Development 122, 3799-3807 (1996)
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DEV0078

Temperature-sensitive mutations that arrest *Arabidopsis* shoot development

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SUMMARY

To identify genes involved in meristem function we have designed a screen for temperature-sensitive mutations that cause a conditional arrest of early shoot development in *Arabidopsis*. We describe the characterization of three mutations, *arrested development* (*add*) *1, 2* and *3*. At the restrictive temperature the *add1* and *add2* mutations disrupt apical meristem function as assayed by leaf initiation. Furthermore, *add1* and *add2* plants exhibit defects in leaf morphogenesis following upshift from permissive to restrictive temperature. This result suggests that proximity to a functional meristem is required for the completion of normal leaf morphogenesis. The *add3* mutation does not have a dramatic effect on the production of leaves by the apical meristem; however, *add3* prevents the expansion of leaf blades at high temperature. Thus, in this mutant the temperature-dependent arrest of epicotyl development is due to a failure of normal leaf development rather than new leaf initiation. While all *add* mutants have a reduced rate of root growth in comparison to wild-type plants, the mutants do not display a temperature-dependent arrest of root development. All *add* mutants display some developmental defects at low temperature, suggesting that these mutations affect genes involved in inherently temperature-sensitive developmental processes.

Key words: shoot development, *Arabidopsis*, meristem, leaf initiation, *arrested development*

INTRODUCTION

Embryogenesis in dicotyledonous plants, such as *Arabidopsis thaliana*, culminates with the formation of a bipolar embryo (see Steeves and Sussex, 1989). The apical pole consists of embryonic leaves (cotyledons) and the shoot apical meristem. The nascent root apical meristem makes up the basal pole. The shoot apical meristem is the source of cells used for both its own perpetuation and for the formation of a shoot composed of stems, leaves and flowers. Slowly dividing cells acting as semi-permanent apical initials occupy the most apical region of the meristem, the central zone (Brown et al., 1964). Immediately basal to the central zone lies the peripheral zone, characterized by more rapid cell divisions and the lateral outgrowth of leaf and flower primordia (Vaughn, 1952).

Fate mapping experiments with *Arabidopsis* indicate that the central and peripheral zones of newly germinated seedlings are specified prior to the completion of embryogenesis (Furner and Pumfrey, 1992; Irish and Sussex, 1992). Clones that occur in the first two true leaves tend to be limited to one or the other leaf, providing evidence that the anlagen for the first two true leaves are also specified prior to germination. However, the occasional occurrence of clones shared between the first two true leaves and later leaves and/or the flowers suggests that sometimes the dry seed is arrested at a less mature developmental stage, when the first true leaves are not yet specified independently from the meristem (Furner and Pumfrey, 1992).

In addition to its role early in leaf primordiagenesis the apical meristem may also promote later stages of leaf development, acting to confer dorsoventrality to developing leaves. Leaf primordia surgically isolated from meristems in a variety of species produced leaves that failed to acquire normal dorsoventrality, suggesting that proximity to a functional meristem is required for normal leaf development (Sussex, 1955; Hanawa, 1961). Mutants lacking apparent apical meristems, including the *shootmeristemless* (*stm*) mutation of *Arabidopsis* (Barton and Poethig, 1993), produce leaves with aberrant morphology. STM is a homeodomain transcription factor with homology to the maize meristem regulator KNOTTED 1, and is transcriptionally expressed only in the shoot meristem (Long et al., 1996; Sinha et al., 1993), suggesting that the defect in leaf development in *stm* mutants is not caused by a requirement for *STM* expression within leaf primordia. While the exact mechanistic process whereby leaves acquire developmental autonomy from the meristem is unknown, mutant phenotypes and surgical results are consistent with the idea that the specification of developmental fates within a leaf is a multi-step process dependent on the continuing function of a shoot apical meristem.

The genetic analysis of shoot meristem function has resulted in the identification of mutations that affect apical development at a variety of developmental stages. Embryonic and early seedling lethal mutations have been identified that disrupt the organization of the apical region of the embryo, resulting in the failure of the cotyledons and/or the apical meristem to form (Barton and Poethig, 1993; Mayer et al., 1991). These

mutations may identify genes involved in the initiation of meristem development during embryogenesis and/or the maintenance of meristem function in later development. To complement these approaches, we have designed a genetic screen to identify temperature-sensitive mutations that arrest *Arabidopsis* shoot development at the seedling stage. Temperature-sensitive mutations facilitate both the analysis of the role of meristem genes in postseedling development and the propagation of mutant lines. Temperature sensitivity can be caused by the structural instability of expressed proteins or because underlying genetic pathways are inherently sensitive to temperature. The latter class is often indicated when complete loss-of-function mutations display temperature sensitivity (Pickett and Meeks-Wagner, 1995; Strome et al., 1995; Tautz, 1992; Thomas et al., 1993). In such situations it is common for a mutant phenotype to be seen even at 'permissive' temperatures. A severe increase in mutant expressivity and/or penetrance, resulting from the combined loss of activity from the mutant gene and its partially redundant partners, occurs after shifting to restrictive temperature.

In this paper we present a screen for temperature-sensitive mutations that prevent the formation of a normal epicotyl at restrictive temperature, and the initial phenotypic characterization of three mutations identified by this screening procedure. The *arrested development* (*add*) *1*, *2*, and *3* mutations prevent normal shoot or leaf development at high temperature. Analysis of the mutant phenotypes indicates that these mutations are sensitive to temperature shifts at various points in development, suggesting that their activity is required throughout development. Concomitant temperature-dependent defects in leaf development suggest a requirement for a normally functioning shoot meristem during *Arabidopsis* leaf initiation and development.

MATERIALS AND METHODS

Plant lines

Mutant screens were performed on M_2 progeny of ethyl-methane sulfonate (EMS)-treated and fast neutron-irradiated plants. Screens were performed on both Columbia and Landsberg *erecta* ecotypes (Lehle Seed Company, Round Rock, TX) and with Wassiliskija ecotype T8 progeny of plants mutagenized by insertion of the *Agrobacterium* T-DNA (Feldmann, 1991; Feldmann and Marks, 1987) from seed provided by the Arabidopsis Genetic Stocks Center at Ohio State University.

