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The *Arabidopsis* embryonic shoot fate map

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SUMMARY

A fate map has been constructed for the shoot apical region of the embryo of the dicotyledonous plant *Arabidopsis thaliana* using spontaneously arising clonal albino sectors caused by the *chloroplast mutator 1-2* mutation. Chimeric seedlings exhibiting albino sectors shared between the cotyledons and first true leaves revealed patterns of organ inclusion and exclusion. Frequencies of clone sharing were used to calculate developmental distances between organs based on the frequency of clonal sectors failing to extend between different organs. The resulting fate map shows asymmetry in the developmental distances between the cotyledons (embryonic leaves) which in turn predicts the location of the first post-germination leaf and the handedness of the spiral of leaf placement around the central stem axis in later development. The map suggests that embryonic leaf fate specification in the cotyledons may represent a developmental ground state necessary for the formation of the shoot apical meristem.

Key words: Fate map, Apical meristem, Clonal analysis, *Arabidopsis thaliana*, *chloroplast mutator*

INTRODUCTION

Embryonic fate mapping experiments using genetic, surgical and dye-marking techniques have provided seminal information guiding the design of genetic and molecular experiments in *Drosophila melanogaster* (Poulson, 1950; Sturtevant, 1927), *Caenorhabditis elegans* (Sulston et al., 1983) *Brachydanio rerio* (Ho and Kimmel, 1993; Kimmel and Law, 1985; Kimmel et al., 1990; Melby et al., 1996; Strehlow et al., 1995) and other animals. For example, the embryonic pattern formation and limb compartmentalization systems active in the *Drosophila* embryo (Garcia-Bellido and Merriam, 1969; Garcia-Bellido et al., 1976), and the relative position and size of individual organ anlage on the embryonic blastoderm were predicted by fate mapping approaches. Recently, comparison of fate maps from a variety of animal species has demonstrated their utility both as characters in phylogenetic reconstruction and in elucidating the evolution of body patterning systems (Félix and Sternberg, 1996; Henry and Martindale, 1998; Sternberg and Félix, 1997).

The pattern of organ fate specification in plant embryos is less well understood than in animal model systems. The physical inaccessibility of embryos in the ovule, the existence of the cell wall and the relative delicacy of plant embryos make surgical and dye-marking fate mapping approaches difficult to establish in plant systems. Histological studies of developing embryos have provided extensive catalogs of cell division patterns occurring during plant embryogenesis. Unfortunately histological characterization of embryonic development does not provide sensu strictu fate maps of embryos as both the spatial orientation and frequency of cell divisions during embryogenesis are likely to depend on prior fate specification and patterning events (Jurgens, 1995). Genetic approaches using marked cell clones in *Arabidopsis* have been extensively used for lineage analyses in the root and shoot and have been used to map organogenic pattern formation in post-embryonic (seed stage, inflorescence and floral) *Arabidopsis* meristems (Bossinger and Smyth, 1996; Dolan et al., 1994; Furner and Pumfrey, 1992, 1993; Irish and Sussex, 1992; Scheres et al., 1994; Schnittger et al., 1996). Lineage analyses have also been performed on the apical regions of cotton and maize embryos (Christianson, 1986; Poethig et al., 1986). However, low numbers of clones bridging both embryonic leaves (either the cotyledons in the case of cotton or the scutellum and coleoptile in the case of maize) and shoot meristem derived organs prevented the construction of high-resolution embryonic organogenic maps. Thus, fate mapping of the early embryo has not been extensively pursued in plants in general or in *Arabidopsis* in particular.

During normal post-embryonic development the emergence of the cotyledons (embryonic leaves) and the production of the first two true leaves quickly follow germination of the mature seed of *Arabidopsis thaliana*. By nine days after germination, depending on growth conditions, the shoot apical meristem has produced true leaves 3 through 5 and the shoot manifests an obvious spiral of leaf placement around the circumference of the meristem, a pattern referred to as spiral/helical phyllotaxis. When comparing seedlings in a population, the handedness of the spiral of leaf placement on the *Arabidopsis* shoot occurs with a right handed or left handed helical twist with an approximate probability of 0.5 (Furner and Pumfrey, 1992; Irish and Sussex, 1992). In post embryonic fate mapping experiments performed to date using *Arabidopsis*, data from all plants regardless of phyllotactic handedness were mapped onto

model plants with either left or right-handed phyllotaxis, permitting the analysis of all chimeras as a single data set (Fig. 1). This approach was based on the expectation that similar, mirror image, pattern formation processes were occurring in individual embryos regardless of eventual seedling phyllotaxis. This assumption appears to be correct because single maps were resolved from combined data sets (Furner and Pumfrey, 1992; Irish and Sussex, 1992).

