Report

Specification of Leaf Polarity in *Arabidopsis* via the *trans*-Acting siRNA Pathway

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Summary

Plants leaves develop proximodistal, dorsoventral (adaxial-abaxial), and mediolateral patterns following initiation. The Myb domain gene *PHANTASTICA* (*PHAN*) is required for adaxial fate in many plants [1, 2], but the *Arabidopsis* ortholog *ASYMMETRIC LEAVES1* (*AS1*) has milder effects, suggesting that alternate or redundant pathways exist [3, 4]. We describe enhancers of *as1* with more elongate and dissected leaves. As well as *RDR6*, an RNA-dependent RNA polymerase previously proposed to influence *as1* through microRNA [5], these enhancers disrupt *ARGONAUTE7* (*AGO7*)/*ZIPPY*, *SUPPRESSOR OF GENE SILENCING3* (*SGS3*), and *DICER-LIKE4* (*DCL4*), which instead regulate *trans*-acting small interfering RNA (ta-siRNA) [6–12]. Microarray analysis revealed that the *AUXIN RESPONSE FACTOR* genes *ETTIN* (*ETT*)/ *ARF3* and *ARF4* were upregulated in *ago7*, whereas *FILAMENTOUS FLOWER* (*FIL*) was upregulated only in *as1 ago7* double mutants. *RDR6* and *SGS3* likewise repress these genes, which specify abaxial fate [13–17]. We show that the *trans*-acting siRNA gene *TAS3*, which targets *ETT* and *ARF4*, is expressed in the adaxial domain, and *ett as1 ago7* triple mutants resemble *as1*. Thus *FIL* is downregulated redundantly by *AS1* and by *TAS3*, acting through *ETT*, revealing a role for ta-siRNA in leaf polarity. *RDR6* and *DCL4* are required for systemic silencing, perhaps implicating ta-siRNA as a mobile signal.

Results and Discussion

ta-siRNA Pathway Mutants Enhance *as1* Leaf-Patterning Defects

In a screen for second-site mutations that enhance *as1* leaf-shape defects, several classes of modifiers were identified (M.E.B and R.A.M., unpublished data). In one class, 18 mutants had similar or identical phenotypes. These mutants fell into at least four complementation groups, and included *rdr6* [18, 19], which has been previously reported to interact with *as1* [5]. Four mutations in *RDR6* were premature stop codons, whereas one was

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an amino acid substitution highly conserved among RNA-dependent RNA polymerases (Figure S1 in the Supplemental Data available online). An enhancer-trap insertion (ET5446) into *AGO7*/*ZIP* (*ago7-m5446*, also known as *ago7-jason*) was also found to enhance *as1* (Figure 1) and triple *as1 rdr6 ago7* mutants were indistinguishable from either double mutant, consistent with these genes being in the same pathway (data not shown). Although leaves largely retained dorsoventrality, lobing was dramatically enhanced such that leaves were dissected by ectopic margins (Figure 1). These margins tended to coincide with the vasculature, whose pattern was severely disrupted in the mutants (data not shown). Floral organs were also affected by lobing of sepals, abaxial curling of petals, and prominently dissected stigma of the gynoecium. The double mutants produced little pollen and were sterile (Figure S2).

Members of the class III HD-ZIP putative sterol/lipid binding transcription factors, including *PHABULOSA*, *PHAVOLUTA*, and *REVOLUTA*, influence leaf shape by specifying adaxial identity [20–24]. Expression is restricted to the adaxial domain by *KANADI* (*KAN*) genes within the GARP family of transcription factors [25–27]. The microRNA *miR165*, which regulates class III HD-ZIP gene expression through transcript cleavage [28], is expressed in the abaxial domain in older primordia and also downregulates *PHB* [29, 30]. In addition to *KAN* genes, further determinants of abaxial fate are the redundantly acting AUXIN RESPONSE FACTOR genes *ETTIN* (*ETT*)/*ARF3* and *ARF4* [17], as well as members of the YABBY gene family, which encode HMG-box zinc-finger proteins, most notably *FILAMENTOUS FLOWER* (*FIL*)/*YAB1* and *YAB3* [13–16].