Screen for temperature sensitive mutations

Mutations were identified by screening for seedlings that either failed to produce true leaf primordia or produced true leaves that were finger-like projections when plants were grown from germination at 29°C. Seedlings were scored for lack of epicotyl development from 6 to 9 days after sowing. For screening, the progeny of EMS- or fast-neutron-mutagenized plants and 4000 T-DNA mutagenized lines were grown in sterile culture on agar medium. Medium consisted of ½×Gamborg's basal medium (Sigma) with 1.0% sucrose and was adjusted to a pH of 5.6 by addition of 0.1 M KOH. Prior to autoclaving, 0.8% agar (Sigma Plant Tissue Culture Grade) was added to the medium. After autoclaving, the medium was allowed to cool to 65°C and filter-sterilized ampicillin was added to a final concentration of 100 μg/ml. To facilitate the systematic scoring of seedling phenotypes, 150×15 mm gridded Petri plates were used (Falcon Intergrid, Fisher Scientific). All sterile manipulations were performed in a laminar flow hood.

Surface seed coat sterilization was performed using a series of sterilizing solutions followed by repeated washes with sterile distilled water. This protocol was kindly provided by Dr Fred Lehle. Seed was placed first in a solution of 0.1% Triton X-100 (Fisher Scientific), vortexed vigorously for 1 minute, and then allowed to hydrate in this solution for 20 minutes. The solution was then decanted and a solution of 95% (v/v) ethanol, 0.1% Triton X-100 was added. Seed were vigorously vortexed for 1 minute and allowed to sit in the solution for 5 minutes. This solution was then decanted and replaced with a solution of 30% commercial chlorine bleach, 0.1% Triton X-100. This solution was vortexed vigorously for 1 minute, vortexed occasionally for 10 more minutes, and then decanted. Sterile water was then used to rinse the seed 5 times by vortexing. Routinely, 10 ml volumes of sterilization solutions and sterile distilled water were used to sterilize up to 50 mg of seed. Sterile seed was plated in top agar made identically to plating medium; plates were then sealed with Parafilm (American Can Co.) and placed into appropriate growth chambers.

To screen for mutations, plants were grown at high temperature (29°C) in a growth chamber with an 18 hour light/6 hour dark cycle under cool white fluorescent lights. Light intensity was 198 μEinsteins at the surface of the growth medium. Plates were screened by observation with dissecting microscopes, and putative mutants were transferred to fresh sterile media. They were then shifted to non-restrictive temperature (21°C) in an attempt to rescue epicotyl development. Seedlings that recovered were transferred to soil to complete their life cycles. Seed from these plants was germinated at low and high temperature in sterile culture to determine the heritability of the temperature-sensitive arrest of shoot development.

Genetic analysis

Mendelian segregation analysis was performed by crossing mutants recovered in this screen with their respective parental lines. The *add1* mutation was crossed to the Wassiliskija (Ws) ecotype, *add2* was crossed to the Landsberg *erecta* ecotype (Ler), and *add3* was crossed to the Columbia (Col) ecotype. Segregation was scored in the F_2 generation. Chi-square analysis was performed on all segregation data. All mutations segregated closely to the expected ratio of 1:3 (mutant:wild-type) for recessive mutations. Crosses between mutations revealed that all are non-allelic.

In genetic crosses the *add1* mutation cosegregated with antibiotic resistance and T-DNA sequences. Recombinant inbred mapping of genomic DNA flanking the insert was performed using lines provided by the Nottingham Arabidopsis Stock Center as previously described (Lister and Dean, 1993). The *add1* mutation has been placed at position 35.2 on chromosome 5 using this approach.

Tissue fixation and scanning electron microscopy

Seedlings were fixed with FAA fixative composed of 3.7% (v/v) formaldehyde, 50% (v/v) ethanol, and 5 % (v/v) acetic acid as previously described (Drews et al., 1991) with the following exceptions. Seedlings harvested from sterile culture were immersed in fixative in 10 ml vials and placed under vacuum for 30 minutes. After slowly releasing the vacuum, vials were capped and placed on a rotary mixer for 24 hours at 4°C. Fixative was removed by vacuum aspiration and tissue was dehydrated and mounted for scanning electron microscopy as previously described (Shannon and Meeks-Wagner, 1993). Polaroid Type 55 Land film was used to record observations; film negatives were transformed into digital positives using a Reli 4618 (Relisys) color scanner with a transparency adapter. Contrast adjustment, size bar addition and cropping of scanned images was performed in Photoshop 3.0 (Adobe).

Temperature upshift experiments

The effect of temperature on the epicotyl development of mutants identified in this screen was determined by growing seedlings of all three mutants with the wild-type parental lines in sterile culture. One set of plates was maintained at 21°C from the beginning of the exper-

iment to act as a control group. Five to ten seedlings were collected and fixed from this population at various times during the 3-26 day period after plating. Seedlings were collected on the same schedule from populations grown at 29°C from the time of plating or grown at 29°C after 3 days of growth at 21°C. *add1* mutant seedlings were also shifted to 29°C after 11 days of growth at 21°C.

Leaf counts were performed using scanning electron microscopy (SEM) to assess both the effect of continuous high temperature and the effect of temperature upshifts at different developmental stages on the initiation and development of leaves. Using etched tungsten needles, critical-point-dried tissue was dissected by hand to reveal the shoot apical meristem. To visualize the shoot apex during dissection, samples were sputter coated prior to dissection and then re-coated prior to SEM analysis. Leaf counts were performed on three to ten seedlings from each time point, depending on quality of tissue fixation and shoot dissection. Data was evaluated by determining the mean leaf number and standard error for each time point.