This report describes a map of the relative location of fate specification events giving rise to the cotyledons and true leaves 1 through 4 in *Arabidopsis*. Similar to fate mapping approaches used in *Drosophila*, a genetic approach has been pursued to generate spontaneous clones sampling cell divisions during early embryogenesis. Plants homozygous for *chloroplast mutator1* (*chm1*) mutations generate albino and albino sectored seedlings following occasional loss of functional chloroplasts in individual cells (Martínez Zapater et al., 1992). The resulting albino sectors may extend both between the cotyledons and between the cotyledons and true leaves, indicating that clone progenitor cells can arise prior to the specification of these organ anlagen. Utilizing previously derived equations (Furner and Pumfrey, 1992) the frequencies with which clones were shared between organs were utilized to calculate developmental distances between organ anlage. This map indicates that fate specification in the early embryo is an asymmetric process, and that asymmetric specification of the cotyledons predicts the eventual phyllotaxis of the shoot meristem. A model of embryonic apical fate specification is presented integrating previously reported observations of the asymmetric expression of a meristematic gene with this map.

MATERIALS AND METHODS

Seed stock

An *Arabidopsis* stock homozygous for the recessive *chm1-2* allele produces albino seedlings at a low rate (8.4%, *n*=305/3627) and produces chimeric seedlings with clonal sectors including the cotyledons and/or the first two true leaves at predictable levels (6.7%, *n*=243/3627). Chimeras were identified in which clonal sectors were shared between the two cotyledons and between cotyledons and true leaves, indicating that the clones arose prior to the commitment of embryonic cells to these different organ fates. These chimeras made up the raw data set of plants used for fate mapping. Seed of the *chm1-2* stock was provided by the *Arabidopsis* Biological Resource Center (Stock No. CS 3246) and was of the Columbia ecotype.

Plant culture and videocapture

Seeds were surface sterilized and plants were cultured in sterile conditions (Pickett et al., 1996). Medium consisted of 0.5× Gamborg's basal medium with 1.0% sucrose adjusted to pH 5.6 by addition of 0.1 M KOH. Prior to autoclaving 0.8% agar w/v was added to media solutions and agar medium was poured into 150×15 mm gridded Petri dishes. Sterile seed was plated onto the surface of the agar medium at intervals of 5 mm using sterile 10 μl mouth pipettes and suction to transfer individual seeds from aqueous suspension. Plated seeds were cold stratified for 3 days at 4°C and then transferred to a growth room maintained at 20°C for the remainder of the experiment. Videocaptures of growing plants were made at 3, 6 and 9 days after transfer to the growth room using an Olympus SMZ stereo dissecting microscope with a Sony RGB color CCD camera. Contrast and brightness enhancements and final assembly of figures were performed using Adobe Photoshop 4.0.

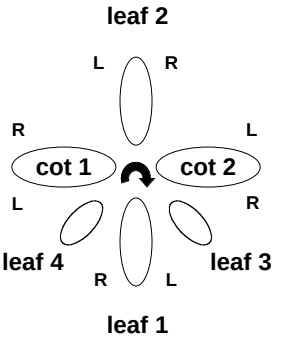

Fig. 1. Model of clockwise phyllotaxis utilized in this study. In accordance with standard practice for fate mapping in *Arabidopsis*, all seedlings analyzed in this study were converted to the same phyllotaxis and analyzed as a single data set. Cot 1 and Cot 2 signify cotyledons 1 and 2. Leaves 1-4 represent true leaves produced during post embryonic shoot formation. The labeling of the right and left margins of cotyledons and leaves is illustrated. For instance, leaf 1 and the right margin of cotyledon 2 are adjacent to the left margin of cotyledon 1. Leaf 2 and the left margin of cotyledon 2 are adjacent to the right margin of cotyledon 1. Arrow indicates direction of the helical spiral of new primordiagenesis.

Developmental landmarks

Developmental landmarks are required as reference points in the map, thus all leaf blades and petioles are designated as having a left margin and right margin when viewed with the petiole (leaf stem) pointing down, the leaf tip pointing up and the adaxial (upper) surface of the leaf facing the viewer. In addition, the spiral handedness of phyllotaxis as seen in leaves 3, 4 and 5 was used to provide a relative identity to the cotyledons and first two true leaves. True leaf 1 is designated as the true leaf closest to leaves 3 and 4, while true leaf 2 is designated as the true leaf farthest away from leaves 3 and 4 and closest to leaf 5. The naming of leaves 1 and 2 is solely derived from the relative positions of leaves 3, 4 and 5 and their position is designated independently from developmental distances calculated in the fate map. Thus in the stereotypic model seedling used for mapping purposes, leaves 1, 3 and 4 point downward and leaf 2 points up (Fig. 1). Cotyledon 1 is designated as the cotyledon adjacent to the right margin of leaf 1 and is the closest cotyledon to leaf 4. Cotyledon 2 is designated as the cotyledon adjacent to the left margin of leaf 1 and is the closest cotyledon to leaf 3.