It was previously proposed that the enhancement of *as1* by *rdr6* was accounted for by misregulation of *miR165* [5], but rather than regulating microRNA, *RDR6* is required for the generation of *trans*-acting small interfering RNA (ta-siRNA) and cooperates with *AGO7*/ *ZIP*, *SGS3*, and *DCL4* [6–11, 31]. We identified, via allelism analysis and sequencing, one additional allele of *ago7* and two alleles of *sgs3* among the enhancers (Figure S3). These interactions were confirmed by crossing *as1* into strains in which the *DCL4* and *SGS3* genes had been disrupted, and by similar crosses with *asymmetric leaves 2 (as2)*, which encodes the LOB-domain interacting partner of AS1 [32, 33] (Figure S3). The phenotypes were indistinguishable between each of the double mutants, although phenotypes in *as1 sgs3* displayed greater expressivity than the other double mutants.

ta-siRNA-Dependent Leaf Defects in *as1* Are Not Mediated by KNOX Genes

The KNOX gene *SHOOT MERISTEMLESS* (*STM*) is required to maintain an indeterminate cell fate in the shoot apical meristem and acts redundantly with the close relative *BREVIPEDICELLUS* (*BP*) [34, 35]. When ectopically expressed in lateral organs, KNOX genes mediate
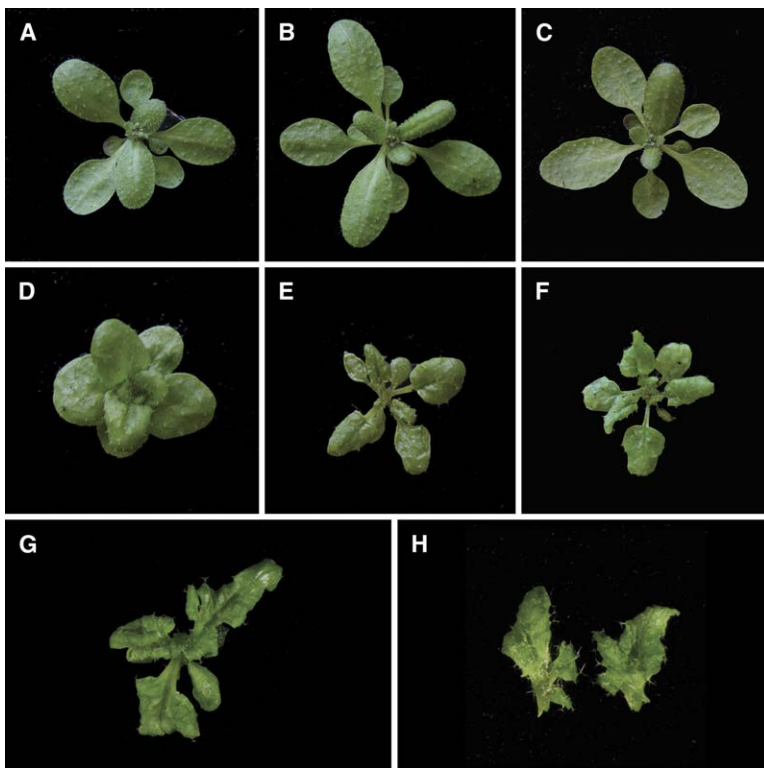
Figure 1. Second-Site Enhancers of *as1* Leaf-Shape Defects Include *rdr6* and *ago7*

Rosettes from 21-day-old plants of wild-type L*er* (A), *ago7-m5446* (B), *rdr6-16* (C), *as1-1* (D), *as1-1 ago7-m5446* (E), and *as1-1 rdr6-16* (F). (G and H) show individual leaves from *as1-1 ago7-m5446*. Double mutants have smaller, more dissected leaves than *as1* alone. Multiple alleles of *rdr6*, *ago7*, and *sgs3* were obtained by scoring for enhanced leaf-shape defects following EMS mutagenesis in an *as1-1* background (Figure S1). Disruptions of *dcl4* and *sgs3* had similar phenotypes (Figure S3).

patterning defects and leaves are highly lobed [36, 37]. *AS1* negatively regulates class I KNOX homeodomain transcription factors in lateral organs [3, 4, 32, 38]. Although loss of *STM* or combined loss of *BP* and *KNAT2* does not suppress the *as1* phenotype [35], increased lobing in *as1 rdr6* plants could be due to enhanced KNOX gene activity, and this was also previously proposed [5].

