaves before finally producing leaves with a full complement of abaxial trichomes (Figure 1C). Similarly, *zip* produced a forward shift in hydathode number and leaf morphology but did not accelerate the rate at which these characteristics developed (Figure 1D). Thus, *zip* mutations appear to trigger an early transition to the adult phase without speeding up the developmental "clock" that regulates vegetative phase change.

Abaxial trichome production is also promoted by mutations that disrupt adaxial/abaxial leaf polarity [5, 6]. Unlike these mutations, *zip-1* does not disrupt the polarity of the mesophyll, nor does it affect leaf expansion in a manner that is consistent with a loss of abaxial cell identity. We observed no obvious difference in the shape of cells in the upper and lower mesophyll layers of leaves 1 and 2 in *zip-1* and wild-type plants (Figure 1H); furthermore, mutations that affect abaxial cell identity typically cause leaves to be flat, upcurled, or even radial [5, 6], not downwardly curled as in the case of *zip*. These observations indicate that the effect of *zip* on leaf morphology reflects a change in leaf identity, not leaf polarity.

Although *zip* mutants express adult vegetative traits precociously, they do not flower early under either long-day or short-day conditions (Table 1). In some experiments we observed a slight delay in the opening of the first flower, whereas in other experiments there was no significant difference in the timing of flower opening in *zip* versus wild-type plants. Furthermore, there was no significant difference in the total number of leaves in mutant versus wild-type plants grown under continuous light, and there was only a small decrease (36.1 $\pm$ 0.9 for *zip-1* versus 39.3 $\pm$ 0.7 for the wild-type) in the total leaf number in plants grown under short days. To determine if *ZIP* regulates reproductive competence, we examined the effect of *zip-1* on the flowering time of plants heterozygous for *35S::LFY*, a transgene that induces plants to flower soon after they enter the adult phase [7]. Although *zip-1;35S:LFY/+* plants produced one less leaf than their *35S:LFY/+* siblings (7.3 $\pm$ 0.2 versus 8.5 $\pm$ 0.2; $p < 0.01$), they actually flowered at approximately the same time as *35S:LFY/+* plants (21.1 $\pm$ 0.1 days versus 20.7 $\pm$ 0.1 days; $p = 0.07$). Because *zip* does not have correlated effects on flowering time and leaf number, we believe that the differences we observed—although statistically significant—are probably an indirect result of the effect of *zip* on vegetative morphology and floral morphogenesis, which may influence the cessation of vegetative growth and the timing of floral bud opening (see below).

Although *zip* has no major effect on the reproductive behavior of the shoot apical meristem, it does accelerate the appearance of leaf traits that are normally associated with floral induction. In particular, the last several rosette leaves in *zip* mutants resemble inflorescence leaves in having a strongly serrate base and a well-defined, dome-like apex (Figure 1C). *zip* plants also displayed an early plateau in hydathode development (Fig-