RESULTS

Distribution of chimera types

The development of 243*chm1-2* seedlings displaying clonal sectors in the cotyledons and/or the first two true leaves was followed over a 7-day period. Thus, the extent of an albino sector could be traced from its initiation in one or both of the cotyledons and then followed throughout later development. Albino sectors were observed that included a widely varying number of leaf margins, ranging from one margin in a single organ to all margins save one in one organ (Fig. 2; Table 1). Based on the total number of marginal regions affected in the cotyledons and the first four true leaves, chimeras exhibiting clones affecting more structures are likely to have been established by albino events occurring earlier in embryonic cell division. If this is the case, 80 chimeras with 1, 2 or 3 marginal regions affected by an albino clone likely arose later in development than the 163 chimeras with 4 or more marginal regions affected. However, in most cases clones were identified that either extended between both cotyledons and other organs or at least one cotyledon and other organs. In addition, similar distributions of sharing patterns within and between the cotyledons were seen regardless of the extent of a clone within

Table 1. Comparison of clone sizes in chimeric seedlings

Margins affected	Number of seedlings	Pattern of clone sharing in cotyledons			
		1 Margin on 1 cotyledon	2 Margins on 1 cotyledon	1 Margin on each cotyledon	3 or 4 affected margins
1, 2 or 3	87	80%	4%	14%	2%
4, 5 or 6	91	44%	13%	32%	11%
7, 8 or 9	54	13%	20%	34%	33%
10 or 11	11	0%	0%	27%	73%

Clone size is based on the total number of right and/or left margins of cotyledons and leaves sharing clones. For each class of clone sizes the type and frequency of clone sharing in the cotyledons is also shown. Twelve possible right and left margins exist for the two cotyledons and first four true leaves. Because albinos were excluded from this analysis, no '12 margins affected class' is represented in the table.

a chimera. Clones tended not to be shared by both margins of a single cotyledon, but instead tended to be shared between the cotyledons (Table 1) regardless of the overall albino proportion of a seedling. Thus, although clones arose at different times during embryogenesis, most must have arisen prior to the unique specification of the cotyledons and apical meristem. In this analysis 179 clones were identified in cotyledon number 1, 180 in cotyledon 2, 223 in leaf 1, 181 in leaf 2, 184 in leaf 3 and 197 in leaf 4; clones were fairly evenly distributed between the left and right side of each organ.

Developmental distance calculations

The frequency with which each marginal region shared albino sectors was determined by counting the number of seedlings with a specific affected sector (for instance the right margin of one cotyledon) and the number of times that sector was shared with other cotyledonary and/or leaf margins. Developmental distances between the marginal regions of any two organs were calculated based on the relative frequencies with which cells derived from a single clonal sector were recruited into the formation of other margins on the same or other organs. Because even smaller clones tended to affect multiple organs, clones were assumed to have arisen prior to the specification of distinct cotyledonary and meristem/true leaf fates. Thus, all chimeras were classed together for the construction of the map. A previously derived equation was used to calculate final map distances (Furner and Pumfrey, 1992).

$$D = 1 - [2 \times (\text{Number of seedlings with clones affecting both margins}) / \text{Total number of seedlings with clones in either margin}] \times 100.$$

With D = the percentage of clonal events in which clones are not shared between two specific organ margins. Thus, if clones are never shared between two particular margins D=100. If clones are always shared between two particular margins, D=0. Distances were calculated between all possible pair-wise combinations of leaf and cotyledon margins (Table 2). All chimeras regardless of the handedness of phyllotaxis were mapped onto a model seedling with clockwise (right-handed) phyllotaxis, in accordance with previous experiments suggesting that phyllotactic handedness is established by a stochastic process (Furner and Pumfrey, 1992; Irish and Sussex, 1992).

Intra and inter-organ distances

The pattern of clone sharing seen between the cotyledons suggests that developmental templating or compartmentalization occurs prior to the establishment of cotyledon organ identity, a result similar to that reported for cotton cotyledons and plant true leaves (Bossinger and Smyth, 1996; Christianson, 1986). Relatively high developmental distances are seen between the right and left margin of each cotyledon and for all but one of the intercotyledonary distances (Fig. 3A; Table 2). Chimeras with wholly white cotyledons make up the lowest frequency class of chimeras among those with clones that occupy 50% or less of seedling organ margins (Table 1). Constraints on division planes imposed by the orientation of existing cell walls could limit the possible patterns of future cell divisions in the early embryo. This type of physical limitation on cell division has been called developmental templating. Under this model a clone arising among the relatively few cells present in a young primordia would establish a static pattern that would expand through subsequent division and growth. Thus, the location and size of the clone in the mature leaf would reflect the proportion of clonal cells in the primordia. Alternatively, limited clonal expansion could indicate that developmental compartments are present in the early embryo (Christianson, 1986; Lawrence, 1992). Most definitions of a compartment share a common idea that cells tend not to cross compartmental boundaries due to regulatory constraints and that all cells derived from a compartment contribute to a single area of the adult body. Thus, the fact that clones tend not to cross the midline of the cotyledons, or cross between the right margin of cotyledon 1 (C1R) and the left margin of cotyledon 2 (C2R) may indicate the presence of compartments.