We first assayed *BP* accumulation in *as1* and *as1 ago7* (Figure S2J), but we could not detect further overexpression in the double mutants, although we could not rule out changes in transcript localization [5]. *STM* was also slightly upregulated in *as1* whole seedlings, in contrast with leaves [35], but no additional upregulation was observed in the *as1 ago7* double mutants, and the level of upregulation was too low to be detected by microarray analysis (see below). Next, we generated triple mutants with *stm* and *bp*. Strong *stm* mutants fail to form a shoot as a result of loss of meristem function, whereas weak alleles, such as *stm-2*, produce leaves from a disorganized shoot [34, 39]. This partial loss of *STM* function did not alter leaf shape in *as1 rdr6 stm-2* triple mutants, which appeared additive (data not shown). *bp* has defects in shoot architecture, as well as in the pedicel [35, 40, 41], but loss of *BP* did not affect leaf patterning in *as1 rdr6 bp* and *as1 ago7 bp*, although sterility in *as1 rdr6* double mutants was occasionally rescued in *as1 rdr6 bp* triple mutants (Figure S2).

as1 ago7 Leaf-Patterning Defects Are Mediated by *ETTIN*

Rather than pursuing additional candidate genes, we sought targets responsible for the double-mutant phenotype by using the *Arabidopsis* ATH1 microarray (Table S1). Transcript levels in vegetative shoots of wild-type plants, *as1*, *ago7*, and *as1 ago7* were compared. As expected, *BP* was strongly upregulated in *as1* mutants compared to wild-type plants, but no additional upregulation was observed in *as1 ago7* double mutants. *ETT* and *ARF4* were upregulated significantly in *ago7* as previously described [8] and were slightly further increased in *as1 ago7* double mutants. *FIL* was not upregulated in *as1* or in *ago7* single mutants, but was strongly upregulated in the double mutant (Tables S1 and S2) as previously described for *as2 rdr6* [5]. Expression levels of *ETT*, *ARF4*, and *FIL* in *as1*, *ago7*, *rdr6*, and *sgs3* single and double mutants were validated by quantitative RT-PCR (Figure 2A). *ETT* and *ARF4* were upregulated in *ago7*, *rdr6*, and *sgs3* single mutants and further increased in all three double mutants with *as1* (Figure 2A). *FIL* was upregulated in all three double mutants but not in single mutants.

ETT is expressed on the abaxial side of the leaf, where it specifies leaf polarity [17, 42], which could account for phenotypic enhancement of *as1* in ta-siRNA-deficient backgrounds. We tested this hypothesis by generating triple mutants between *as1 ago7* and *ett*. The *ett* mutation has no impact on leaf development alone, but strongly suppresses the enhancement of *as1* by *ago7/ zip*, so that triple mutants have smoother leaves with normal margins, resembling those of *as1* (Figures 2B– 2D). Thus misexpression of *ETT* in *ago7* leads to enhancement of the *as1* phenotype. *ETT* may act via downregulation of adaxial genes such as *PHABULOSA (PHB)* or upregulation of abaxial genes such as *FIL* [17]. We identified an auxin response element (AuxRE) 1136 bp upstream of the ATG in the *FIL* promoter (TGTCTC), indicating that *FIL* may be directly activated by ETT via binding to the AuxRE, although deletion analysis suggests this element is not required alone [43]. Nonetheless,
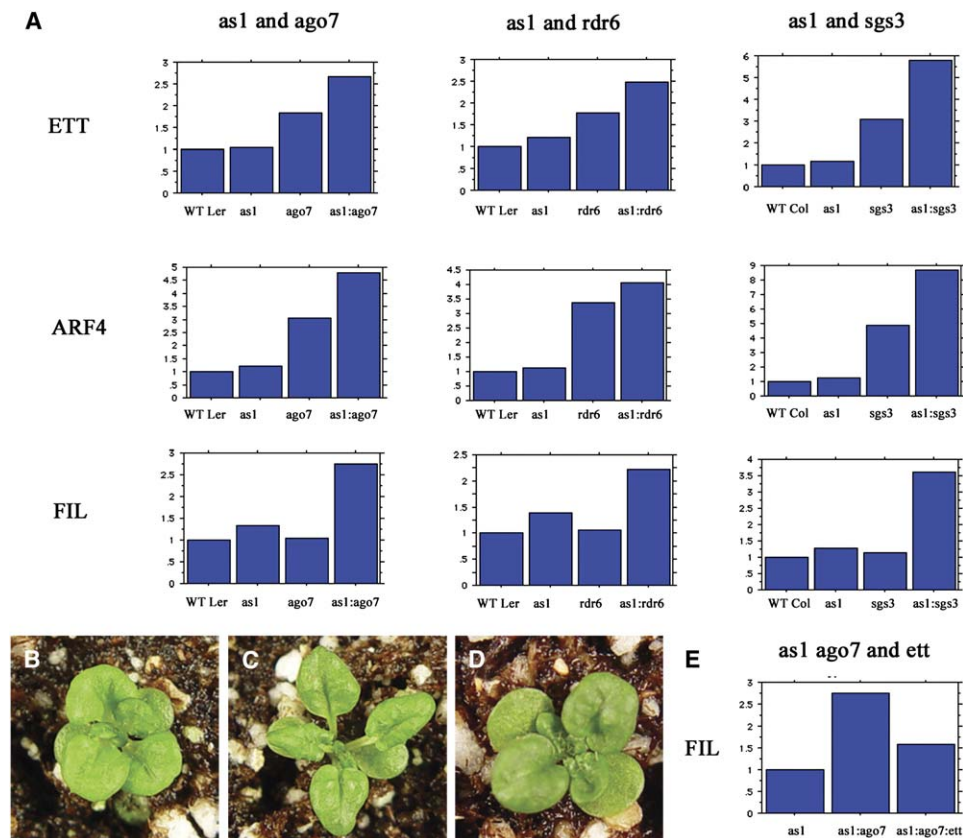
Figure 2. *ETT*, *ARF4*, and *FIL* Are Regulated by *AS1* and *trans*-Acting siRNA Pathway