To assay the effect of temperature upshift on root growth, mutant and wild-type plants were grown on agar medium in sterile culture as described for mutant isolation. Seed was placed in a single row in the upper 1/3 of each Petri dish, and plates were oriented such that a vertical agar surface was provided for root growth. Germination and growth was allowed to proceed for 3 days at 21°C, then plates were moved to 29°C. Root lengths were determined at the point of upshift (day 3), then again at day 5 and day 8. Rates of root growth were determined between day 3 and 5 and then between day 5 and 8 for each root, then mean rates were determined for all wild-type and mutant populations. Standard errors for these mean rates were also determined.

RESULTS

Identification of temperature-sensitive seedling mutants

In order to identify temperature-sensitive mutations that cause reversible arrest of apical meristem function, we screened for seedlings that displayed arrested shoot development when grown in sterile culture at 29°C. The progeny of approximately 114,000 fast-neutron- and EMS-mutagenized seed were screened. Additionally, T-DNA-mutagenized lines were screened in 400 pools of seed with each pool containing seed drawn from 10 different T-DNA insertion events. From the combined screen, 18 mutants were identified that showed delayed or arrested epicotyl development at 29°C, and good recovery of epicotyl development when shifted to 21°C. These were self crossed, and their progeny were retested for the temperature-sensitive arrest phenotype.

Mutants recovered from one EMS-mutagenized line, one fast-neutron-mutagenized line, and one T-DNA-mutagenized line displayed temperature-sensitive arrests of epicotyl development upon retesting. The remaining mutants showed non-conditional seedling arrest phenotypes with variable expressivity within a population. The three temperature-sensitive mutations recovered segregate as recessive non-complementing mutations that cause a temperature-dependent arrest or delay of epicotyl development (Fig. 1). We have designated these mutations as *arrested development* (*add*) *1, 2* and *3*.

add1, *2* and *3* mutants display different responses to growth in continuous low and high temperatures

The production of leaf primordia by *add1, 2* and *3* was assessed relative to that of wild type to determine if epicotyl develop-

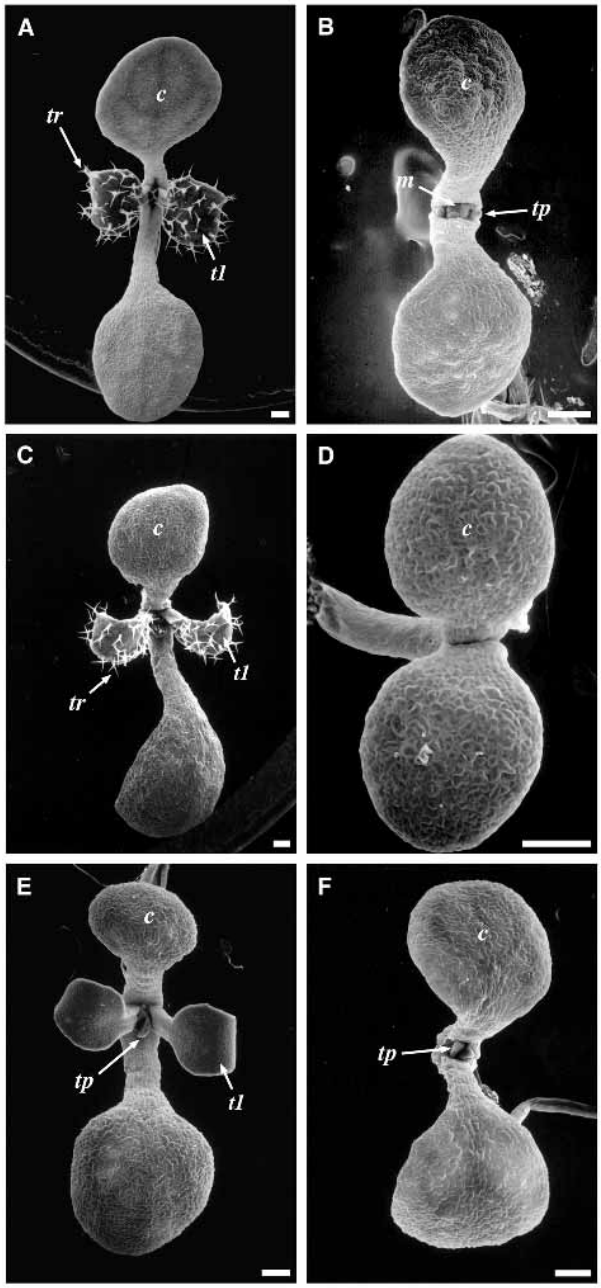

Fig. 1. Wild-type and mutant seedling development after germination and growth at 29°C for 6 days. (A) Wild-type Wassilewskija seedling with cotyledons and two true leaves. The apical region of an *arrested development 1* (*add1*) seedling lacking true leaves (B). Wild-type Landsberg *erecta* seedling (C) with an epicotyl displaying the first 2 true leaves. Both leaves have developed leaf blades and trichomes. (D) *arrested development 2* seedling with cotyledons, true leaf primordia are not visible. (E) Wild-type Columbia *glabrous1* (*gl1*) seedling with 3 true leaves. The *gl1* mutation prevents trichome development in this line, however, all true leaves have acquired dorsoventrality. (F) The apical region of an *arrested development 3* mutant. Epicotyl development is delayed in this mutant because leaf blade expansion has not occurred. However, primordial leaves are asymmetric and blade development is apparent, suggesting that these leaves have acquired dorsoventrality. Scale bar, 250 μm. *c*, cotyledon; *tp*, true leaf primordium; *t1*, leaf from first set of true leaves; *tr*, trichome; *m*, meristematic region.