The cotyledonary fate map does show one obvious distance asymmetry, however. The developmental distance between the left margin of cotyledon 1 (C1L) and the right margin of cotyledon 2 (C2R) is 48.7 D, between 8.8 and 19.1 D shorter than any other developmental distance seen within or between the cotyledons (Fig. 3A; Table 2). The asymmetry seen in cotyledon fate specification as shown by the close mapping of the C1L and C2R margins is also reflected in later organogenesis. The right and left margins of leaf 1 (L1R and L1L) map relatively closely to C1L and C2R respectively (Fig. 3B; Table 2). The fate distance between leaf 1, left (L1L) and cotyledon 2, right (C2R) is the closest seen between any two regions in the first four organs produced by the plant (39.2 D). In comparison to the close association between C1L and C2R, leaf 1 maps between 6.9 and 9 D closer to these adjacent cotyledonary regions than the cotyledons map to each other. Leaf 2 maps farther away from adjacent cotyledonary regions (C1R and C2L) than leaf 1, although it also maps closer to these regions than they do to each other (Table 2). Leaf 1 and leaf 2 map relatively far away from each other, with no intermarginal distances less than 55 D.

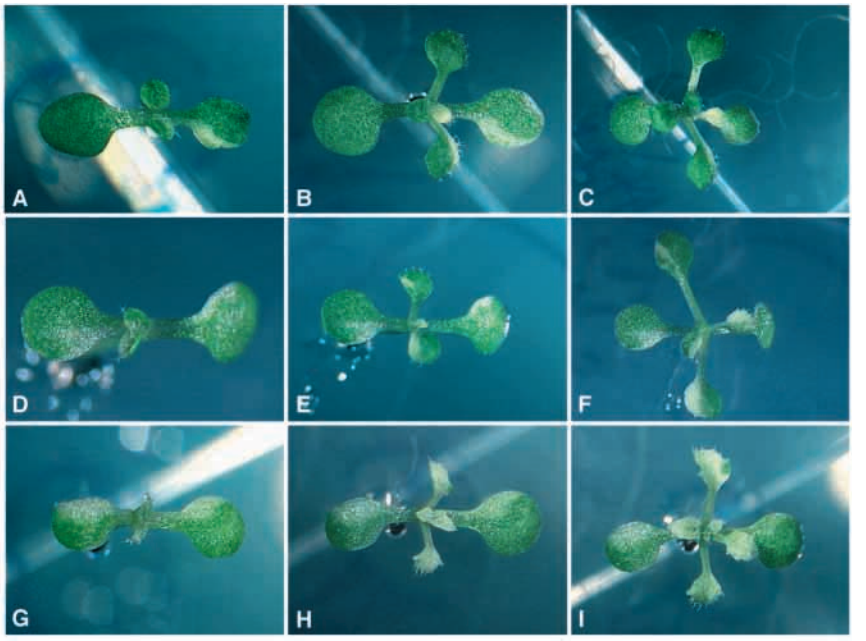

Fig. 2. Patterns of albino clone sharing between cotyledons and true leaves in *chm1-2* plants. Populations of *chm1-2* plants were grown in sterile culture (24) and videocaptures were taken 3 (A,D,G), 6 (B,E,H) and 9 (C,F,I) days after seeds were transferred from cold stratification. All seedlings are oriented as the model seedling in Fig. 1. (A-C) Clones affecting one cotyledon margin generally extended into the adjacent margin of leaf 1 and included either leaf 3 or 4. In this seedling albino progenitor cells provided daughters that contributed to the right margin of cotyledon 2, left margin of leaf 1 and all of leaf 3. (D-F) The left margin of cotyledon 1 shares a clone with the non-adjacent left margin of cotyledon 2. This pattern of sharing between the cotyledons was rare, most intercotyledonary clone sharing occurred between cotyledon 1L and cotyledon 2R, however it highlights the regulative nature of cotyledon fate specification in relation to the pattern of cell divisions in the early embryo. (G-I) Clone shared between the right margin of cotyledon 1 and the left margin of cotyledon 2 is shared with all later true leaves, indicating that precursor cells in both of the cotyledons can be developmentally isolated from the entire post-germination shoot.