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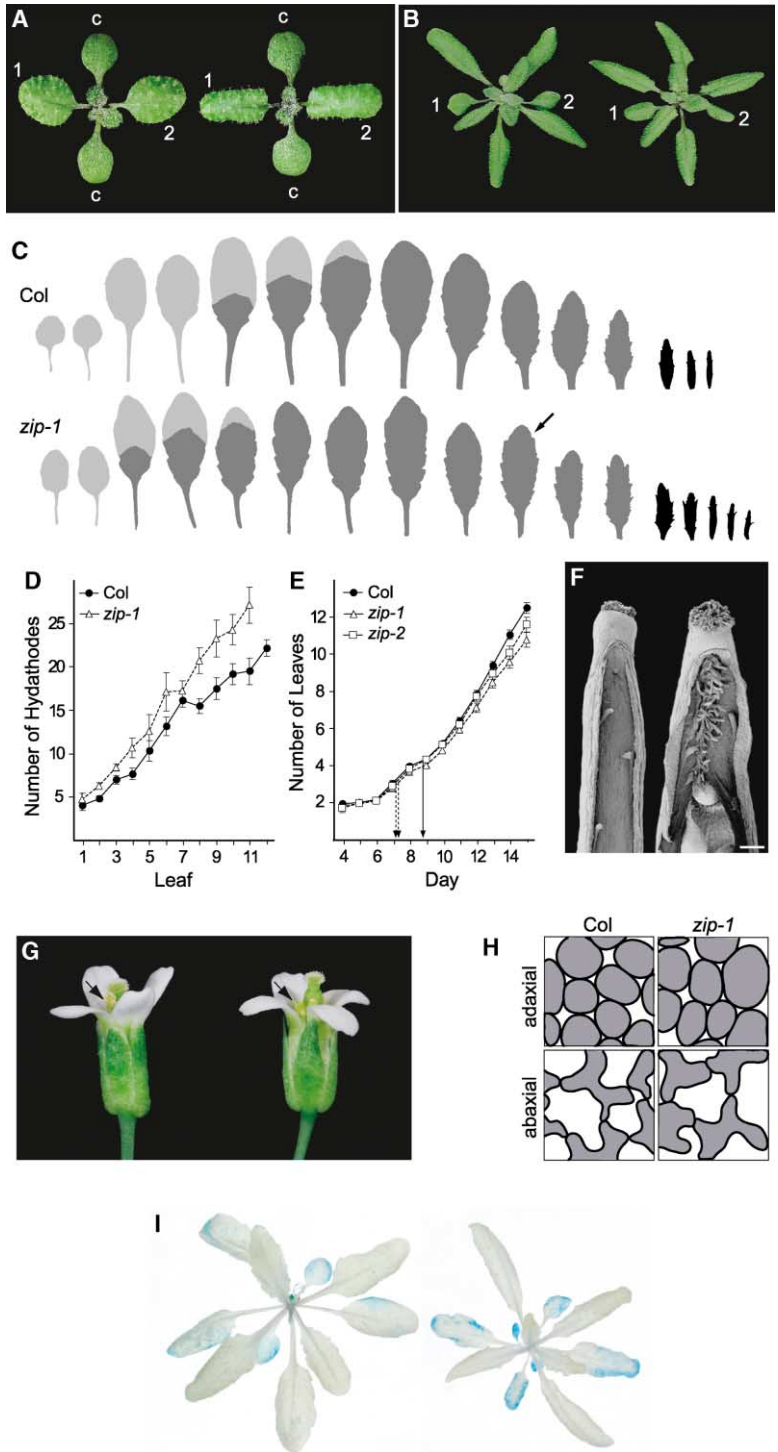

Figure 1. Phenotype of *zippy*

(A) Ten-day-old Col (left) and *zip-1* (right) plants. Cotyledons and the first two leaves are indicated.

(B) Eighteen-day-old Col (left) and *zip-1* (right) plants; the first two leaves are indicated.

(C) *zip-1* accelerates the appearance of leaves with an adult shape and adult pattern of abaxial trichomes. Leaves are shown in order of production, from left to right, and shaded to indicate no abaxial trichomes (light gray), abaxial trichomes (dark gray), or bracts (black). An arrow indicates a bract-like leaf tip.

(D) *zip-1* increases the number of hydathodes per leaf and accelerates the appearance of a brief plateau in hydathode number; this plateau occurs between leaves 6 and 7 in *zip-1* plants and between leaves 7 and 8 in wild-type plants.

(E) Col, *zip-1*, and *zip-2* plants have a similar rate of leaf initiation, but abaxial trichomes (arrow) appear earlier in *zip* plants.

(F) *zip-1* carpels (right, with valve and seeds removed) have a split septum and ectopic stigmatic tissue. The scale bar represents 200 μm.

(G) Col (left) and *zip-1* (right) flowers; arrows indicate stamens.

(H) *zip-1* does not affect mesophyll cell morphology in the first two leaves.

(I) *zip-1* (right) only slightly increases GUS expression in the L2 transgenic line.

ure 1D). These results may indicate that *zip* causes rosette leaf primordia to prematurely adopt a reproductive fate, without causing the shoot apical meristem to adopt a similar fate, and suggest that these tissues can respond independently to induction of the reproductive phase. Although it has no effect on flowering time, *zip* is required for floral morphogenesis; *zip* plants produce stigmatic tissue in the middle of the septum, which is often accompanied by splitting of the septum (Figure 1F). In addition, *zip* plants have variable seed-set because stamens frequently fail to contact the stigma (Figure 1G). We have not determined the basis of this defect, but because *zip-1* has no significant effect on mature stamen length (long stamens are 38.2 ± 0.1 mm in *zip-1* plants and 38.2 ± 0.1 mm in the wild-type; $n = 100$, $p = 0.54$), it probably results from a change in the timing of carpel or stamen elongation.