Quantitative RT-PCR was used to assay expression levels of *ETT*, *ARF4*, and *FIL* in the following genotypes: wild-type L*er*, *as1*, *ago7*, and *as1 ago7*; wild-type L*er*, *as1*, *rdr6*, and *as1 rdr6*; and wild-type Columbia, *as1*, *sgs3*, and *as1 sgs3* (A). Results were normalized to *ACTIN2*, with the value from wild-type plants arbitrarily set to 1.0. Lower panel shows rosettes from 21-day-old *as1* (B), *as1 ago7* (C), and *as1 ago7 ett* (D). Level of *FIL* expression in *as1*, *as1 ago7*, and *as1 ago7 ett* was measured by quantitative RT-PCR (E).

overexpression of *FIL* in the *as1 ago7* double mutant was reduced in the *as1 ago7 ett* triple mutant (Figure 2E), supporting the idea that *FIL* is a downstream target of *ETT*. The level of *FIL* expression in *as1 ago7 ett* is still higher than in *as1*, which may be due to upregulation by *ARF4*. Misexpression of *ETT* in the single ta-siRNA-deficient mutants does not activate *FIL* (Figure 2A), because of repression by *AS1* and *AS2* [16, 33].
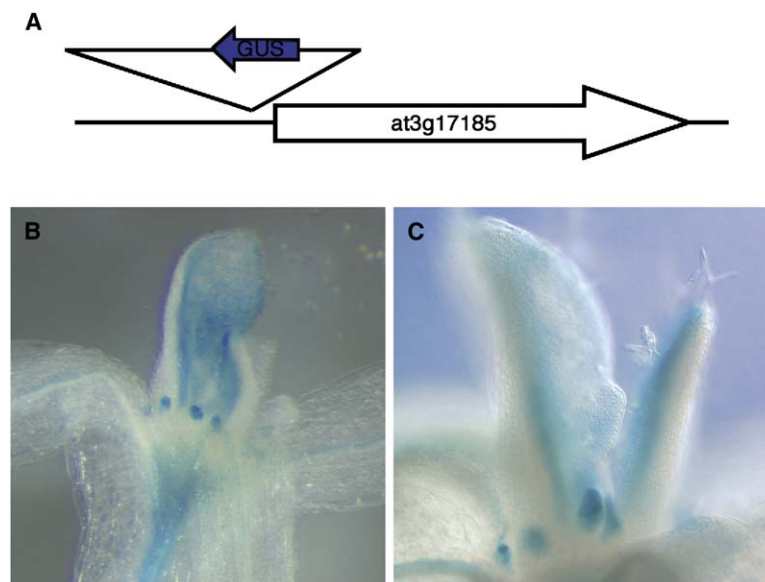