Leaves 3 and 4 map most closely to the adjacent margins of leaf 1 (L1L for leaf 3 and L1R for leaf 4) and leaf 2 (Both L4L and L4R map closely to L2L) although they also map closely to their associated cotyledons (Table 2). However, leaf 3 and 4 also show displacement in relation to the close adjacent mapping seen between the cotyledons and between the cotyledons and leaves 1 and 2. Leaf 3L and R both map most closely to C2R, although L3R maps the most closely. Thus, leaf 3 appears to arise from cells partitioned to a region of the embryo that also contributes to the right side of C2. Leaf 4 however displays close mapping in register with its closest cotyledon, C1. The L4L margin maps most closely to C1L while L4R maps most closely to C1R (Table 2). Thus, the tendency of adjacent regions to map closely in the cotyledons and first two true leaves appears to be lost for leaves 3 and 4. It is likely that regulatory interactions occurring between the cotyledons and first true leaves do not directly affect the placement of leaves 3 and 4, while interactions with leaves 1 and 2 are more important.

DISCUSSION

Compartments or templates in cotyledon development

The pattern of clonal exclusion seen within and between the cotyledons could indicate that embryonic compartmental boundaries correspond to a plane running down the midline of both cotyledons in the mature seed. This plane has been described as the frontal longitudinal plane by other authors (Long and Barton, 1998). However, this apparent fate limitation is clearly not absolute at the resolution of the current map. Clones are found which are shared between distantly mapping cotyledonary regions (Fig. 2D-F) indicating that clones can be shared between all regions of the cotyledons, and entirely white cotyledons were seen in some chimeras regardless of number of organ margins affected in total (Table 1). This may suggest instead that early cell division patterns in the embryo and spatial constraints imposed by the cell wall matrix on cell lineages tend to bias the location and direction of later patterns of cell division and expansion during the

Table 2. Developmental distances between marginal regions arising in both the same and different organs

	C1L	C1R	C2L	C2R	L1L	L1R	L2L	L2R	L3L	L3R	L4L
C1R	57.5										
C2L	65.4	62.5									
C2R	48.7	65.6	67.8								
L1L	50.7	59.8	65.2	39.2							
L1R	41.3	52.8	62.8	58.0	45.3						
L2L	65.1	45.9	63.5	65.3	63.9	57.9					
L2R	65.3	55.6	54.4	65.4	59.6	55.9	55.8				
L3L	57.7	58.8	54.1	51.6	41.2	56.0	57.8	53.6			
L3R	57.5	55.2	59.8	43.3	35.4	47.4	56.5	58.9	32.6		
L4L	41.6	50.8	65.0	56.7	49.8	33.3	41.6	62.7	50.3	51.3	
L4R	55.4	47.1	57.5	59.8	52.5	47.4	41.3	54.6	48.9	47.9	34.0

Developmental distances represent the percentage of times clones arising within any two marginal regions fail to be shared between regions. All distances are calculated using the equation in the text. Purple numbers represent developmental distances across an organ, red numbers indicate mapping distances of less than 50 D. To read distances, find the column and row intersection between regions of interest, for instance the distance across leaf 1, the L1L to L1R distance, is 45.3 D.

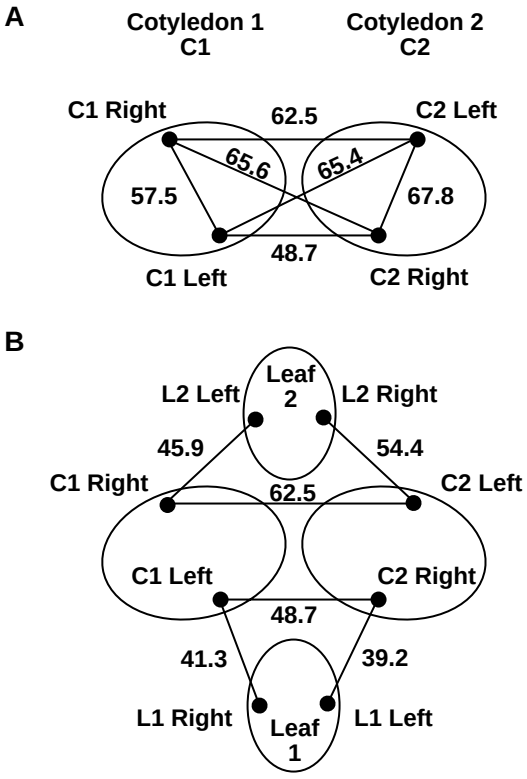

Fig. 3. Schematized seedlings showing calculated intermarginal developmental distances between and within (A) the cotyledons alone and (B) the cotyledons and the first true leaves. All distances are determined as described in the text, and are written adjacent to lines indicating two adjacent marginal regions. Lines are not drawn to scale. Asymmetric close mapping seen between C1 Left (C1L) and C2 Right (C2R) (A) predicts the location of the first true leaf (B). The distance between C2R and L1L (B) is the closest developmental distance seen between any two regions in the first four organs.

