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 primordiogenesis 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 shoot meristemless (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 M2 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 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 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 replaced with 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 F2 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 401B (Reliys) 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. 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 primordiation, 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. (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 primordiation, 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...
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).
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*, *add2*, and *add3* 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 desiccation, the existing *Arabidopsis* 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 de-
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 primordiogenesis, 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 waschel (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 primordiogenesis. 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.

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