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Comparison of Leaf Plastochron Index and Allometric Analyses of Tooth Development in *Arabidopsis thaliana*

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Abstract

Two methods of analyses were used to investigate tooth development in *serrate* (*se*) mutant and wild-type Columbia-1 (Col-1) *Arabidopsis thaliana* leaves. There were almost twice as many teeth with deeper sinuses and two orders of toothing on the margins of *serrate* compared with Columbia-1 leaves. The main objective of this study was to test three hypotheses relative to the source of polymorphism in tooth development: (i) Teeth share similar growth rates and initial sizes, but the deeper teeth are initiated earlier in leaf development. (ii) Teeth share similar timing of initiation and growth rates, but the deeper teeth have a larger initial size. (iii) Teeth share similar timing of initiation and initial sizes, but the deeper teeth have a faster growth rate. Leaf plastochron index (LPI) was used as the time variable for leaf development. Results showed teeth in *se* were initiated at −27 LPI, 15 plastochrons earlier than those of Col-1. *Serrate* leaf expansion was biphasic, with the early phase expanding at half the relative plastochron rate of the later phase, which equaled the constant relative expansion rate of Col-1 leaves. Allometric analyses of tooth development obscured the interactions between time of tooth and leaf initiation and the early phase of leaf expansion characteristic of *serrate* leaves and teeth. Timing of developmental events that allometric analysis obscured can be readily detected with the LPI as a developmental index.

Key words: Leaf plastochron index; Allometric analysis; Tooth development; Relative rates of leaf growth; *Arabidopsis thaliana*; *Serrate*; Columbia-1

Introduction

In *Arabidopsis thaliana*, many mutations exist that can be used for the genetic dissection of development in this model plant (Meyerowitz 1989). Because the mechanisms underlying the morphogenesis of leaf margins is poorly understood, the *serrate (se)* mutant affords an excellent opportunity to investigate alterations of the leaf margin. The gene affected by the *se* mutation has recently been isolated and characterized to a limited extent, and it contains a C2H2 zinc finger motif (M. Prigge, personal communication). Leaf margins ranging from deeply lobed to entire have been identified in several groups of dicot species, but nothing is known about polymorphism in depth of toothing. We do not

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know whether the same processes influencing depth of lobing operate with regard to tooth formation; therefore, the morphologic differences between the development of lobed and toothed margins need to be quantified. In addition, we wish to introduce the leaf plastochron index (LPI), a robust and precise measure of leaf age, because it has not previously been applied to growth analysis of teeth or lobes.

The leaf margins of *A. thaliana* are defined as toothed on the basis of depth of their sinuses. Teeth have sinuses less than one fourth of the distance to the midvein. Lobes have sinuses extending one fourth or more of the distance to the midvein (Hickey 1973). The *se* mutation of *A. thaliana* results in deeper and an increased number of teeth on the leaf margin.

The structure of the leaf margin is usually apparent before the young leaf unfolds from the apical bud. Leaf primordia exhibit bilateral dorsiventral symmetry very soon after initiation on the peripheral region of the shoot apical meristem (Steeves and Sussex 1989). Development of the structure of the margin begins before the phase of major expansion of the lamina, a phenomenon noted in early research of leaf development, " . . . the basic architecture of the leaf originates very early in ontogeny, . . ." (Foster 1936). Although few studies address tooth initiation in leaves, published micrographs of developing leaves show teeth at an early stage (for example, Merrill 1979; McLellan 1990). The form of the mature leaf lamina arises through spatial gradients of expansion that change over time as the leaf grows to maturity.

It has been proposed that allometric analysis of growth can be used to assess whether regions of the leaf expand isometrically or anisometrically (Dolan and Poethig 1991). In allometric analysis, two dimensions of a growing structure are plotted on log-log axes (for example, log sinus length versus log leaf length). When the plot is a straight line, the relationship is represented by:

$$\log y = k\log x + \log b, \qquad (1)$$

where k = slope, and b = y-intercept.

The allometric constant, k, represents the ratio of relative growth rates of the two dimensions, and the y-intercept, b, represents the value of y when x = 1. The constant, b, is often interpreted to represent the initial shape of the structure, especially when the units of measurement are sufficiently small (for example, millimeters). Time is implied by the log leaf length coordinate (Causton and Venus 1981). It has been determined that the sinus regions of young leaves for deeply lobed genotypes initially expanded more slowly relative to leaf length than did leaves of more shallowly lobed genotypes, but expansion was later closer to isometric (Dolan and Poethig 1991; Hammond 1941a,b; Jones 1993; McLellan 1990).

Another approach to analyzing leaf expansion is through the use of the plastochron index (PI), which is a precise method to determine the developmental age of plants and organs derived from the shoot. The use of the PI in the quantitative description of leaf growth, including growth rates and timing of events, is amply illustrated in the monograph by Maksymowych (1990). The use of the PI in growth analysis of leaves was previously formalized for *A. thaliana* (Groot and Meicenheimer, in press). The PI defined by Erickson and Michelini (1957) is a continuous function representing the age of a plant in units of plastochrons, the time period between initiation of two successive leaves. In practice, the plastochron is more broadly defined as the time period between two successive leaves reaching a conveniently measurable reference length (for example, 3 mm). Its calculation requires the measurement of two successive leaves: one just longer (leaf n) and one just shorter (leaf n+I) than the reference length:

$$Pl = n + (\log L_n - \log \lambda)/(\log L_n - \log L_{n+1}), \qquad (2)$$

where n = number of leaves longer than the reference length, λ; L_n is the length of leaf n; and L_{n+1} is the length of leaf n+1.

For studies of leaf development, the leaf plastochron index (LPI) is used (Erickson and Michelini 1957):

$$LPI_a = PI - a, \qquad (3)$$

where a is the serial number, counting from the first true leaf, of the leaf in question.

This function represents the age of a leaf in plastochrons, with zero set to when the leaf is at the index length. The LPI has advantages over allometric analyses by being able to age leaves and determine rates of growth and timing of developmental events. Allometric analysis is unable to determine whether an altered morphogenetic program is due to a difference in timing of developmental events.