Mutations of *HASTY* (*HST*) [8, 9] and *SQUINT* (*SQN*)

Table 1. Effect of *zip* on Leaf Identity and Flowering Time

	Leaves without Abaxial Trichomes	Leaves with Abaxial Trichomes	Rosette Leaves	Bracts	Days to Flowering	n
Continuous Light						
Wild-type (Col)	3.3 ± 0.1	8.4 ± 0.2	11.8 ± 0.2	3.3 ± 0.1	22.8 ± 0.2	46
zip-1	2.1 ± 0.1[a]	10.1 ± 0.3[a]	12.2 ± 0.2	4.3 ± 0.1[a]	24.7 ± 0.3[a]	45
zip-2	2.1 ± 0.1[a]	9.9 ± 0.2[a]	12.0 ± 0.2	4.4 ± 0.1[a]	24.7 ± 0.3[a]	46
+/+ and +/*zip-1*(Col)[b]	3.4 ± 0.1	8.5 ± 0.2	11.9 ± 0.2	3.8 ± 0.1	22.4 ± 0.2	30
zip-1[b]	2.2 ± 0.2[a]	9.1 ± 0.3	11.3 ± 0.3	4.0 ± 0.1	23.0 ± 0.2	16
Short Days						
Wild-type (Col)	7.9 ± 0.1	31.3 ± 0.8	39.3 ± 0.7	7.3 ± 0.3	58.9 ± 0.7	21
zip-1	4.5 ± 0.2[a]	31.5 ± 1.0	36.1 ± 0.9[a]	7.9 ± 0.3	58.1 ± 1.1	21

[a] Significantly different from wild type (t test, $p < 0.01$). Values are ± SEM.

[b] Siblings from the cross +/*zip-1* × +/*zip-1* were characterized phenotypically, and their genotype was subsequently determined with a PCR assay, as described in the Experimental Procedures; because +/+ and +/zip-1 plants were not significantly different from one another ($p < 0.1$), these genotypes were pooled for this analysis.

[10] have a precocious phenotype like that of *zip* and appear to operate in independent pathways [9]. In order to determine the functional relationship between *ZIP* and these two genes, we examined the phenotype of double mutants. We found that *zip-1;sqn-1* plants had a stronger mutant phenotype than either single mutant parent (Figure 2A), whereas the phenotype of *zip-1;hst-1* plants was no more severe than that of *hst-1* (Figure 2B). *zip-1;sqn-1* plants produced abaxial trichomes on leaf 1.1 ± 0.1 (n = 24), compared to leaf 3.4 ± 0.3 (n = 22) for either single mutant. *hst-1;zip-1* double mutant plants produced abaxial trichomes on leaf 2.7 ± 0.3 (n = 20), which was not significantly different from findings with *hst-1* alone (2.9 ± 0.2, n = 20). These results suggest that *zip* acts in parallel to *SQN* and in the same pathway as *HST*.

ZIP maps near the marker *nga111* on chromosome 1; the approximate breakpoints of the fast-neutron-induced deletion mutations *zip-2* and *zip-3* limited *ZIP* to a 32 kb BAC F10D13 region containing seven predicted transcripts (Figure 3A). Sequencing of these genes in *zip-1* revealed a single base pair change that generated a premature stop codon in the predicted transcript of At1g69440 (Figure 3B). This allele is likely to be functionally null; it is predicted to delete more than half of the C-terminal conserved domain (see below) and is phenotypically indistinguishable from two deletions (*zip-2* and *zip-3*) that remove the entire gene. A T-DNA insertion (SALK_037458) in the first intron of At1g69440 has a phenotype similar to that produced by these three alleles. Transformation with an 8.4 kb genomic fragment containing At1g69440 (Figure 3A) rescued the phenotype of *zip-1* and *zip-2*, confirming that this gene corresponds to *ZIP*.

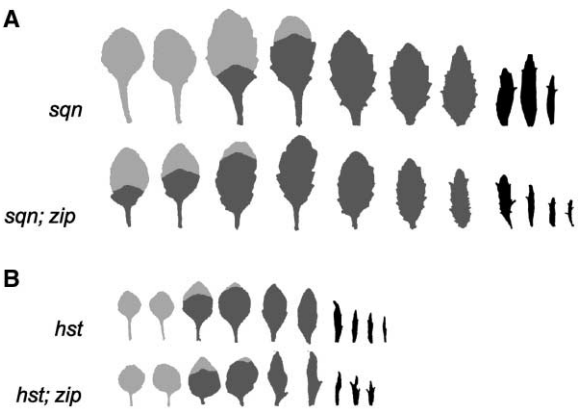

Figure 2. Genetic Interaction between *zip-1* and Other Mutations Affecting Vegetative Phase Change

(A) *zip-1* enhances the early adult phenotype of the *sqn-1* mutant, causing precocious serration of the leaf margin and abaxial trichome production.