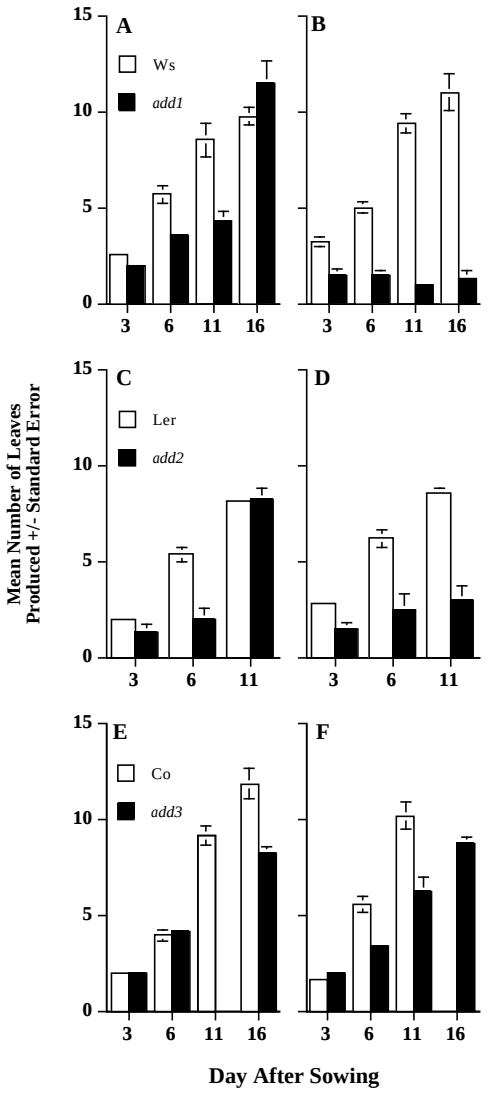

Fig. 2. The *add1* and *add2* mutations have temperature dependent effects on leaf initiation, while *add3* does not dramatically affect primordia initiation. Seedlings were removed from populations on the days indicated after sowing and leaves were counted by scanning electron microscopy (see Materials and Methods). Bars represent the mean values for number of leaves produced, and error bars represent the standard error of the mean. The *add1* mutation has an effect on leaf production when compared to its wild-type background line Wassilewskija (Ws) whether plants are germinated and grown at low (21°C) (A) or high (29°C) (B) temperature. The *add2* mutant also shows a temperature dependent arrest of shoot development when plants grown at low (C) and high (D) temperature were compared to each other and to wild-type La plants. All wild-type La plants had flowered by day 11, ending new leaf primordiagenesis, so further counts were not performed. The *add3* mutation does not cause a dramatic temperature dependent arrest of leaf production (E,F), however, *add3* leaf production is generally slower than wild type at either temperature.

ment is altered during continuous growth from germination at low (21°C) or high (29°C) temperatures. Leaves were counted on *add1* and Ws wild-type plants at 3, 6, 11 and 16 days after seed were sown in sterile culture. After 3 days of growth at 21°C both *add1* and Ws plants had initiated two leaf primordia (Fig. 2A), but the leaf initiation profile of the two populations then quickly diverged. During the interval from 3 to 11 days after sowing, an average of two leaves were produced by *add1* plants, while an average of six leaves were produced by wild type (Fig. 2A). These results indicate that epicotyl development at 21°C is delayed in *add1* plants. However, leaves were eventually produced by *add1* plants grown at 21°C and, following floral induction between 16 and 26 days after germination, inflorescences and flowers were produced.

In contrast, growth from germination at high temperature revealed a tight arrest of *add1* epicotyl development while wild-type plants continued normal development (Fig. 2B). *add1* seedlings collected from populations of plants grown continuously at high temperature never produced more than one or two true leaves. This indicates that there is a postgermination temperature-sensitive requirement for *add1* function for the initiation of epicotyl development and the formation of true leaves.

Similar to *add1*, the *add2* mutation delayed epicotyl development at low temperature and arrested it at high temperature. When grown at 21°C, *add2* plants initially produced leaves more slowly than wild-type seedlings, but the total number of rosette leaves formed by *add2* and wild type was equivalent by 11 days after sowing (Fig. 2C). Plants grown at 21°C bolted between 11 and 16 days after sowing and produced normal flowers. Germination and growth of *add2* plants at high temperature resulted in an arrest of epicotyl formation early in vegetative development. Seedlings grown from germination at high temperature and harvested 3, 6 or 11 days after sowing all showed an arrest of epicotyl formation after the production of two to four leaves (Fig. 2D). Plants grown at 29°C for 11 days after sowing produced two true leaves that acquired dorsoventrality, as reflected in the presence of adaxial trichomes and leaf blades, and one leaf which arrested as a young primordium. Inflorescence and flower development did not occur in *add2* plants grown at high temperature, indicating that a tight and enduring developmental arrest is caused by this mutation.

In contrast to the other mutations, the *add3* mutation did not appear to have a dramatic conditional effect on the production of leaves. Leaf initiation is subtly delayed in later development (Fig. 2E, F) with plants producing one to two fewer leaves than wild type at both 21°C and 29°C. However, *add3* did have a dramatic temperature-sensitive effect on leaf morphology, increasing marginal serration and inhibiting leaf blade development (Fig. 6F). Thus, in this mutant the delay of epicotyl development is not attributable to a lack of new primordiagenesis, but is due instead to a loss of the normal expansion of leaf blades.

add mutant shoot development is sensitive to temperature upshift during postgermination development

Establishment of the temperature-sensitive period of a mutation requires the determination of the effect of temperature upshifts at various stages of development. To determine if the *add1* mutation had a temperature-dependent effect on later development, wild-type Ws seedlings and *add1* mutants were shifted from 21°C to 29°C at 3 or 16 days after sowing. Wild-type plants shifted after 3 days of development showed approximately the same shoot developmental profile as plants grown

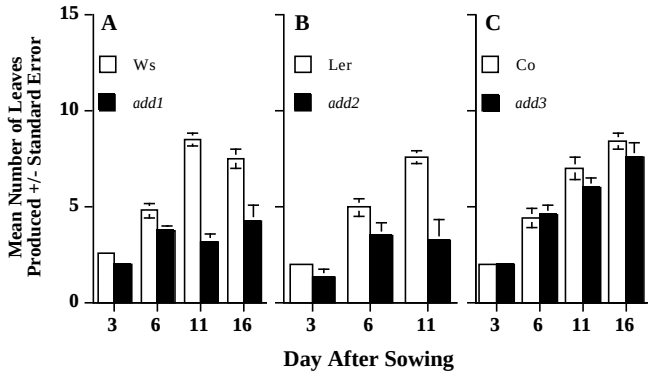

Fig. 3. Shifts from low to high temperature after germination arrest *add1* and *add2* leaf production, but do not alter *add3* leaf initiation. To determine if the mutations identified in this screen have distinct temperature sensitive periods, plants were shifted from 21°C to 29°C after 3 days of growth at the lower temperature. All bars represent mean number of leaves, error bars are the standard errors of those means. (A) *add1* and Ws, (B) *add2* and La, (C) *add3* and Co.