The main objective of this investigation was to analyze differences in tooth growth between *se* and the wild-type using the LPI and allometric analysis. Use of the LPI enables us to test the following hypotheses relative to the source of tooth polymorphism in *A. thaliana*. Teeth share similar growth rates and initial sizes, but the deeper teeth are initiated earlier in leaf development (Figure 1A). Teeth share similar timing of initiation and growth rates, but the deeper teeth have a larger initial size (Figure 1B). Teeth share similar timing of initiation and initial sizes, but the deeper teeth have a faster growth

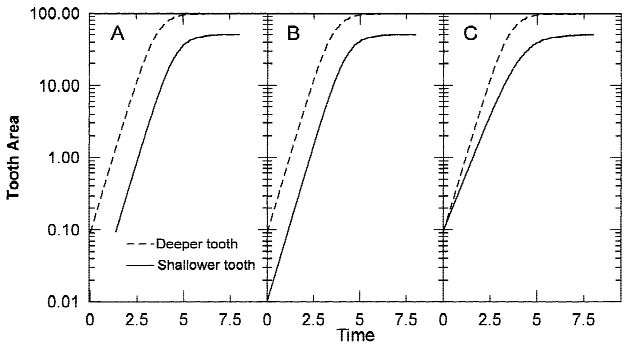


Figure 1. Graphical representation of three hypotheses that could explain differences in tooth size. **(A)** Deeper teeth initiate earlier than shallower teeth. **(B)** Deeper teeth are larger at initiation than shallower teeth. **(C)** Deeper teeth expand at a higher relative rate than shallower teeth.

rate (Figure 1C). In the preceding hypotheses higher allometric constants (k) would yield deeper teeth. Similar initial size coefficients (b) in allometric analysis would lend support to hypothesis 1C, but different initial size coefficients would not discriminate between hypotheses 1A and 1B because this method of analysis does not involve a direct temporal variable. We used allometric analysis of the data to provide a means of comparison of our *Arabidopsis* data with that published in the literature. Allometric analysis permits tests of the following two hypotheses: A. The sinus of deeper teeth grows slower (smaller k) relative to shallower teeth. B. Deeper teeth are larger (larger b) than shallower teeth early in leaf development. We provide an objective assessment of the relative merits of the LPI and an allometric approach to the growth analysis of polymorphisms in depth of toothing.

Materials and Methods

Plant Material

Arabidopsis thaliana seeds were obtained from the Arabidopsis Biological Resource Center at Columbus, Ohio. From the Columbia-1 (Col-1) genotype, one mutant was studied: *se*, with deeper and increased number of teeth per leaf than Col-1. The *se* mutant was generated by x-ray irradiation, and the investigator involved considered it "essentially isogenic" with the wild-type (George P. Rédei, personal communication). It was important that the mutant and wild-type were isogenic to ensure that the developmental differences observed would be due only to the mutation. *A. thaliana* seeds were soaked in distilled water 24 h before sowing. The seeds were dispensed in the center of 4-inch pots filled with 1:1 Garden Magic Topsoil (Michigan Peat Co., Houston, TX):Sakrete White Sand (Sakrete Inc., Cincinnati, OH), using a Pasteur pipette (Meinke and Sussex 1979). The pots were placed in a growth chamber (model CEC38-15, Rheem Manufacturing Co., Montgomery, AL), and covered with plastic wrap to retain moisture. Once the seedlings emerged, the plastic wrap was removed and they were watered as needed. The plants were fertilized with full-strength Hoagland's solution once per week. When the first true leaves were visible, the seedlings were thinned to one per pot. The growth chamber conditions were maintained at a 10 h/14 h photoperiod and 22°C/22°C temperature (light/dark) cycle, and illuminated by fluorescent bulbs (General Electric, Cool White, 1500). The light intensity was 600 μmol s^{-1} m^{-2} photosynthetically active radiation (PAR) at soil level. Growth chamber humidity was maintained at 70% RH.

Sampling

When seedlings were established, they were numbered, and individuals for sampling tooth development were chosen with the aid of a random number table. Just before sampling, the leaf lengths of all the visible leaves on a plant were recorded. The PI of each plant sampled was determined at a reference length of 3 mm according to Eq. (2). In addition, the LPI was determined for each leaf analyzed according to Eq. (3).

Statistical validation of the assumptions for use of the PI were evaluated in a previous work (Groot and Meicenheimer, in press). It was determined that under the preceding growth conditions, the growth of leaves 11–37 of Col-1, and leaves 7–37 of *se* could be accurately described by the LPI. Only those leaf serial numbers were used in the subsequent growth analyses of teeth.

Leaves of *A. thaliana* that were longer than about 6 mm were detached and placed on 1-mm grid paper. Digitized images of the leaves were acquired by means of a charge coupled device, using the Image 1 image analysis hardware and software (Universal Imaging Corp., Philadelphia, PA) of a personal computer. The remainder of the rosette was vapor fixed for 1 h with 2% aqueous OsO_4 in a sealed, moist chamber. This was followed by 8 h postfixation in 1% aqueous OsO_4 at room temperature. The samples were rinsed two times with distilled water for a total of 30 min, three times with 30% ethanol (EtOH) for a total of 45 min, and then stored at 4°C in the dark until needed. Subsequently, each rosette was carefully dissected in a Petri dish of 30% EtOH to remove the leaves and leaf primordia, exposing the shoot

apical dome. The dissected organs were individually dehydrated for 30 min each in a 30%, 50%, 70%, and 95% EtOH series, and then rinsed three times for a total of 3 h in absolute EtOH. The samples were critical-point dried, mounted on an SEM stub with silver paint, and coated with approximately 23 nm of gold in a Denton Vacuum, model Desk II sputter coater (Moorestown, NJ). The mounted samples were viewed at 20 keV in a Philips model 505 SEM. To ensure consistency of magnifications; all the samples were viewed at the same working distance and fixed electronic focus. All focusing was accomplished by use of the fine Z-movement control of the specimen stage. Digitized images were acquired by means of a cable connecting the composite video output of the SEM to the image analysis hardware (Data Translation model DT3152, Marlboro, MA) of a personal computer. The surface of the tooth to be imaged was oriented normal to the direction of view before acquiring the image.

Data Analysis

By use of Image 1 image analysis software, several measurements were made on the digitized images: midrib length (M), tooth apex to midrib length (T), tooth sinus to midrib length (S), and tooth area (A) (Figure 2). Midrib length was the curve length of the midrib plus the petiole of the leaf. Tooth-to-midrib distance was the length of the line perpendicular to the midrib, from the midrib to the apex of a tooth. The apex of a tooth was the point at which the curvature of the tooth outline reversed direction. Tooth sinus–to–midrib distance was the length of the line perpendicular to the midrib, from the midrib to the apical sinus of a tooth. Tooth area was the area of the tooth formed by the line from its apical sinus to its basal sinus and the perimeter of the leaf margin. In the *se* mutant, the smaller, secondary teeth were omitted from growth analysis because Col-1 lacked such teeth.

Growth analysis was based on mean tooth measurements of each leaf, with each leaf representing a particular point in time of leaf development. Analyses of mean tooth measurements of a population of plants were performed both chronologically and allometrically. In chronologic growth analysis mean tooth area per leaf was plotted on a log axis versus LPI, the time variable. The slope of a straight line fitted to the data represented the relative plastochron rate of tooth expansion in area. The slopes between the genotypes Col-1 and *se* were compared with the heterogeneity of slopes test in PROC GLM of SAS (SAS Institute, Carey, NC). Because successive leaves were not always available to determine