(B) *zip-1* has little or no effect on the *hst-1* phenotype. Leaves are shown in order of production from left to right and are shaded to indicate no abaxial trichomes (light gray), abaxial trichomes (dark gray), or bracts (black). Both the *sqn-1* and *hst-1* mutations reduce the number of rosette leaves.

The *ZIP* transcript consists of three exons totalling approximately 3.2 kb and is predicted to encode a 990 amino acid protein containing a central PAZ domain and a C-terminal PIWI domain [11]. These two domains define the PPD class of proteins, of which there are ten in *Arabidopsis*. Phylogenetic analysis indicates that PPD proteins can be divided into a subgroup related to the *Drosophila* protein PIWI and a subgroup related to the *Arabidopsis* protein AGO1 [2]. All ten of the predicted *Arabidopsis* PPD-proteins (including AGO1 [12], AGO4 [13], and PNH [14, 15]) are in the AGO subgroup (Figure 3C); of these, ZIP is the only one with no close relatives.

We analyzed *ZIP* expression by using real-time RT-PCR (Table S1) because its mRNA was undetectable by Northern analysis. As controls for primer specificity, we measured expression in *zip-1,* which contains a premature stop codon, and *zip-2*, a deletion of the entire locus; expression was reduced by half in *zip-1* and was undetectable in *zip-2*. Very little *ZIP* transcript was detected in the roots of wild-type plants, consistent with the observation that *zip* has no phenotype in roots. Expression was highest in mature rosette leaves and slightly less in floral buds. The shoot apices of plants grown under short days to delay flowering showed a 2-fold increase in expression between 8 and 22 days after planting. This latter result is somewhat unexpected because the loss-of-function phenotype of *ZIP* suggests that it is required in the juvenile phase. However, the observation that *ZIP* is expressed throughout shoot development is consistent with its mutant phenotype and may indicate that

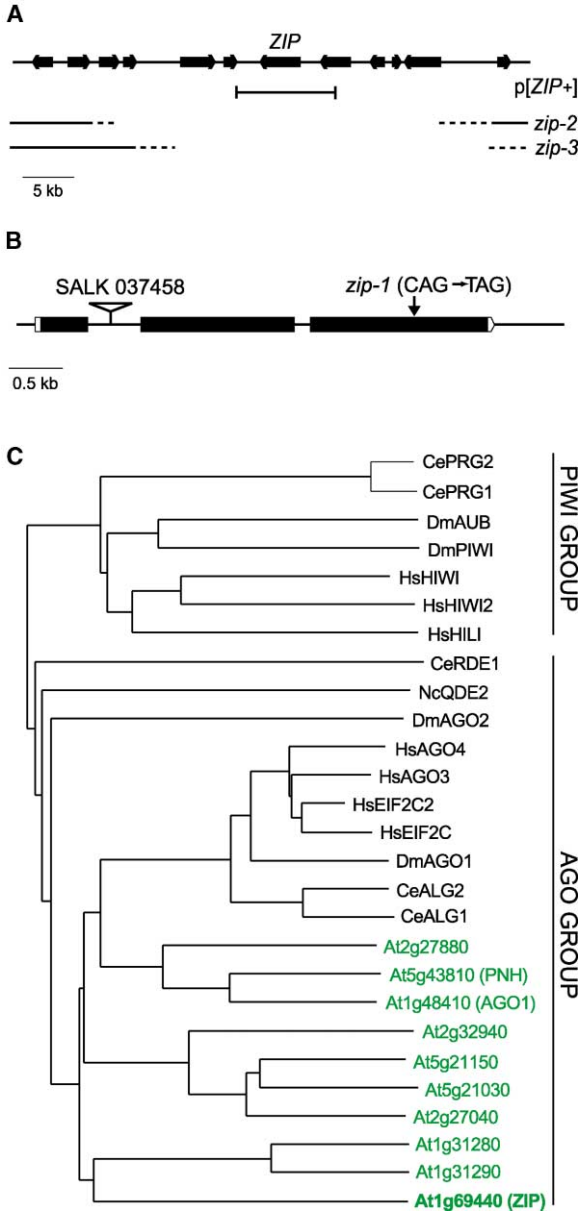

Figure 3. *ZIP* Is a Member of the *AGO* Family

(A) Chromosomal region surrounding *ZIP*. Approximate breakpoints of the *zip-2* and *zip-3* deletion alleles are shown, along with the genomic fragment, p[*ZIP*+], that was used to generate a rescue construct.