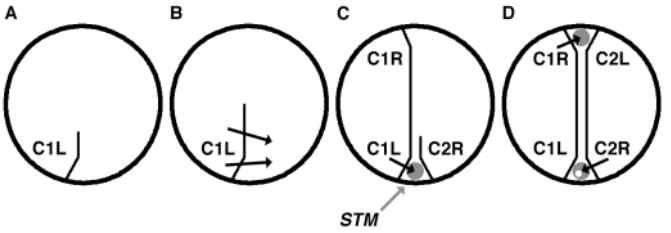

Fig. 4. A model of the organization of the embryonic apex by cotyledon fate acquisition and subsequent induction of *shootmeristemless.* (A) The left region of cotyledon 1 is likely to be the first organ fate induced in the embryo, C1 is the smaller cotyledon anlage signifying that it likely arose in an embryo composed of fewer cells. (B) C1L fate arises as a primordial initial that exerts a negative influence on adjacent tissue causing a lateral displacement of cotyledon 2 fate to the opposite site of the embryo. (C) C1 develops from an initial to a primordium and acquires dorsiventrality. With the onset of adaxial fate specification an adjacent local region of *shootmeristemless* expression develops and the first epicotyl meristem fate is acquired. (D) As dorsiventrality spreads to C1R it induces the second localized region of *STM* expression. C2R begins development as a leaf initial, causing a suppression of the leaf morphogenetic potential of adjacent *STM*-expressing tissue. The older C1L region may now play a role in the negative regulation of *STM* expression, promoting the emergence of the first true leaf. Displacement of the position of the Leaf 1 initial (indicated by the lacunae in *STM* expression) by C2R causes the onset of phyllotaxy. Because the left or right handedness of the phyllotactic spiral is stochastic from plant to plant, acquisition of C1 fate must also be stochastic under this model. Either cotyledonary region must have the potential to become C1.

development of cotyledon and leaf blades. Position-dependent biasing of later development by patterns of cell division in immature tissue has been referred to as developmental templating (Dawe and Freeling, 1992). Competing hypotheses based on templating or compartmentalization could both explain the high developmental distances seen between most of the cotyledonary regions, but can not be differentiated using fate mapping approaches alone. In *Drosophila*, fate compartments were revealed in imaginal discs by utilizing a genetic background that gave clonal regions a significant growth advantage over other cells in the embryo (Garcia-Bellido et al., 1976). When clones of these rapidly growing cells arose adjacent to compartment boundaries, they failed to overgrow the boundary region, indicating an informational restraint on their proliferation. It is possible that wild-type clones of certain *Arabidopsis* auxotrophic genes might be useful in shedding further light on this issue.

Asymmetric cotyledon mapping predicts the location of true leaf 1

The cotyledon fate map is asymmetric in terms of the relative fate distances seen within and between the cotyledons. The C1L:C2R region of the cotyledons is the most closely mapping region between the cotyledons, suggesting that specification of the C1L:C2R region occurs with different timing in relation to the specification of the C1R:C2L region. The fate distances seen between the cotyledons are also intriguing in light of the eventual establishment of phyllotactic handedness shown by later leaves and the likely timing of primordiagenesis in the embryo and young seedling. Tracing the clockwise phyllotactic spiral backward from leaf 4, the leaf order 3, 2, 1, cotyledon 2, cotyledon 1 is suggested going from youngest to oldest leaf. By this criterion, the close mapping of the C1R:C2L region appears to predict the location of the first true leaf, and by implication the establishment of the first region of shoot meristematic activity. The identity of leaf 1 as the oldest true leaf is also suggested based on it's close mapping with the cotyledons, and is consistent with the fact that leaves 3 and 4 map most closely to it and arise between leaf 1 and the cotyledons.

The physical location of older leaves has been demonstrated to play a role in determining the placement of younger leaf primordia in a number of seed plants and ferns (reviewed by Steeves and Sussex, 1989). Surgical ablation or physical isolation of adjacent older leaf primordia in meristems showing spiral phyllotaxis often causes the younger primordium to emerge in close proximity to the adjacent older leaf position. These experiments helped establish a model suggesting that leaves produce a proximal inhibitor that prevents the specification of leaf primordia in nearby meristematic tissue. Thus it is possible that leaf 2 displaces the position of leaf 3 toward leaf 1 by exerting an inhibitory effect on proximal meristematic regions. This displacement again suggests that leaf 1 is likely to be the oldest true leaf in the plant, as leaves

lose their ability to suppress adjacent primordiagenesis as they age. Currently several models exist concerning the physical or molecular nature of the adjacent leaf signaling system (Green, 1994), experimental confirmation of one or the other model has yet to be reported. However, evidence of chemical signaling between organ primordia has been documented in several instances (Van Der Schoot et al., 1995).