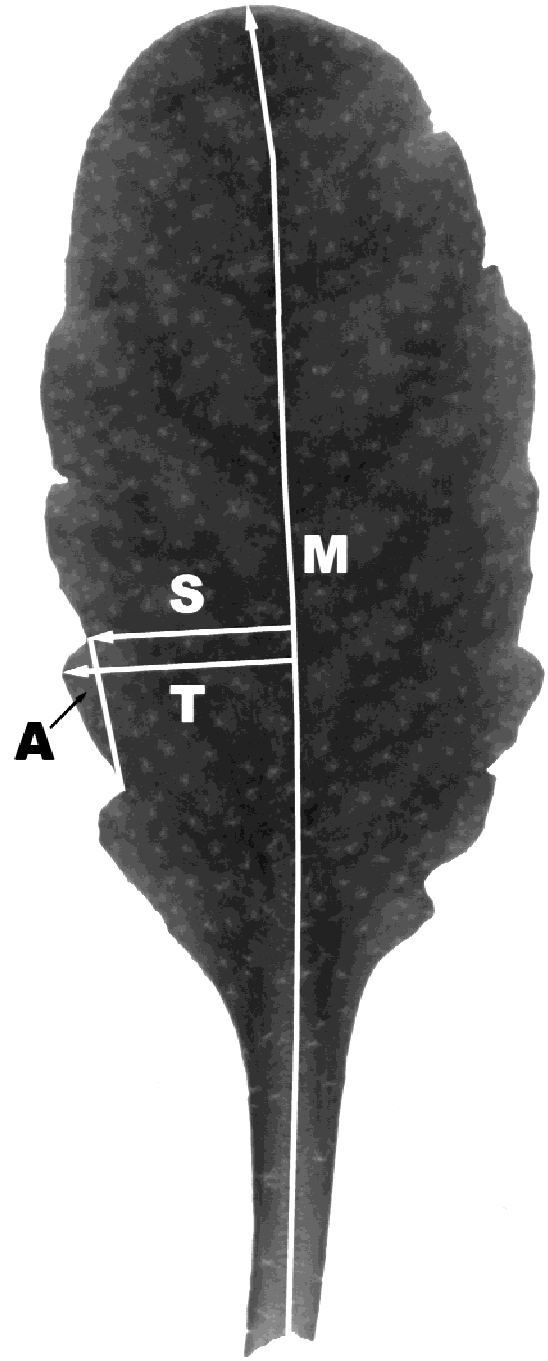


Figure 2. Digitized image of an *Arabidopsis thaliana* leaf, illustrating the measurements made on each tooth. *M,* midrib plus petiole; *S,* tooth sinus-to-midrib distance; *T,* tooth apex-to-midrib distance; *A,* tooth area.

the LPI of tooth initiation, the mean LPI was taken of the youngest leaf primordium with a tooth and the oldest leaf primordium without teeth. The mean LPI of tooth initiation was compared between the two genotypes using Student's t test. Solving for tooth area at the LPI of tooth initiation yielded the

mean tooth area at initiation, which was compared between the two genotypes by determining whether their 95% confidence intervals of prediction overlapped.

Allometric analysis on lobe growth by previous investigators used lobe area, sinus-to-petiole distance, and lobe-to-petiole distance as the "dependent" variables on species that were palmately lobed. Because *A. thaliana* was not palmately toothed, mean tooth area, mean tooth sinus-to-midrib distance, and mean tooth apex-to-midrib distance were used as the "dependent" variables. These "dependent" variables were plotted versus leaf length on log-log axes. From least squares regression of a straight line on these log-log plots the slope (allometric constant) and the intercept were determined. The heterogeneity of slopes of ANCOVA was performed on the data by using the SOLUTION option of PROC GLM in SAS to compare the allometric constants and the intercepts between the genotypes Col-1 and *se*. In addition, PROC REG was used to test the allometric constants for isometric growth, which would equal 1 for linear dimensions of growth and 2 for area dimensions of growth.

Voucher Specimens

Selected plants of the populations raised in the growth chamber were chosen to document their identity. At least two rosette and two bolted plants of each genotype were pressed and mounted on herbarium sheets. The specimens were deposited in the Willard Sherman Turrell Herbarium at Miami University, Oxford, OH.

Results

Columbia-1

The vegetative phase of the wild-type produced a rosette of leaves arranged in a (3,5) contact parastichy pattern of spiral phyllotaxis (Figure 3a,b). Teeth were not readily apparent on expanded leaves because leaf margins were revolute (Figure 3a). Teeth appeared to initiate in a basipetal sequence on the leaf margin (Figure 3c–d) because the largest teeth were usually apicalmost. The young leaf (Figure 3e) was broader in the basal region and the basalmost four teeth occupied the basal third of the leaf compared with the mature leaf on which these four teeth occupied half of the lamina length (Figure 3f). The teeth of Col-1 in the mature leaf, according to the terminology of Hickey (1973), had rounded apices, and their apical and basal sides ranged from convex to straight (Figure 3f).

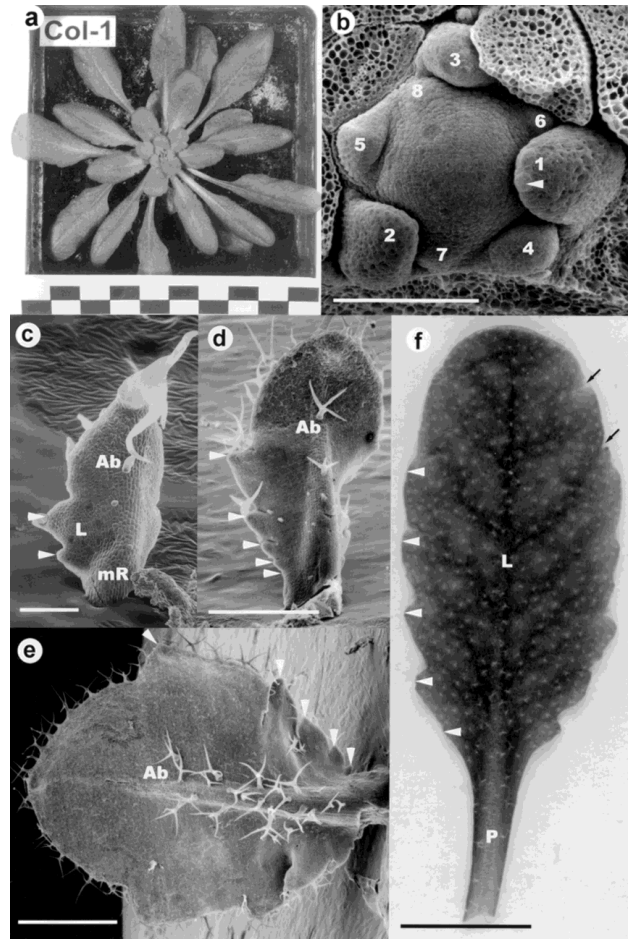


Figure 3. Ontogeny of the leaf of Columbia-1 genotype. **(a)** Rosette leaves of *Arabidopsis thaliana*. Col-1 teeth are not evident because the leaf margins are revolute. Ten centimeter scale is in 1-cm increments. PI = 32.8 plastochrons. **(b)** Leaf primordia initiate in a spiral pattern from the shoot apex; numbered from oldest to youngest. A trichome *(arrowhead)* is initiating at the apex of the oldest primordium. Plastochron index of shoot = 12.6 plastochrons. Scale bar = 100 µm. **(c)** Abaxial *(Ab)* view of leaf primordium with teeth *(arrowheads)* on the margin. Lamina *(L)* is differentiated from the midrib *(mR)* at this stage. LPI = −8.4 plastochrons. Scale bar = 100 µm. **(d)** Abaxial *(Ab)* view of leaf primordium where all teeth *(arrowheads)* have initiated. LPI = −2.4 plastochrons. Scale bar = 500 µm. **(e)** Abaxial *(Ab)* view of young leaf. *Arrowheads* indicate teeth along a margin. LPI = 1.1 plastochrons. Scale bar = 1 mm. **(f)** Adaxial view of mature leaf showing the lamina *(L)* and petiole *(P)*. *Arrowheads* indicate teeth along a margin. Flattening of the leaf for photography tore the leaf margin *(arrows)*. LPI is between 10 and 15 plastochrons. Scale bar = 10 mm.