from germination at 21°C or 29°C (Compare Fig. 2A and B with Fig. 3A). *add1* plants upshifted on day 3 halted further shoot development after the production of an average of 4.2±1.7 (SE) leaves (Fig. 3A). After day 6, no further leaf primordia were initiated and flowering never occurred. The epicotyl of *add1* plants shifted after 3 days of growth at low temperature and maintained at high temperature for 8 more days possessed leaf primordia that arrested development as long, fingerlike projections (Fig. 4B). Although younger leaf primordia did not produce trichomes or other obvious aspects of dorsoventrality, larger (presumably older) leaves did possess occasional adaxial trichomes (Fig 4B). Older *add1* plants shifted from 21°C to 29°C at day 11 and then grown for 10 days at 29°C also showed an arrest of apical development and never produced an inflorescence or flowers (Fig. 4F). However, leaves produced by these plants had significant laminar development and produced adaxial trichomes. This suggests both that shoot meristem function is arrested in late upshifts and that the leaves had progressed past the stage where *ADD1* gene function and/or a functional shoot apical meristem is required for dorsoventral development.

Analysis of the development of *add2* seedlings shifted to 29°C after 3 days of growth at 21°C suggests that *ADD2* function is required for normal postgermination development (Figs 3B, 5). The first two leaves formed by *add2* plants in this experiment produced trichomes and leaf blades, suggesting that they had acquired dorsoventrality (Fig. 5D). The third leaf formed did not produce adaxial trichomes but did show asymmetry and blade flattening consistent with the acquisition of dorsoventrality. After day 6, further development of the epicotyl did not occur in upshifted plants, indicating that *ADD2* gene function is required during early development for shoot meristem function and/or epicotyl development. Individual *add2* plants shifted to 29°C after 11 days of growth at 21°C also displayed an arrest of epicotyl formation (data not shown).

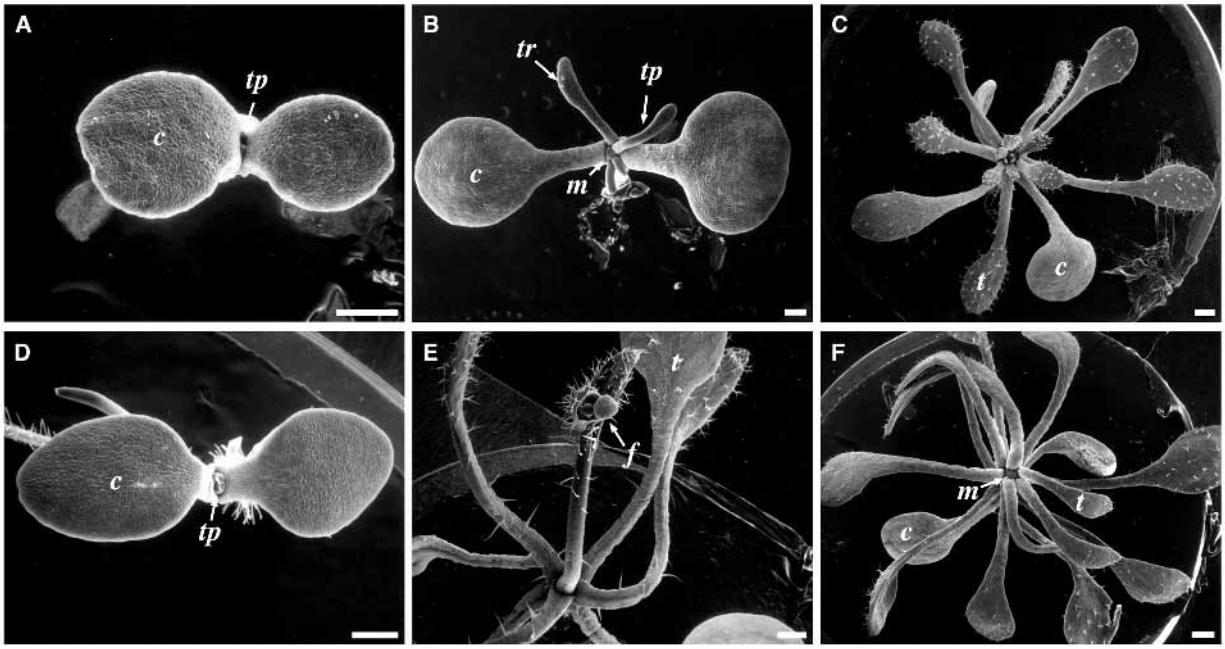

Fig. 4. Effect of temperature shift on seedling morphology. *add1* (A) and Ws (D) seedlings were grown for 3 days at permissive temperature. Plants at this developmental stage were shifted to 29°C and allowed to grow for 8 days, at which point they were harvested for SEM analysis. *add1* mutants showed an arrest of epicotyl development, enlargement of the meristem and finger-like leaf primordia (B) suggesting that meristem function has been lost from these seedlings. Wild-type Ws plants maintained in the same regimen produced normal leaves and flowered (E). To assess the effect of temperature on later stages of *add1* development, *add1* plants grown for 11 days at permissive temperature (C) were also shifted to restrictive temperature and grown for 10 more days. Unlike younger *add1* plants shifted from low to high temperature early in development (B), the temperature shift at this stage did not have a dramatic effect on leaf morphology, but did cause an arrest of further epicotyl development (F). *c*, cotyledon; *f*, flower; *tp*, true leaf primordium; *t1* leaf from first set of true leaves; *tr*, trichome; *m*, meristematic region. Scale bars for A, B and D, 250 μm; scale bars for C, E and F, 500 μm.