It is possible that the asymmetric close mapping of the cotyledons and leaf 1 could be due to the non-random initiation of spontaneous clones in the *chm1-2* mutant line. Non-random induction could skew the map by reducing developmental distances in regions in which they arose, while expanding distances in regions with clone scarcity. However, the range of clone numbers in organs seen in this study (see Results) is low in relation to the ranges seen in induced clone maps (Furner and Pumfrey, 1992), suggesting that variation in clone number between organs is an inherent element of fate mapping, regardless of the mode by which clones are induced. Non-random clonal distribution could also arise if subsequent clonal events tended to occur adjacent to existing clones. However, the low rate at which clones arose in the population (6.7%) and the fact that clones used in the study were entire, lacking interdigitation of green tissue, suggests that clones arose from single cells. Any local increase in clone number within a single organ must also be accompanied by an increase in clone sharing between organs to reduce fate distance, otherwise the distance equation would return a larger distance due to an increase in the denominator without an increase in the numerator. Thus not only must clone distribution be inherently non-random, but so also must clone sharing be non-random in specific regions, a circumstance that seems unlikely given the relatively small variation in clone number seen in this study.

Based on the total organ area occupied by clones, it is likely they arose within the first four to five divisions of the embryonic cells that eventually contribute to the green tissue of leaves. The fact that clones could be generated at various times could also contribute to asymmetry, if the subsequent rate of expansion of albino and green tissue varies based on location in the embryo. The observation that asymmetric clone sharing is seen in the cotyledons, regardless of total area of organs affected (Table 1) suggests that the size of the clone does not affect its likelihood of capturing specific inter-organ sharing events. Thus, data in Table 1 indicate that clones arose within a small number of cell divisions prior to the unique specification of cotyledon and true leaf fates, tend to be large and range over multiple organs, and sampled a similar pattern of sharing events regardless of size and time of initiation. Currently new approaches are being pursued in our laboratory to induce clones at specific stages from the first cell division through the late heart stage embryo. The comparison of two maps constructed using two independent clone generation systems should help to determine if non-random patterns of clone distribution are impacting one map or the other. If non-random clone expansion is seen it is possible that a process similar to developmental templating is limiting the direction of clone expansion, or that physiological aspects predispose some cell populations toward clone generation in *chm1-2* embryos.

Opposite or alternate phyllotaxis of the cotyledons and first true leaves

The placement of organs within the cotyledon and first true leaf whorls of the *Arabidopsis* seedling has been described by a number of authors as manifesting opposite phyllotaxis (Callos and Medford, 1994; Medford et al., 1992). Under this phyllotactic model the cotyledon anlagen are specified at the same time, across the main axis of the embryo from each other, while later in development leaves one and two also are specified at the same time across the main axis of the embryo from each other. One important implication of this model is that *Arabidopsis* seedlings undergo a regulated developmental transition from opposite to helical phyllotaxis with the development of the third true leaf. The within-organ fate distances seen in this study suggest however that cotyledon 1 and 2 and leaf 1 and 2 do not arise with true opposite (decussate) phyllotaxis at the same time in development but instead show alternate (distichous) phyllotaxis. In the case of both cotyledon 1 and leaf 1 the right margin to left margin distances are shorter than those seen in cotyledon 2 and leaf 2 respectively (Table 2). The general trend from cotyledons through leaves 1 and 2 to leaves 3 and 4 is that older organ anlagen have smaller within-organ developmental distances, suggesting that older organs are more likely to arise entirely within a white or green clonal region. This general trend has been reported in post-germination fate maps as well (Furner and Pumfrey, 1992). One possible explanation of the observed pattern could be that C1 fate arises earlier in embryogenesis than C2 and that fewer progenitor cells contribute to C1. If C2 arises later in embryogenesis, from an anlage composed of more cells, C2 might be more likely to capture green/albino clone boundaries than C1.