Serrate

Leaf primordia of *se* also initiated in a spiral phyllotaxis but were arranged in a (5,8) contact parastichy

pattern (Figure 4a, b). After initiation of the primary teeth (Figure 4c), secondary teeth initiated on the basal side of the primary teeth (Figure 4d). The primary teeth had a strongly convex apical side and a concave to straight basal side, causing their apices to be inclined basally (Figure 4e, f). The secondary teeth were smaller than the primary teeth, and they tended to have straight apical and basal sides. The leaves and teeth of *se* had pointed, rather than rounded apices (Figure 4f). There were almost twice as many teeth per leaf of *se* than there were for Col-1 (Table 1).

The distinguishing feature of *se* was the deeper and more numerous teeth than those of Col-1 (see Figures 4a,f and 3a,f). Heteroblasty in the *se* mutant is accelerated relative to Col-1. There were fewer round primary leaves, and teeth first appeared on the third leaf (Table 1). The teeth sinuses were about twice as deep as those of Col-1 (Table 1). The *se* mutant had fewer rosette leaves, many more cauline leaves, but a similar number of total leaves compared with Col-1 (Table 1). In the range of leaf serial numbers used for the LPI, the mean relative growth rate (RGR) was about 1.25 times larger for Col-1 (Table 1). The mean plastochron of *se* was about 1.5 times longer than that of Col-1 (Table 1).

Growth Analysis of Development

Comparison of the LPI functions of leaf elongation for Col-1 and *se* revealed significant differences (Figure 5). Serrate leaves were initiated at an earlier LPI and elongated at a lower relative rate up until −3 LPI. Thereafter, the relative rates of leaf elongation of *se* and Col-1 were similar.

Plotting mean tooth area versus LPI for both *se* and Col-1 indicated that *se* initiated teeth about 15 plastochrons earlier in leaf development than did Col-1 (Figure 6). Each point on the plot represented the mean tooth area of all the teeth on a particular leaf. A t test for time of initiation indicated that the difference was significant ($Pr > |T| = 0.0004$, 7 *df*). The RGR in tooth area per plastochron of Col-1 was greater than that of *se* (Figure 6). The heterogeneity of slopes test in ANCOVA indicated that the difference was significant ($Pr > F = 0.0001$). The tooth areas at initiation were determined by solving the regression lines in Figure 6 for area at LPI of tooth initiation. The 95% confidence intervals for prediction of the mean tooth areas at initiation did not overlap; therefore, teeth of Col-1 had a greater initial size than that of *se* (Figure 6). The lower RGR of *se* teeth offset the earlier time of initiation, so that average tooth area was similar in the expanded leaves for the two genotypes.

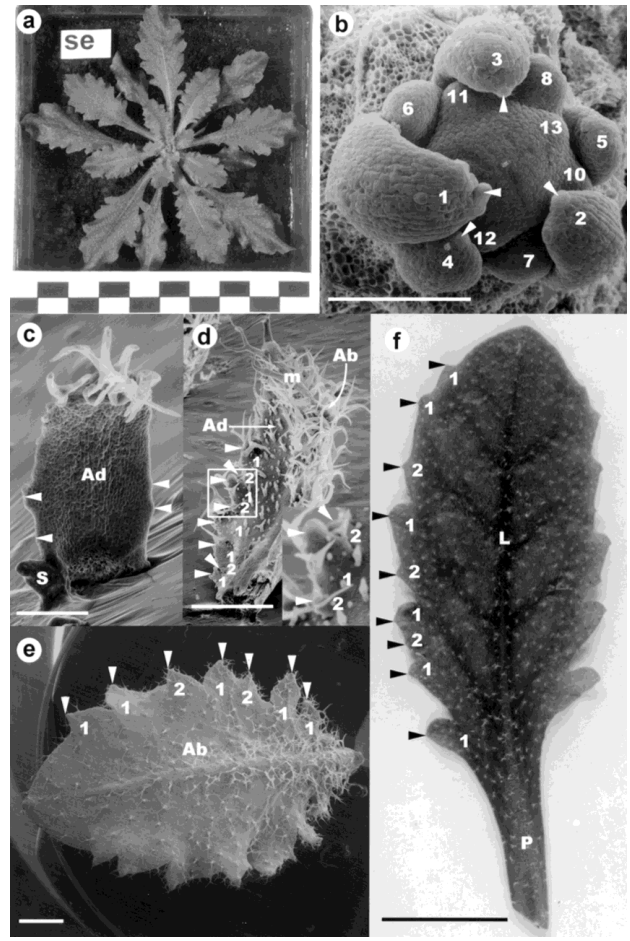


Figure 4. Ontogeny of the leaf of *serrate* genotype. **(a)** Rosette leaves of *Arabidopsis thaliana*. *Se* teeth are prominent. Ten centimeter scale is in 1-cm increments. PI = 25.5 plastochrons. **(b)** Leaf primordia initiate in a spiral pattern from the shoot apex; numbered from oldest to youngest. Primordium nine is hidden beneath primordium one. Trichomes *(arrowheads)* are initiating at the apices of the oldest primordia. PI = 14.7 plastochrons. Scale bar = 100 µm. **(c)** The first teeth *(arrowheads)* have initiated on a leaf primordium, seen from its adaxial *(Ad)* surface. Structure projecting exmedially from base is a stipule *(S)*. LPI = −25.1 plastochrons. Scale bar = 100 µm. **(d)** Several teeth *(arrowheads)* are apparent on the margin of the leaf primordium. Teeth are compound, with secondary teeth *(2)* being borne on primary teeth *(1)*. Secondary teeth are borne on the basal side of the primary teeth (*inset*, enlargement of the boxed area). The apical portion of leaf margin *(m)* is visible, separating the adaxial surface *(Ad)* of the leaf from the abaxial surface *(Ab)*. Trichome density is greater on the abaxial surface. LPI = −7.1 plastochrons. Scale bar = 500 µm. **(e)** Abaxial *(Ab)* view of young leaf. Teeth *(arrowheads)* are either primary *(1)* or secondary *(2)*. LPI = 3.7 plastochrons. Scale bar = 1 mm. **(f)** Adaxial view of mature leaf. Teeth *(arrowheads)* are either primary *(1)* or secondary *(2)*. LPI is between 10 and 15 plastochrons. Scale bar = 10 mm.