(B) The genomic structure of the *ZIP* transcript (oriented 5′ to 3′) showing the position of the T-DNA insertion and the *zip-1* point mutation. Exons are represented by thick lines; open areas represent the 5′ and 3′ UTRs.

(C) A bootstrap cladogram showing the relationship of ZIP to the predicted *Arabidopsis* PPD proteins (green) and other selected members of this family.

ZIP plays a permissive role in vegetative phase change, rather than a regulatory one.

PPD proteins have been shown to be involved in both the regulation of developmental pathways and RNAi-like phenomena such as posttranscriptional gene silencing (PTGS) of transgenes in plants [2]. In *Arabidopsis*, *AGO1* is required for development and PTGS [1, 12], *PNH* has developmental functions but is not required for PTGS [14–16], and *AGO4* is required for transcriptional gene silencing but not development [13]. To determine if *ZIP* plays a role in PTGS, we introduced *zip-1* into the L2 transgenic line. This line contains a 35S::GUS transgene that is silenced posttranscriptionally by a mechanism that depends on *AGO1* [1]. *zip-1* had no effect on the pattern of gene silencing during shoot development (Figure 1I) and produced little increase in GUS activity. Spectrophotometric analysis of *zip-1* and wild-type segregants in F2 families homozygous for the L2 transgene revealed that *zip-1* plants produced 39 $\pm$ 12 ng GUS/μg protein (n = 28), whereas their wild-type siblings produced 23 $\pm$ 10 ng GUS/μg protein (n = 27). By comparison, *ago1-1* plants in an L2 background had nearly ten times as much GUS activity as wild-type plants (275 $\pm$ 25 ng GUS/μg protein; n = 6). We conclude that *ZIP* plays little or no role in PTGS.

The phenotype of *zip* is significant for several reasons. The observation that *zip* has little or no effect on either flowering time or reproductive competence is consistent with the phenotype of other phase change mutations in *Arabidopsis* [10] and maize [17, 18] and indicates that vegetative maturation and floral induction are regulated by different pathways. This is similar to the situation in *C. elegans*, for which somatic maturation and reproductive development are regulated independently [19]. The phenotype of *zip* is also interesting because of its specificity. Other genes involved in vegetative phase change in *Arabidopsis* are required for many different processes in shoot development [8–10, 20–22]. Although *ZIP* plays a role in floral development, during vegetative growth its only obvious mutant phenotype is a defect in the regulation of the juvenile-to-adult transition. It is of course possible that the lack of additional phenotypes reflects a functional overlap between *ZIP* and other *PPD* genes in the *Arabidopsis* family. We think this is unlikely, however, because *ZIP* has no close relatives within this family (Figure 3C).

Members of the PPD protein family participate in a variety of regulatory mechanisms, including transcriptional silencing, posttranscriptional silencing, and both positive and negative translational regulation, with some PPD proteins having more than one of these functions [2]. Both genetic and biochemical evidence indicates that PPD proteins may act in conjunction with small regulatory RNAs, such as miRNAs and siRNAs [2]. In light of the genetic evidence that *ZIP* and *HST* act in the same pathway (Figure 2B), it is interesting to note that the mammalian ortholog of *HST*, *Exportin 5*, promotes the nuclear export of small double-stranded RNAs [23].

Although we have not yet identified the targets of ZIP, many of the miRNAs isolated from *Arabidopsis* display temporal variation in their level of expression [24–26] and could therefore play a role regulating vegetative phase change. Temporal fate in *C. elegans* is controlled by the miRNAs *lin-4* and *let-7* [27, 28]. Although there are no *Arabidopsis lin-4* or *let-7* homologs [29], there is an intriguing similarity between the phenotype of *zip* and the phenotype of mutations in the *C. elegans* Argonaute-like genes, *ALG-1* and *ALG-2*; animals mutant for both

of these genes have a heterochronic phenotype associated with a reduction in levels of the *lin-4* and *let-7* miRNAs but do not display a defect in PTGS (RNAi) [30]. Whether ZIP is also required for the biogenesis and/or activity of miRNAs should become apparent from future studies of this and other genes that have similar effects on vegetative phase change in *Arabidopsis*.