Table 1. Rates of root growth for mutant and wild-type seedlings at low and high temperature

	Mean rates of root elongation in mm/day for ten different plants ±s.e.			
	Rates between 3 and 5 days after germination		Rates between 5 and 8 days after germination	
Plant line	Roots grown continuously at 21°C	Roots shifted from 21°C to 29°C 3 days after germination	Roots grown continuously at 21°C	Roots shifted from 21°C to 29°C 3 days after germination
add1	2.5±0.4	2.8±0.5	2.8±0.4	1.7±0.6
Wassiliskewja	3.9±0.3	4.0±0.6	3.8±0.3	3.3±0.5
add2	1.1±0.2	1.2±0.3	1.1±0.3	0.9±0.2
Landsberg	2.2±0.3	2.1±0.2	3.7±0.3	6.9±0.7
add3	2.8±0.2	3.0±0.1	2.8±0.4	1.5±0.3
Columbia	4.5±0.3	4.4±0.4	4.7±0.5	3.5±0.5

The *add3* mutation did not display a dramatic effect on the rate of leaf production by plants held at constant high temperature. Temperature upshifts performed after 3 days of growth at 21°C showed that the *add3* mutation does not have a dramatic temperature-sensitive effect on leaf initiation (Figs 3C, 6). However, *add3* leaf blade development was arrested following the upshift to 29°C. Leaves arrested early in development with small, spiked leaf blades (Fig. 6B,F), while plants grown at low temperature produced more normal leaf blades (Fig. 6C). Thus, temperature upshift experiments suggest that the *add3* mutation confers temperature sensitivity during later stages of leaf development, but not during the early stages of leaf primordium initiation.

Root development is not arrested by temperature upshift

To determine if root development is also sensitive to upshift, root growth of mutant and wild-type plants was assayed in plants upshifted after 3 days of growth at low temperature. The average rate of root growth was then determined from days 3 to 5 and days 5 to 8. At all temperatures the rate of root growth of the mutants is slower than that seen in wild type. However, the rate of root growth is similar at low and high temperature for *add1*, *2* and *3* plants (see Table 1). These data indicate that shoot defects seen in mutants following upshift are not accompanied by a dramatic temperature-dependent decrease in root growth.

DISCUSSION

Interpretation of mutant phenotypes with reference to the *Arabidopsis* seed fate map

The embryo within the dried seed represents the developmental start point for the temperature-sensitive screen we have performed. Because embryogenesis is completed in a normal fashion prior to seed dessication, the existing *Arabiodpsis* fate map (Furner and Pumfrey, 1992) leads us to predict that mutants primarily affected in the organization of the postgermination meristem would form cotyledons and two true leaves or leaf primordia prior to an arrest of epicotyl development. Two of the mutations recovered in our screen, *add1* and *add2*, meet these criteria. At high temperature both mutants arrest devel-

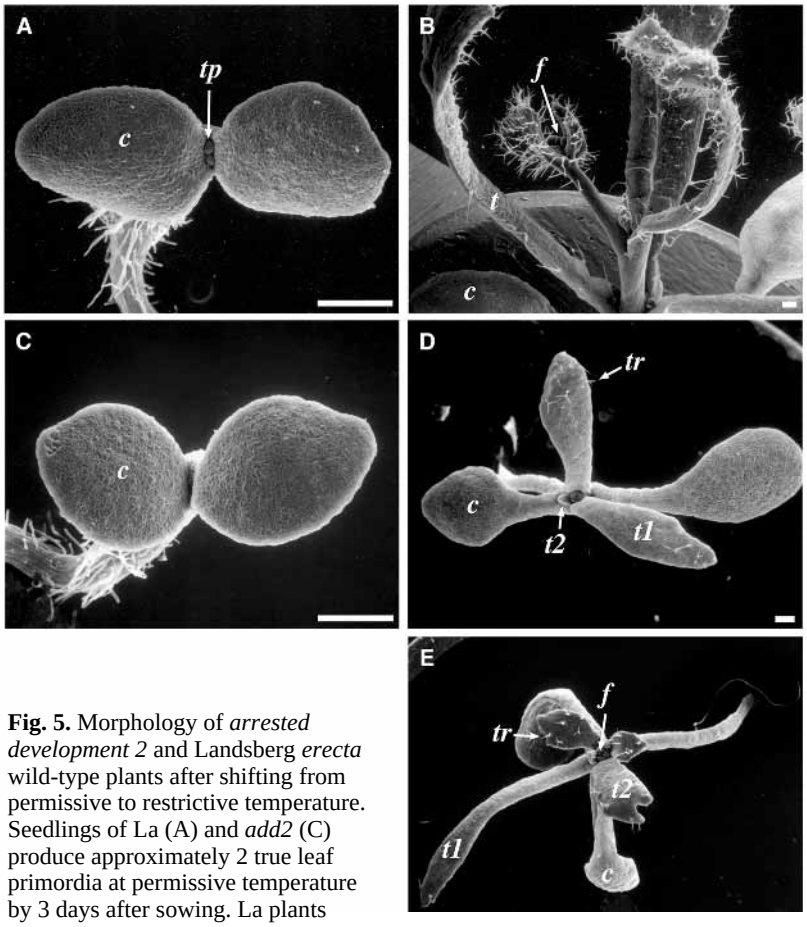

Fig. 5. Morphology of *arrested development 2* and Landsberg *erecta* wild-type plants after shifting from permissive to restrictive temperature. Seedlings of La (A) and *add2* (C) produce approximately 2 true leaf primordia at permissive temperature by 3 days after sowing. La plants shifted after 3 days growth at 21°C to restrictive temperature and grown for 8 more days produced a normal vegetative rosette of true leaves and flowered (B). However, *add2* plants similarly shifted from 21°C to 29°C showed an arrest of apical development. *add2* plants arrested after producing 2 true leaves with trichomes and leaf blades, and 1 primordium that was arrested prior to the production of adaxial trichomes (D). *add2* plants grown for 11 days at low temperature produced a rosette and initiated flowering, however, the first set of true leaves generally showed reduced leaf blade formation and decreased numbers of adaxial trichomes (E). *t1*, leaf from first set of true leaves; *t2*, leaf from second set of true leaves; *tp*, true leaf primordium, *tr*, trichome; *c*, cotyledon; *f*, flower. Scale bars, 250 μm.