Table 1. Comparison of Developmental Parameters Between Col-1 and *se*

Developmental Parameter	Col-1	*se*	Pr>|T|	*df*	*n*
Serial number of first toothed leaf	4.5 ± 0.2	3.2 ± 0.2	0.0010[a]	9	12
Depth of tooth sinus[b]	0.11 ± 0.03	0.23 ± 0.01	0.0025[a]	16	78
Mean tooth number per leaf	8.0 ± 0.2	15.4 ± 0.3	0.0001[c]	54	62
Total leaves per main shoot	51.5 ± 0.3	59.8 ± 3.9	0.1010	4	11
Cauline leaves per main shoot	7.3 ± 0.2	25.8 ± 2.0	0.0008[c]	4	11
Days from sowing to bolting	41.3 ± 0.4	49.4 ± 1.2	0.0013[a]	5	11
RGR (d^{-1})	0.64 ± 0.009	0.51 ± 0.009	0.0001[c]	265	267
Plastochron (d)	0.49 ± 0.028	0.74 ± 0.056	0.0013[a]	147	227

Mean data ± standard error of the mean. The probability that the means are similar between the two genotypes, using a t test is given by Pr>|T|, and the degrees of freedom is indicated by df. n = total number of observations in the t test.
[a] $p < 0.01$.
[b] *Measured for leaves older than LPI + 4.0. A value of 0.25 means that the sinus extends one fourth of the distance to the midrib.*
[c] $p < 0.001$.

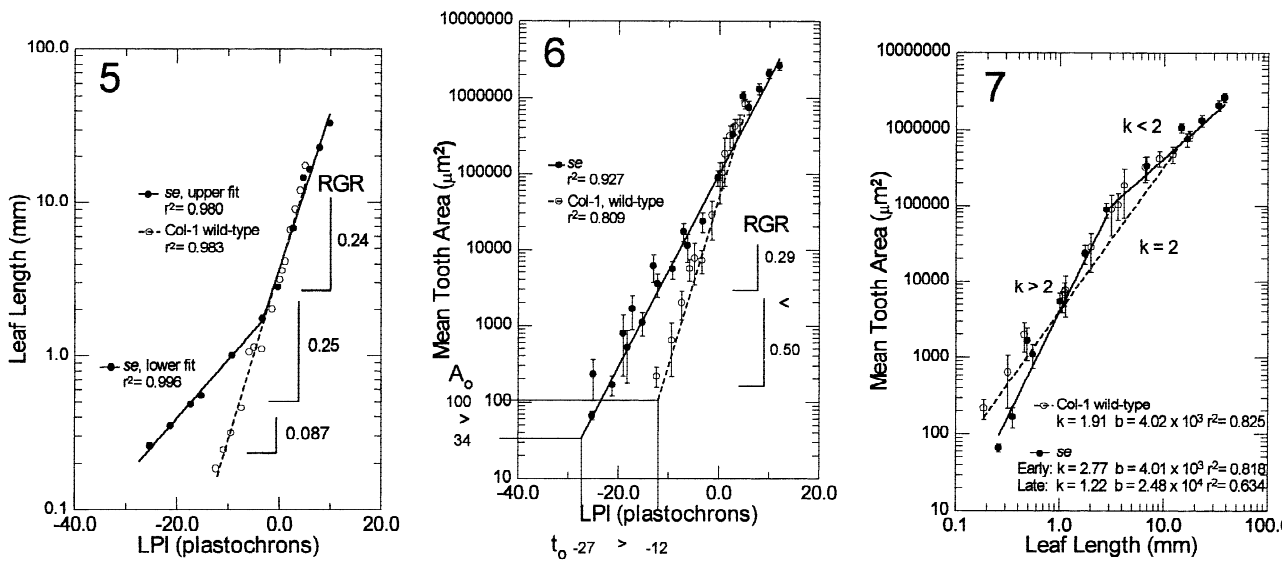


Figure 5–7. Growth and allometric analyses of leaf length and tooth area for *se* and Col-1. 5. Length of leaves as a function of LPI. The *se* mutant leaves are initiated earlier and expand at a lower relative plastochron growth rate *(RGR)* during early phases of elongation. RGR of *se* and Col-1 are equal during the later phase of leaf expansion. (6) Mean tooth area as a function of LPI. Smaller *se* teeth are initiated earlier and expand at a lower relative plastochron growth rate *(RGR)* than Col-1. Each point represents the mean of two to 14 tooth areas in one leaf. (7) Mean tooth area as a function of leaf length. Tooth area increases isometrically for Col-1, but for *se* there is a complex biphasic response. Each point represents the mean of 2 to 14 tooth areas in one leaf. Allometric data for best fit straight lines are indicated in annotations.

Allometric analysis of mean tooth area indicated that teeth of *se* had two phases of relative growth, but not Col-1 (Figure 7). Early relative growth of teeth in *se* exceeded that of Col-1 but later slowed so that mature tooth areas of the two genotypes were similar (Figure 7). There was no statistical support for two phases of relative tooth growth in Col-1 because analysis of the allometric relationships bounded by the same early and late boundaries of leaf length showed no significant difference in slope (Pr > F = 0.9319): k = 1.88 for the early phase versus k = 1.77 for the late phase. In addition, such partitioning decreased the coefficient of determination (0.624 for the early phase, and 0.378 for the late phase) relative to the full data set. The allometric constant of Col-1 was similar to two (Pr > F = 0.3206), indicating isometric growth of the teeth (Figure 7). In contrast, the early phase of *se* had an allometric constant greater than 2 and greater than that of Col-1 (Pr > F = 0.0022 and 0.0013, respec-

tively). The later phase of *se* had an allometric constant less than 2 (Pr > F = 0.0001). *Se* teeth had smaller initial area than Col-1 teeth but became equal in area when leaves reached 1 mm in length. Thereafter, *se* teeth exceeded Col-1 area, reflecting the higher allometric constant during early tooth expansion.

The LPI functions of mean tooth apex (Figure 8) and mean tooth sinus (Figure 9) distance from the midrib were similar in overall form to that of leaf length (Figure 5) for both genotypes. That is, both apex and sinus *se* metrics were clearly biphasic with early growth of se being significantly less than that of Col-1. There was no significant difference between *se* and Col-1 in regard to these metrics in later stages of tooth development. Comparison of the RGR constant characterizing apex and sinus growth in the early stage of se tooth development revealed that sinus expansion was 3% less than apex expansion (Figures 8 and 9).

In the allometric analysis of tooth apex to midrib distance, the best fit lines of the two genotypes almost superposed (Figure 10). There was no significant difference between either the allometric constants (Pr > F = 0.6376) or the intercepts (Pr > F = 0.4591). Both of the allometric constants were similar to one (Pr > F = 0.8624 for Col-1, and 0.1683 for *se*), indicating isometric expansion of the tooth apex region with that of leaf length. An allometric analysis of sinus-to-midrib distance showed a similar condition (Figure 11). There was no support for two phases of differing relative growth in sinus-to-midrib distance of the two genotypes. There were no significant differences between the allometric constants (Pr > F = 0.2311) or the slopes (Pr > F = 0.3991) of Col-1 and *se*. Both of the allometric constants were similar to 1 (Pr > F = 0.5161 for Col-1, and 0.2422 for *se*), indicating isometric expansion of the sinus region with that of leaf length. Allometric analysis of sinus length versus tooth apex length (Figure 12) resulted in allometric constants equal to 1 and similar intercepts for both Col-1 and *se* teeth. Thus, using allometric analyses, the hypotheses that deeper teeth have a lower RGR in the sinus region or are larger than more shallow teeth were not supported.