Figure 3. *TAS3* Noncoding RNA Is a Polarity Gene

The gene-trap insertion GT19682 (Experimental Procedures) is located 37 bp upstream of the transcription start of the *TAS3* gene AT3g17185 (A) and results in GUS reporter-gene expression in the adaxial domain of emerging leaves (B and C). Strong staining is also observed in the stipules, and in the vasculature of older leaves.
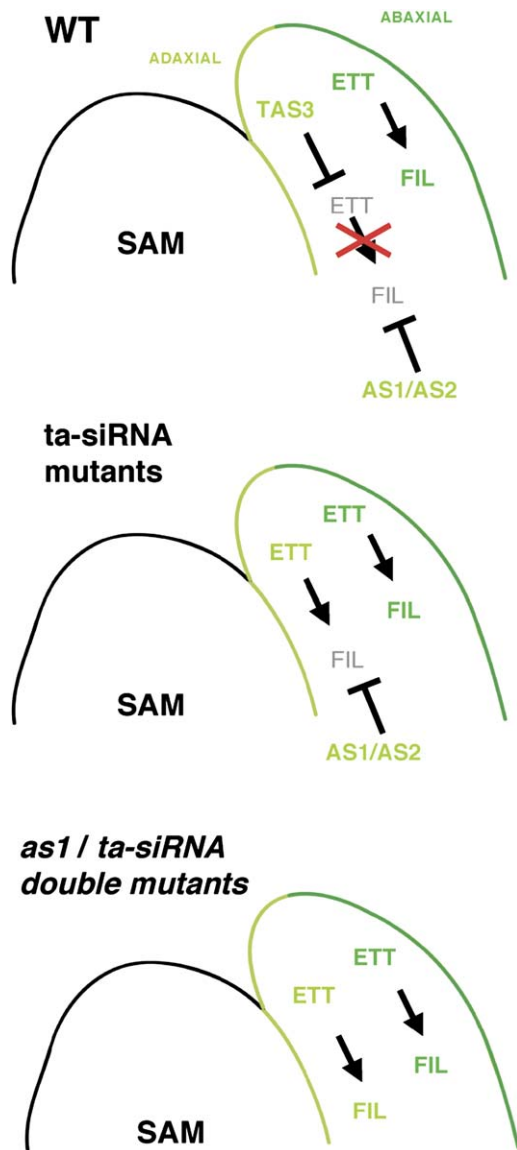
Figure 4. The Role of *trans*-Acting siRNA *TAS3* in Leaf Polarity

In wild-type plants, *ETT* and *ARF4* positively regulate *FIL* in the abaxial domain of the leaf. Downregulation of *ETT* and *ARF4* by *TAS3* on the adaxial side is abolished in *ta*-siRNA mutants, leading to misexpression of *ETT* and *ARF4*, but *FIL* expression remains repressed by *AS1/AS2*. Double mutants between *as1* and the *ta*-siRNA pathway promote ectopic expression of *FIL* via *ETT* and *ARF4*, resulting in polarity defects and smaller, dissected leaves.

Adaxial Expression of *TAS3* Confers Leaf Polarity

Small 21 nt ta-siRNAs are derived from noncoding RNA precursors that are initially targeted for cleavage by a miRNA. Cleavage products are converted into dsRNA by RDR6, and subsequently into 21 nt siRNAs by DCL4. RDR6 requires SGS3 for ta-siRNA production. AGO7 is not required for ta-siRNA production, but may instead utilize the 21 nt ta-siRNA for target-mRNA cleavage [8, 11, 12]. *ETT* mRNA is targeted by the ta-siRNA *TAS3*, explaining enhancement of *as1* in ta-siRNA-deficient backgrounds. TAS3 also matches one of six precursor genes for the abaxial microRNA miR166, which may account in part for miR166 upregulation in

as2 rdr6 [5]. However, suppression of *as1 ago7* by *ett* indicates that the phenotypic consequences of this match are small relative to targeting of ETT. We found that a gene-trap reporter gene integrated into the promoter of *TAS3* was expressed on the adaxial side of early leaf primordia, as well as in stipules (Figure 3). Thus, *TAS3* likely downregulates *ETT* and *ARF4* in the adaxial domain. *AS1/AS2* on the one hand, and ta-siRNA on the other, acting through *ETT*, redundantly repress the polarity gene *FIL* (Figure 4). In double mutants, misexpression of *FIL*, as well as the related gene *YAB5* (Table S1), likely results in the observed polarity defects.