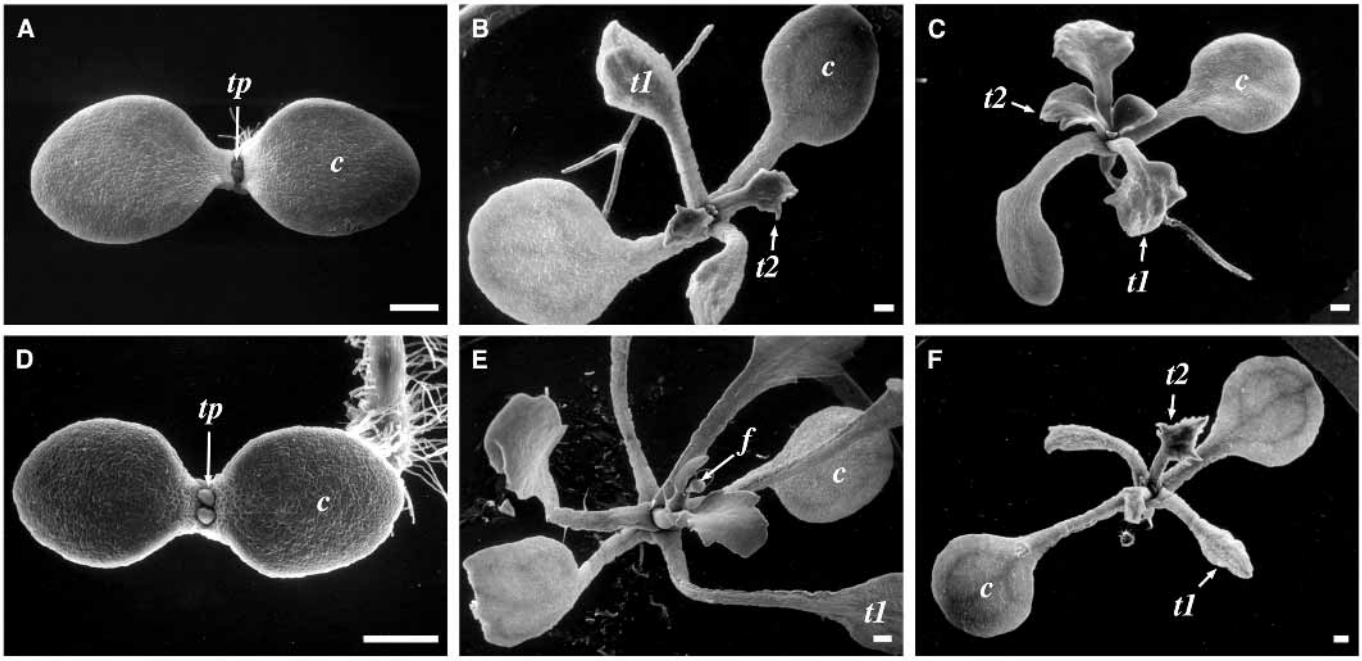

Fig. 6. Morphological effects of temperature upshifts on *add3* plants. Mutant *add3* (A) seedlings and seedlings of the wild-type background line Columbia (Co) *glabrous1* (D) produced approximately two leaves during growth at 21°C for 3 days after sowing. Wild-type plants shifted to 29°C on day 3 and grown for 8 more days at the high temperature produced a rosette of vegetative leaves and flowered after temperature upshift (E). *add3* plants also produced a vegetative rosette after upshift to 29°C, but leaves formed highly serrated leaf margins (B) when compared to *add3* plants grown 21°C (C). This effect is even more pronounced in *add3* plants held at restrictive temperature continuously for 11 days after sowing, with both the first two true leaves and the second two true leaves displaying severe inhibition of leaf blade expansion and increased serration (F). *tp*, true leaf primordia; *t1*, leaf from the first set of true leaves, *t2*, leaf from the second set of true leaves, *f*, flowers; *c*, cotyledons. Scale bars, 250 μm.

opment after germination with the production of two to three true leaves.

The phenotypes of *add1* plants germinated at high temperature indicate that shoot apical meristem function ceased after embryonic leaf primordia were produced. This suggests that one role of the wild-type *ADD1* gene is the initiation and/or maintenance of normal epicotyl meristem function in *Arabidopsis.* The *add1* mutation co-segregates with DNA sequences and antibiotic resistance carried by an engineered T-DNA (Feldmann, 1991) which should facilitate the molecular characterization of the *ADD1* locus in order to determine the role this gene plays in development.

In contrast to *add1* mutants, *add2* mutants germinated at high temperature arrest development after three true leaves are formed. Interpreting this result in light of the *Arabidopsis* fate map it would appear that normal embryogenesis is completed in *add2* mutants and that some postembryonic meristem function continues briefly at high temperature, as the primordial initial for the third true leaf is not fully specified prior to germination. Thus, it is likely that wild-type *ADD2* function is more directly involved in the maintenance of meristem function than in the initiation of meristem activity during germination.

The *add3* mutation reveals that mutations that disrupt normal leaf expansion can also be recovered in this mutant screen. Epicotyl development of *add3* plants appears to be reduced, even though shoot apical meristem function, as assayed by leaf production, is not dramatically affected by this mutation. Because primordia formed by *add3* plants at high temperature produce rudimentary leaves with petioles and small blades, it is likely that the specification of leaf identity is normal in this mutant, while the growth required for the expansion of the leaf is inhibited. The interstitial expansion of leaves is thought to require the presence of diffuse meristematic activity across the entire surface of the expanding leaf (Poethig and Sussex, 1985). It is possible that wild-type *ADD3* function is required for the generation or maintenance of this general meristematic activity in the developing leaf.

add1, add2 and *add3* confer temperature sensitivity throughout development

The fact that older *add1* plants arrest development when shifted from low to high temperature indicates that *ADD1* is required not only for the organization or initiation of the shoot apical meristem, but also for normal meristem function throughout the *Arabidopsis* life cycle. A similar result is seen with *add2* plants, although primordia initiated prior to the temperature upshift form leaves capable of more normal development than is evident in *add1* mutants. These results suggest that normal epicotyl formation, including leaf primordiagenesis, is sensitive to the effect of the *add1* and *add2* mutations throughout vegetative development, as no specific temperature sensitive period was identified.