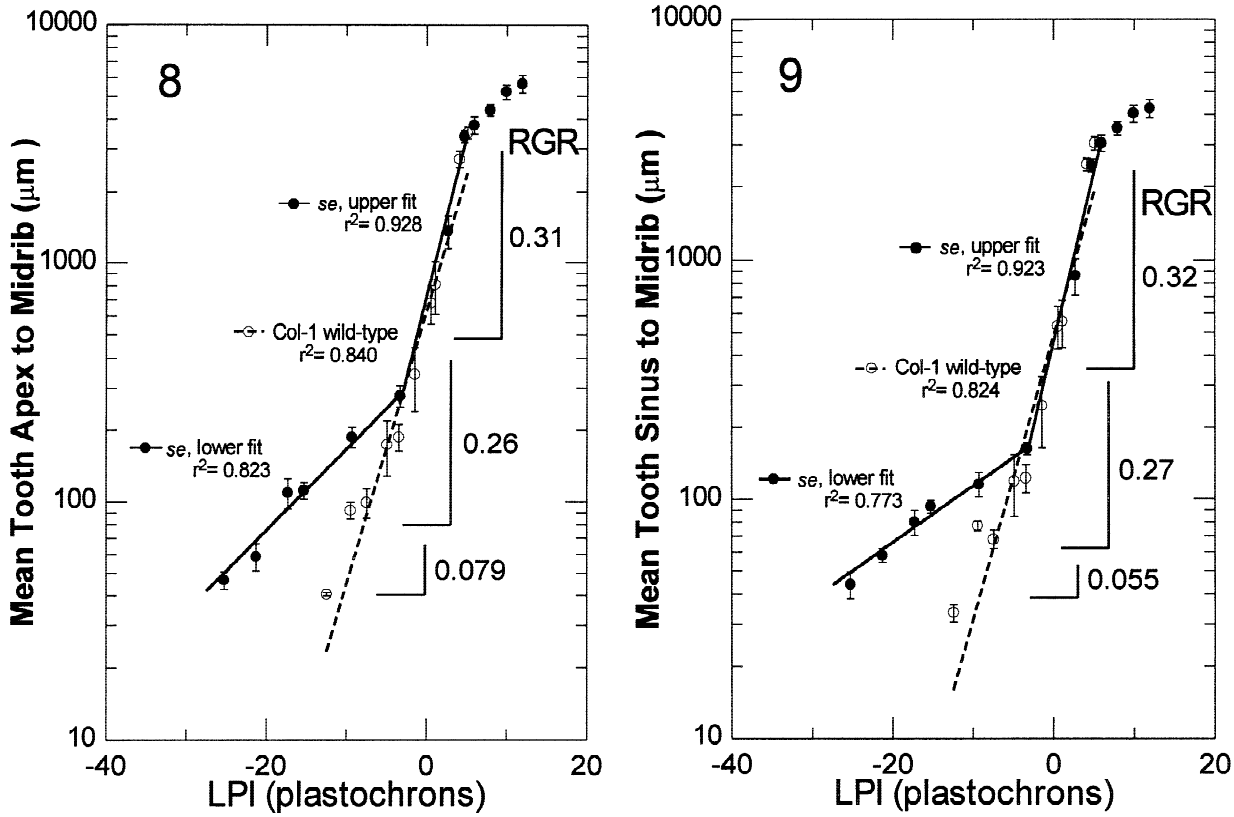
Discussion

The *se* mutant is pleiotropic, which indicates that the *SE* gene is expressed in tissues besides that of the presumptive teeth in leaf primordia. Not only is the leaf margin affected, but also the flowering time is delayed (Table 1), leaf RGR is initially slower (Figure 5), there are more bifurcate than trifurcate leaf trichomes, the cauline internode length is irregular, and the siliques appear shorter than wild-type. The delay of flowering may be indicative of lowered constitutive levels or responsiveness to gibberellins (GAs), but this is probably not the case in *se*. Under short day conditions, reduced levels of, or insensitivity to, GAs results in dwarf *A. thaliana* plants with delayed flowering (Wilson and others 1992). On the other hand, increased levels of, or hypersensitivity to, GAs results in spindly plants with premature flowering (Jacobsen and Olszewski 1993). Neither of these phenotypic suites are characteristic of *se*.

Leaf development in *A. thaliana* was similar to that of other simple-leafed dicots. The leaves initiated as primordia from the shoot apex in a spiral phyllotactic pattern (Figures 3b and 4b). Col-1 exhibited a (3,5) and *se* a (5,8) contact parastichy pattern of phyllotaxis. This suggests that *se* leaf primordia were expanding at a lower relative plastochron rate of radial expansion than Col-1, although data to test this hypothesis were not collected in this study. Typical of most simple dicot leaves differentiation was basipetal (Maksymowych 1990), although Tomlinson and others (1991) noted an exception of acropetal leaf differentiation in *Quercus rubra* (northern red oak). Basipetal leaf maturation was first reported in *A. thaliana* by the observation of a basipetal gradient in cell expansion (Pyke and others 1991).

The increased number and depth of toothing was not due to a more "adult" heteroblasty in *se*. The leaves of *se* did not resemble the late leaves and inflorescence leaves of Col-1, its background genotype. The number and depth of teeth in Col-1 leaves did not appear to change after the early leaves of the heteroblastic series were present.