Experimental Procedures

Plant Materials and Phenotypic Analysis

All plants are in the Columbia background unless otherwise indicated. *zip-1* was generated by EMS mutagenesis; *zip-2* and *zip-3* were generated by fast-neutron mutagenesis as described [31]. The sequence-indexed T-DNA insertion SALK_037458 was generated by Jose Alonso and Joe Ecker (Salk Institute) and was obtained from the *Arabidopsis* Biological Resource Center. *zip-1* was crossed to *sqn-1* [10] or *hst-1* [9]; *zip* plants were selected in the F2 generation and scored for the *sqn* or *hst* phenotype in the F3 generation. Plant growth, phenotypic analysis, and microscopy were carried out as described [9]. The interaction between *zip-1* and *35S::LFY* was examined with siblings from the cross +/*zip-1* × +/*zip-1;35S::LFY*/+ (*35::LFY* was a gift from D. Wiegel). Plants heterozygous for *35S::LFY* were identified by the dominant inflorescence phenotype of this transgene; the allelic constitution at the ZIP locus was determined by a PCR assay that took advantage of the BsaI polymorphism introduced by *zip-1*. Hydathodes were visualized by a cross of *zip-1* to the GFP enhancer trap line E340 (http://enhancertraps.bio.upenn.edu). The statistical significance of differences between genotypes was determined with T tests.

Cloning and Genomic Rescue

We mapped *zip* near the marker *nga111*by using an F2 population from a cross of *zip-1* to Landsberg *erecta* (Ler). Additional SSLP markers generated with Ler sequence information provided by Cereon allowed us to further refine its position to a region encompassed by the BAC F10D13. PCR analysis showed that *zip-2* and *zip-3* represent overlapping deletions. Sequencing of candidate genes within the overlapping region revealed a C-to-T change at position 2308 of the ORF of At1g69440 in the EMS-induced allele, *zip-1*. *zip-1* is distinguishable from the wild-type allele by the BsaI polymorphism generated by this mutation. cDNA isolation was carried out as described [31]; an additional cDNA clone, SQ053e1, was obtained from the Kazusa DNA Research Institute. Because the cDNAs did not span the entire ORF, RT-PCR products were used for determining the sequence of the remaining coding region and a portion of the 5′ UTR. The 3′ UTR was isolated by RACE as described in [31]. We created the genomic rescue construct by isolating an 8.4 kb PstI-KpnI fragment from the BAC F10D13 and inserting it in the PstI and KpnI sites of pCAMBIA2300 (Cambia). Plant transformation and selection was carried out as described [31]. Alignments were generated by bootstrap analysis (1000 replications) with Clustal X 1.81. *Arabidopsis* sequences are available from www.tigr.org/tdb/e2k1/ath1/LocusNameSearch.shtml, all other sequences are as in [2].

Expression Analysis

Tissue isolation and RNA extraction was done as described [31]. PolyA RNA was purified with the MicroPoly(A)Purist kit (Ambion), and amplification was carried out in a DNA Engine Opticon (MJ Research) with the QuantiTect SYBR Green RT-PCR kit (Qiagen). *eIF4*α was amplified with the primers 5′-CTCAGGGGTATTTATGCT TATGGTTTTGAGAAGCC-3′ and 5′-CCAGCTGCTGCAAGACACCA GAGC-3′, and *ZIP* was amplified with the primers 5′-CCTCAATCTT GTTGCCTCAAATCTTCCTTCTAGC-3′ and 5′-GGTGTTGCTGCTGTT CTCTCCACAACTTGAG-3′. Expression was assayed in at least two 100 ng samples and four 50 ng samples for each tissue. Quadruplicate standard curves were prepared for both primer sets by determination of the threshold cycle (C_T) for 5-fold serial dilutions of Col RNA from 2 ng to 250 ng. For *ZIP* the log (units) were $-0.404C_T+9.58$ and for eIF4α the log (units) were $-0.255C_T+4.93$.

Transgene Silencing Assay

We assayed transgene silencing by crossing *zip-1* or *ago1-1* [12] to the L2 line [1] (a gift of H. Vaucheret) and then selecting F3 families that were homozygous for *35S::GUS* and segregating these mutations. Protein was extracted from individual plants in 5 mM Na_2HPO_4, 10 mM β-mercaptoethanol, 10 mM EDTA, and 0.1% SDS. Protein concentration was determined with the Bio-Rad Protein Assay, and 10 μg of protein was added to 1 ml of 1 mM PNPG in 50 mM Na_2HPO_4 and 10 mM β-mercaptoethanol and incubated at 30°C for 5 min before the OD^{405} was measured. A standard curve for the PNPG assay was prepared with *E. coli* β-glucuronidase (Roche).

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