Temperature upshifts performed with *add3* mutants demonstrated that leaf primordia are produced at approximately the same rate as observed for plants held at low temperature. This implies that the *add3* mutation does not have a dramatic effect on the initiation of true leaf primordia. However, in older *add3*

plants the development of the leaf blade is extremely sensitive to temperature with the youngest leaves showing the greatest inhibition of laminar development. This indicates that the *add3* mutation has an independent effect on the development of each leaf and that *ADD3* must have a reiterative function that regulates the development of each successive leaf.

Temperature-sensitive increases in mutant expressivity and/or penetrance may indicate mutations in genes that participate with other genes in biological processes that can proceed at low temperature in the absence of one gene's function (Pickett and Meeks-Wagner, 1995; Strome et al., 1995; Tautz, 1992; Thomas et al., 1993). Higher temperatures reveal a requirement for the combined activity of all genes in a process. For example, mutants in *polycomb* group genes, which act as transcriptional repressors for a wide variety of developmental regulators in *Drosophila*, display inherent temperature sensitivity, temperature dependent increases in expressivity, and pleiotropic effects on developmental gene regulation. The *add1*, *add2* and *add3* mutations all show a temperature-dependent increase in expressivity, rather than a sharp transition from a completely normal to a mutant phenotype. Thus, the *add* mutations may be revealing a widely applied scheme for the regulation of meristem identity genes.

Autonomy of leaf development in apically arrested mutants

Arabidopsis mutants that display loss of shoot apical meristem functions, including *shootmeristemless* (Barton and Poethig, 1993) *schizoid* (Medford et al., 1992), and *wuschel* (Laux et al., 1996), also tend to produce leaves with altered morphology and/or dorsoventral defects. Similar to other shoot meristem mutants, the first one or two true leaves in *add1* plants raised at high temperature produce finger-like projections that lack dorsoventrality. These mutant phenotypes, and the studies performed on surgically isolated primordia, suggest that a functional meristem is required for both the initiation and completion of the leaf morphogenetic program. It is currently unknown if the shoot apical meristem generates a specific signal required for the completion of leaf morphogenesis, or if shoot apical meristems and young leaf primordia are partially analogous structures sharing genetic regulation.

Later defects in *add1* leaf morphogenesis may indicate that *ADD1* functions only in regulating apical meristem development, which is in turn required for the completion of leaf morphogenesis. Alternatively, *ADD1* may be a pleiotropic regulator that acts both in the shoot apical meristem and leaves. The molecular characterization of *ADD1* and localization of the ADD1 gene product should help to address this issue by determining if *ADD1* acts solely in the meristem or in both the meristem and in leaves.

In contrast to *add1*, *add2* seedlings shifted from permissive to restrictive temperature 3 days after sowing produce leaves that undergo morphogenesis after initiation. In addition, *add2* seedlings grown from germination at the restrictive temperature produce true leaves with partial leaf blades and some trichomes at the distal tip of the leaf, suggesting that this mutation does not prevent the acquisition of dorsoventrality by already initiated leaves. The production of adaxial trichomes occurs first in the distal-most tip of the leaf of wild-type plants (Lloyd et al., 1994). The distal-most portions of leaves from *add2* plants grown at high temperature also acquire dorsoventrality and accumulate adaxial trichomes, while proximal portions fail to produce adaxial trichomes, suggesting that this portion of the leaf has not acquired normal dorsoventrality. Thus, either shoot meristem function is not as completely inhibited in *add2* plants as it is in *add1* plants, or *add1* and *add2* affect different aspects of meristem function and/or leaf primordiagenesis. An analysis of the epistatic interactions between these two mutations will be a first step in determining whether these mutations act in overlapping or independent biological processes.

Shoot specificity of mutations

In all cases *add* mutant seedlings grown at high or low temperature have slower rates of root growth than wild type. However, temperature sensitive arrest of root development was not observed in any case, and root morphology (i.e., branching and superficial appearance using SEM) were normal (data not shown).

We have also assessed root growth in wild-type seedlings grown on basal medium with sucrose, in which the shoot apical meristem and cotyledons were removed 3 days after germination. In this experiment, the rate of root growth by 'decapitated' wild-type seedlings is 5- to 20-fold less than that observed for intact *add* mutant seedlings at either high or low temperature (data not shown). This result suggests that root meristems do not enjoy complete physiological autonomy from normal shoot development even when grown in rich medium. Furthermore, this raises the likely possibility that the *add1*, *add2* and *add3* mutations delay root growth because they cause diminished shoot growth when maintained at high temperature. Many of the 'shootless' mutations described to date reinitiate shoot meristematic activity during seedling growth (Barton and Poethig, 1993; Laux et al. 1996), which may account for reports of normal root growth in these mutations. However, the quantitative characterization of root growth in these mutants has not been presented, making comparison with the *add* mutations difficult. Given the complicated physiological interplay between shoot and root (Letham, 1994), the available data supports a specific role for the *ADD* genes in regulating aspects of shoot meristem function or leaf development.

We wish to thank Bruce Bowerman, Vicki Chandler, Steve Johnson, Paula Martin, Susan Shannon and Chris Shelton for critical review of the manuscript. This research is supported by grants from the U.S. Department of Agriculture National Research Initiative Competitive Grants Program (Nos. 93-37100-9189 and 93-37304-9040) to D. R. M.-W. F. B. P. was supported by an American Cancer Society Postdoctoral Fellowship (No. PF-3794) and by a Postdoctoral Fellowship from the National Institute of General Medical Sciences, NIH (No. 1 F32 GM17629-01). M. M. C. was supported by a National Institutes of Health Developmental Biology Training Grant (No. 5 T32 HD07348).

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(Accepted 3 September 1996)