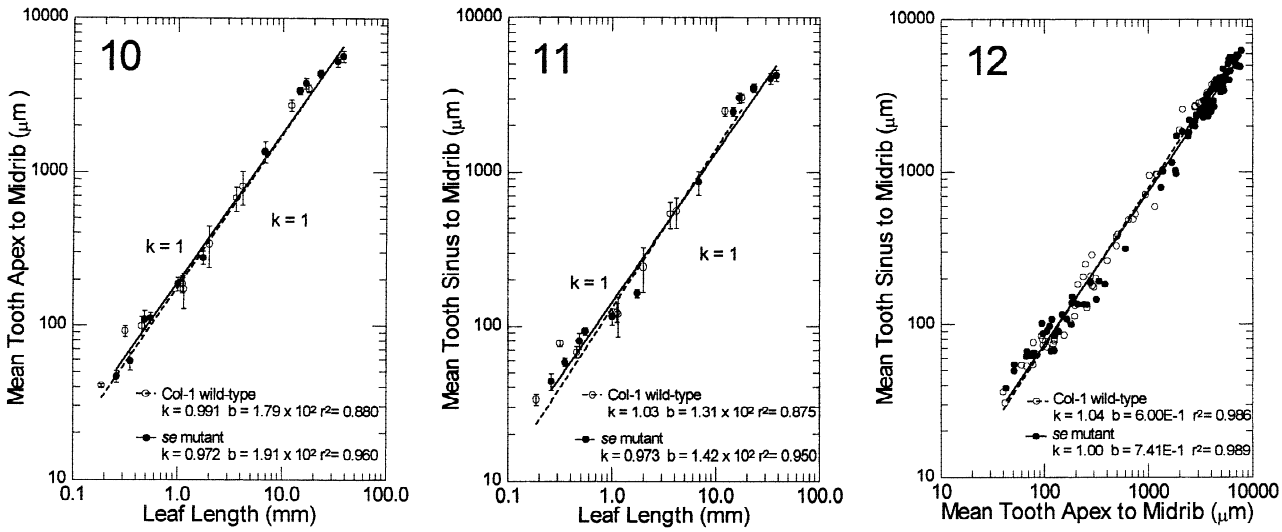
Growth analysis of teeth by use of the LPI permitted us to evaluate our three working hypotheses on tooth development. Hypothesis 1A (deeper teeth are initiated earlier) was supported in terms of LPI of initiation (Figures 6, 8, and 9) and in terms of serial number of first toothed leaf (Table 1). There was no support for hypothesis 1B (deeper teeth are initially larger). In fact, on a tooth area basis the reverse was indicated by the data (Figure 6). There were no differences in tooth apex and sinus lengths between Col-1 and *se* (Figures 8 and 9). There was no support for hypothesis 1C (deeper teeth grow faster) as well. In terms of tooth area, *se* expanded at close to half the relative rate of Col-1 (Figure 6). In terms of tooth apex and sinus elongation the early stage of *se* elongation was less than Col-1 elongation, whereas elongation rates were similar between both genotypes in the later stage of tooth elongation (Figures 8 and 9).



Figures 8 and **9.** Growth analyses of mean lengths of tooth apices and tooth sinuses relative to midrib in *se* and Col-1 as a function of LPI. (8) Mean tooth apex length. (9) Mean tooth sinus length. Both *se* tooth apices and sinuses are initiated earlier and expand at a lower relative plastochron growth rate *(RGR)* than Col-1 during the early phase of development. RGR of *se* and Col-1 apices and sinuses are equal during the later phase of tooth apex expansion.

Deeper lobing in palmately lobed species reportedly arises primarily through an allometric relationship in which sinus regions elongate at lower relative rates compared with apical regions (Dolan and Poethig 1991; Hammond 1941a,b; Jones 1993; McLellan 1990). Allometric analyses of our data permitted the following conclusions to be drawn in regard to depth of pinnate toothing. The allometric constant between mean *se* tooth area and leaf length was greater than Col-1 during the early phase but was less than Col-1 during the later phase of tooth development (Figure 7). Allometric analyses of mean length of tooth apices and sinuses as functions of leaf length revealed no differences between the genotypes (Figures 10 and 11). Likewise, allometric analysis of sinus length and apex length revealed no differences between the two genotypes (Figure 12). LPI analysis did, however, indicate that the RGR of tooth apices exceeded the RGR of tooth sinuses in *se* during the early stage of tooth expansion (Figures 8 and 9)

Both *se* and Col-1 exhibited constant but different relative rates of tooth area increase with se teeth expanding at about half the rate of Col-1 (Figure 6). The temporal functions of *se* and Col-1 leaf elongation were significantly different from one another in terms of form. Col-1 leaves elongated at a constant rate from their time of initiation, whereas *se* exhibited a biphasic growth function, in which older leaf primordia expanded at about three times the rate of younger leaf primordia (Figure 5). The relative rate of Col-1 leaf elongation corresponded to the relative rate of the *se* leaf elongation in the later phase of development. The temporal interplay of these relative rates results in dissimilar allometric relationships between tooth area and leaf length of the two genotypes. Whereas Col-1 exhibited an allometric constant equal to the expected 2.0 throughout leaf expansion, *se* had an allometric constant significantly greater than 2.0 in the early phase of leaf expansion and significantly less than 2.0 in the later phase of leaf expansion. The average of the allometric constants for se tooth area was similar to the average Col-1 allometric constant. The main effects of the *se* mutation therefore appeared to be threefold; earlier initiation of leaf primordia and tooth



Figures 10–12. Allometric analyses of tooth apices and sinuses to midrib distance in *se* and Col-1 genotypes. (10) Tooth apex length as a function of leaf length. (11) Tooth sinus length as a function of leaf length. Each point represents mean of two to 14 teeth in one leaf in 10 and 11. (12) Tooth sinus length as a function of tooth apex length. Each point represents one tooth. Expansion is isometric in all cases and there is no significant difference between the genotypes. Allometric data for best fit straight lines are indicated in annotations.

primordia, a uniform decrease in the relative rate of tooth area expansion, and a decrease in leaf expansion during the early phases of leaf development. The decrease in leaf length expansion was threefold, whereas tooth area expansion was twofold. The combination of these two dissimilar rates resulted in more numerous teeth on *se* leaves that are similar in total mature area to those of Col-1.

Given that the stage of leaf development in Figures 3d and 4d represented the end of the tooth production period, the total production interval lasted approximately 20 plastochrons in *se* and 10 plastochrons in Col-1. Using the mean plastochrons reported in Table 1, this translated to about 15 days in *se* and 5 days in Col-1. This expanded "window" of tooth initiation in *se* could have allowed for more teeth to be initiated, assuming that the two genotypes produced teeth at similar rates. With Col-1 data it is estimated that tooth production was 1.6/day. On the basis of the preceding assumption, this would predict that *se* leaves would produce 24 teeth in 15 days. This prediction is greater than the total mean number (15) of teeth produced on *se* leaves. Thus the earlier timing of tooth initiation in *se* could also explain why there were more teeth per leaf than there were in Col-1 (Table 1). The double teeth in *se* (Figure 4f) may have represented a temporal reiteration of the tooth initiation program. Observations of developing leaves supported this view because primordia of secondary teeth appeared to initiate subsequent to those of the primary teeth (Figure 4d).

When the area data in Figure 6 were analyzed allometrically (Figure 7), the differences in growth were masked. In allometric analysis of area growth, one would expect deeper teeth to be explained by either a greater allometric constant or a higher intercept. The greater allometric constant for deeper teeth would have resulted in larger teeth relative to leaf length, and the higher intercept would have resulted in larger teeth at all stages of leaf development, assuming that the allometric constants were similar. As illustrated in Figure 7, the allometric relationships between tooth area and leaf length were complex. The allometric constant for *se* was initially greater than that of Col-1, but later it was smaller. The two phases of growth were peculiar to allometric analysis of tooth area and were not evident when tooth area was plotted versus LPI (Figure 6). The early phase in Figure 7 was probably not due to differences in sample preparation (SEM samples versus fresh leaves) because the data points of the early phase included both SEM and fresh samples. The two phases probably represented a change in the RGR of the leaves in *se* (Figure 5). The allometric constants in Figure 7 could be derived by dividing the relative plastochron rate of growth of tooth area (Figure 6) by the relative plastochron rate of growth in leaf length (Figure 5). Because relative plastochron rate of growth in leaf length is in the denominator when deriving allometric constants, the early allometric constant for *se* in Figure 7 was greater than that of the later allometric constant. The change in leaf RGR occurred at about LPI -3, or

about 2 mm, coinciding with the change in the allometric constant in Figure 7. Meicenheimer (1981) observed two phases of relative plastochron rate of leaf growth in *Epilobium hirsutum*, but the trends were opposite from that of Figure 5, initially a higher rate and later a lower rate. Meicenheimer (1981) explained the differing rates on the basis of mechanical constraint—that leaves older than a particular LPI grew slower because they were in contact with older primordia. Release from mechanical restraint could explain the increase in RGR for 2-mm leaves of *se* (Figure 5) because leaves greater than about 1 mm left the confines of the apical bud. However, Col-1 did not display this behavior; therefore, the changing RGR in *se* may have been an expression of the mutant phenotype independent of mechanical constraint.