Harmonious leaf development relies on a complex overlap between several genetic pathways [44]. The involvement of ta-siRNAs in leaf polarity was not previously evident because of redundancy with *AS1/AS2*, although phase-change defects in *ago7/zip* include weak defects in polarity, especially in the epidermis [8]. Redundancy may maintain phenotypic robustness in *Arabidopsis* [45], but why should plants utilize ta-siRNAs for axis specification? In contrast to microRNAs, which appear to be cell autonomous, siRNA-induced gene silencing in *Arabidopsis* spreads from cell to cell in a "relay" mechanism that depends on *RDR6*, *DCL4*, and the putative RNA helicase *SDE3* [31, 46]: the very same genetic pathway responsible for processing and amplifying ta-siRNA. Along with the plant hormone auxin, which activates ARF3 in the abaxial domain, it is possible that *TAS3* contributes to the "Sussex signal": a hypothetical molecule thought to pass from the meristem to the adaxial domain of the incipient leaf primordium, conferring leaf polarity [29, 44, 47].

Experimental Procedures

Plant Materials

Mutant alleles of *as1-1*, *as2-2*, *stm-11*, *stm-2*, and *bp-2* were described previously [48]. Gene and enhancer trap lines were generated as previously described [49, 50]. Plants were grown either on soil or on MS medium supplemented with sucrose, with a minimum day length of 16 hr. Ethyl methanesulphonate (EMS) mutagenesis was carried out as previously described [35]. Briefly, seeds from *as1/as1 stm-1/+* or *as1/as1* plants were treated with 0.5% EMS for 8 hr. Seeds were collected from 1200 single fertile M1 plants (*as1/as1 stm-1/+*) or from 150 pools of five plants (*as1/as1*), and progeny were screened for leaf development defects. All mutants were in the Landsberg *erecta* background, except for *sgs3* and *dcl4* single mutants, which were in the Columbia background. Double mutants between *as1-1* and mutants in the L*er* ecotype were made with *as1-1* backcrossed six times into L*er*. The gene-trap insertion GT19682 was isolated and stained as previously described [51]. The insertion-site sequence was determined following PCR amplification. The GUS reporter gene is in the opposite orientation to the *TAS3* precursor and acts as an enhancer trap because of the presence of a weak promoter in the Ds element [51].

Microarray Analysis

RNA was harvested from whole rosettes from L*er*, *as1-1*, *ago7-m5446*, and *as1-1 ago7-m5446* plants grown under 24 hr light at 15°C to promote vegetative development. Samples were frozen in liquid nitrogen prior to RNA extraction. Total RNA was extracted with Trizol reagent (Invitrogen) from tissue ground with a mortar and pestle. Following purification with an RNAeasy column (QIAGEN), labeling and hybridization of the ATH1 Genome array and scanning were carried out at the Cold Spring Harbor Microarray Facility with Affymetrix protocols and reagents.

GeneSpring software (Agilent Technologies) was used for statistical analysis of the data by ANOVA followed by multiple testing corrections with a False Discovery Rate of 5% with a p value cutoff of

0.05 [52]. For each genotype, two independently obtained RNA extractions from plants grown at different times (biological replicates) were hybridized to the ATH1 microarray in duplicate (technical replicates). Only *n*-fold changes above 1.3 are reported in Tables S1 and S2, although smaller changes were sometimes significant.

Quantitative RT-PCR

RNA was extracted from 21 day seedlings. First-strand cDNAs were made by using oligo dT primers (Invitrogen). Quantifications were performed on a QPCR ABI 7700 Real Time PCR System with the SYBR green PCR master mix (Applied Biosystems). PCR was carried out in 96-well optical reaction plates heated for 10 min to 95°C to activate hot-start Taq DNA polymerase, followed by 40 cycles of denaturation for 30 s at 95°C and annealing-extension for 45 s at 60°C. Target quantifications were performed with specific primer pairs. The following primers have been used: for AT2G33860 / ETT, CAACACTTGTTCGGATGGTG and CCCACACCAAATGTTCCTCT; for AT5G60450 / ARF4, ATACTACCCCACCCGGAAAC and TGAGA CTGCATCGCAAAATC; for AT2G45190 / FIL, ACCGGACCACTTC TCTCCTT and CTGGAGCTGGAGCTGGTTAG; and for At3g18780/ ACTIN2, primers have been described [9].

For each genotype, quantifications were made in triplicate, on two independently obtained cDNA samples. For each quantification, a melt curve was realized at the end of the amplification experiment by steps of 0.5°C from 55°C to 95°C, to ensure that quantification was not caused by primer self-amplification but by a pure and common PCR product. Results were normalized to that of ACTIN2. Corresponding wild-type values have been used as a control and arbitrarily set at 1.0.

Supplemental Data

Supplemental Data include three figures and two tables and are available with this article online at: http://www.current-biology. com/cgi/content/full/16/9/933/DC1/.

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