Allometric analyses conducted by other investigators produced clearer conclusions relative to more deeply lobed leaf mutants or varieties. McLellan and Dengler (1995) were the only researchers to conduct an allometric analysis of lobe growth using area as the dimension in question. Among several varieties of *Begonia dregei,* the deeply lobed genotype had a lower allometric constant than that of more shallowly lobed genotypes. This result was opposite to what was expected for the *se* mutant because *B. dregei* leaves were palmately lobed. In such leaves, the sinuses of deeper lobes were closer to the petiole and had shorter arc distances from each other than those of shallower lobes; therefore, deeper lobes were narrower and smaller than shallower lobes. Other allometric analyses of sinus and lobe-to-petiole distances indicated that the sinus regions expanded more slowly relative to lobe regions in mutants or varieties that had deeper lobes than normal (Dolan and Poethig 1991; Hammond 1941a, b; Jones 1993; McLellan 1990; Whaley and Whaley 1942). From allometric analysis of sinus and tooth growth in *A. thaliana* (Figures 10 to 12), the results were inconclusive. Both tooth and sinus-to-midrib distance expanded isometrically with that of leaf length for both genotypes. There were also no significant differences between the intercepts. Chronologic analysis of tooth growth explained why teeth were deeper in *se* where allometric analysis was unable to do so. Allometry does not provide a direct method of assessing temporal changes of growth parameters during leaf development.

This report is the first to address growth analysis of toothed leaves. Because previous research on lobe development used only allometric techniques, this work is also the first to address growth analysis of teeth on a plastochron basis to precisely determine the timing of events. Each data point in the plots illustrating growth analysis of teeth (Figures 6, 8 and 9) represents one leaf, and its value is the mean of all the tooth measurements on that leaf. Because the number of teeth per leaf varies through leaf ontogeny, data points on the plots represent differing numbers of measurements, hence differing weights. For that reason it is important that the statistical tests and regressions pertaining to those plots make use of all the measurements used to produce the mean values rather than the mean values alone. The result of this strategy lowers the coefficients of determination of regressions and decreases the significance of statistical comparisons relative to using mean values alone, but it more fairly represents the data.

Further characterization of the *se* mutation is necessary to understand how they alter normal tooth development. Because altering GA levels affects depth of toothing or lobing (Chandra Sekhar and Sawhney 1991), such an effect should be investigated. It is expected that GA application to leaf primordia of the *se* mutant may phenocopy the wild-type. Genetic characterization would probably afford greater insight into depth of toothing or lobing because *se* is a single-gene, recessive mutation that is isogenic with the wild-type. Results of crosses of the mutant with those of similar and contrasting phenotypes can indicate the presence of allelism and genetic interaction.

Other genes associated with leaf development can be studied in the *se* mutant. The KNOTTED1-like homeodomain (*knox*) proteins appear to be involved with specifying indeterminacy of shoot apical meristems. In species with simple leaves, *knox* expression is strong in shoot apical and floral meristems but absent in presumptive locations of leaf and floral organ primordia (Chuck and others 1996). In *Lycopersicum esculentum*, a species with compound leaves, however, the expression is also found in leaf, leaflet, and floral organ primordia (Hareven and others 1996). Further research by Hofer and others (1997) has shown that the *unifoliata* mutant of pea is caused by an alteration of an ortholog of the *FLORICAULA/LEAFY* gene. Its homologue in *A. thaliana* is expressed at high levels in developing lobes of *Lepidium* (Neelima Sinha, personal communication). The expression of *knox* and *leafy* genes may be correlated with tooth and lobe initiation and may interact with the *se* mutation, resulting in deeper lobes and teeth. For example *FLO/LFY* genes may be expressed during tooth and lobe initiation and may be expressed earlier in leaf development of *se* than in the wild-type.

When allometric analysis is used to compare growth behaviors among leaf parts or among taxa,

the allometric constants typically are tested first for differences (McLellan and Dengler 1995). If the allometric constants are found to be similar, the intercepts are tested for differences. Comparisons of allometric constants illustrate trends of growth relative to that of leaf length, which do not always reflect trends in the size of the variables over chronologic time. Because the allometric constant is a function of the RGRs of leaf length and the variable of interest, one can make true comparisons between allometric constants only when the RGRs in leaf length are similar among the entities being compared. Leaf RGR can vary among taxa and among leaf serial numbers of the same taxon. Thus, one can validly compare the growth among lobe area, lobe length, or sinus distance in the *same* leaf serial number of the *same* taxon or genotype but not necessarily among different leaf serial numbers, taxa, or genotypes. Thus, the allometric constant can reflect growth behavior of a variable only under special conditions, which can be determined only by use of the PI.

Interpretation of the intercept derived from allometric analyses is more problematic because it could represent differences in timing of initiation, in initial size, in RGRs, or a combination of all three. This adds complexity to the assumption by Dolan and Poethig (1991) that the intercept " . . . represents the initial shape of the structure." Furthermore, the intercept in allometric analysis represents the size of the variable when the leaf length is *one*, but rarely is a structure under investigation initiated at this value (see Figure 7), even if the units of measurement are adjusted. In allometric analysis, a difference in timing of initiation will shift the trend line along the log length axis, but a difference in initial size would cause a shift in the direction of the variable under question. A lower leaf RGR coupled with earlier leaf initiation tends to cause a shift toward greater leaf lengths. True differences in the growth parameters (timing of initiation, initial size, or RGR) would be detected by allometry only when two of the three parameters are equal and only when the leaf RGRs are similar. Because differences in intercepts are tested only when allometric constants are similar, the RGRs of the variable under question must also be similar.

Use of the LPI to analyze growth of teeth is recommended. Allometry can be used when the PI assumptions fail for the range of leaf serial numbers desired; however, when allometric parameters are being compared among taxa or leaf serial numbers, equality of leaf RGRs among the entities being compared must first be tested. The advantages of using LPI over allometry is that the timing of initiation and initial size of teeth can be precisely determined, and the growth behavior of teeth is accurately described. Plotting tooth area versus LPI yields their growth rate, timing of initiation, and initial size. Plotting sinus and tooth apex length versus LPI shows whether there are significant differences in relative growth between the two regions. Plotting lobe area, lobe length, or sinus distance versus LPI also has the advantage of being able to derive the relative plastochron rates of growth of the variables. These RGRs can then be divided by the relative plastochron rate of growth in leaf length (Figure 5) to yield the respective allometric constants.

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