The different within-organ distances seen between leaf 1 and 2 might also result from slightly different times of initiation and/or numbers of cells contributing to each anlage. Leaf 1 maps most closely to C1 and C2, and arises within a region shown to map closely between the cotyledons. Thus, white clones shared between cotyledons in this region would be expected to contribute most of the cells to the leaf 1 anlage. This compartmentalization is also interesting in the light of the placement of leaves three and four in close proximity to leaf 1. The C1L, leaf 1, C2R region has a high frequency of clone sharing occurring during early embryogenesis. Thus, it is not surprising that L3 and L4 anlagen arising later in this region tend to be generated within cell fields that are already established as either white or green clones, accounting for their small within-organ developmental distances. The green/albino clone boundaries established earlier in embryogenesis are likely to be physically distant and developmentally inaccessible to organs arising on the leaf 2 side of the embryonic frontal plane. Thus, for leaves 3 and 4 it is likely that their small relative within-organ sizes are a reflection of the fact that they arise relatively late in development, after mitotic division cycles have increased the size of early albino clones in the C1L:C2R region. The continual expansion of the embryo through mitotic cell division and the establishment of early asymmetry in the embryo suggests that organs with small intra-organ developmental distances are older organs when comparing the cotyledons or the first true leaves. The pattern seen also suggests that phyllotaxis in the early embryo is best described as alternate, with the sequential development of the cotyledons and first two true leaves at slightly different times. This has already been proposed for the first and second true

leaves, one of which tends to emerge from the meristem slightly earlier than the other (Medford et al., 1992).

In total this fate map supports the idea that phyllotaxis is established by a generative program of overlapping regions of inhibition initiating within developing leaf primorida, rather than by a descriptive developmental pattern of primordia placement originating in the meristem. It also suggests that the alternate pattern of phyllotaxis seen between the cotyledons and between leaves 1 and 2 influences the phyllotactic placement of leaf 3, due presumably to the negative influence of leaf 2 on the possible close placement of leaf 3. It is interesting to speculate that the transition from alternate to helical phyllotaxis by the seedling is a result of the continuing placement of single leaf nodes coordinated with increases in size and alterations in shape of the meristem. In maize, conversion of phyllotaxis from a normal, alternate pattern to an opposite pattern occurs in plants homozygous for the recessive mutation *abphyl1* (Jackson and Hake, 1999). The embryonic meristems of *abphyl1* plants are significantly larger than wild type, suggesting that a change in meristem size causes a later alteration of phyllotaxis. A number of *Arabidopsis* mutants also have a similar coupling of alteration of meristem size and phyllotaxis (Clark et al., 1993; Leyser and Furner, 1992) which may support the idea that the progression to helical phyllotaxis depends on a system of alternate leaf induction acting continuously as meristem size and morphology changes.

A model of apical fate specification

The close developmental association of the first true leaf with marginal regions of the cotyledons seen in this experiment is predicted by a recent analysis assaying the relative timing of meristem gene expression in the early embryo (Long and Barton, 1998). The *shootmeristemless* (*STM*) gene of *Arabidopsis* is required for the production of a shoot apical meristem, and cells destined to form true leaves first accumulate and then lose *STM* mRNA (Long et al., 1996). Plants homozygous for loss-of-function mutations in the *STM* gene produce seedlings with cotyledons that possess fused marginal regions on their petioles and produce no true leaves. Thus, *STM* does not appear to be required for the specification and development of the cotyledons, but all subsequent leaf primordiagenesis depends on the transient expression of *STM*. Analysis of the pattern of STM mRNA accumulation in the early, globular stage embryo shows that gene expression first occurs in cells that will contribute to one of the two adjacent marginal regions of the cotyledons (i.e. either the C1L:C2R or C1R:C2L region) (Long and Barton, 1998). Thus, *STM* expression is asymmetric in the early embryo, and only later in development does expression arise near the opposite presumptive intercotyledonary marginal region. This asymmetric expression suggests a difference in the timing of the specification of meristematic identity in the C1L:C2R and C1R:C2L region and by inference the likely difference in timing of specification of the first two true leaves.

The expression of the *shootmeristemless* gene also occurs during post-embryonic meristem formation, when branch shoot meristems are induced at the base of leaves. Branch meristems are initiated on the upper (adaxial) surface of leaves, suggesting that adaxial leaf fate is capable of inducing *STM* expression and subsequent meristem organization. Recent analysis of *STM* expression in the *phabulosa* mutation also suggests that adaxial leaf surfaces can induce *STM* expression in a manner independent of the shoot apical meristem (McConnell and Barton, 1998). This mutation adaxializes the lower surface of leaves and cotyledons, causing the production of leaves that are entirely encased in adaxial epidermal cells. These leaves express *STM* in a band surrounding the entire leaf base, and can produce branches that project from the bottom surface of leaves. Thus, *STM* is required in the embryo for meristem formation and subsequent formation of true leaves 1 and 2, but also is induced by leaf cells with adaxial leaf fates. Stimulation of *STM* expression by existing leaves, and the asymmetric expression of *STM* in the embryo raises an interesting possibility in light of the asymmetric close mapping of the C1L and C2R cotyledonary regions. The close mapping of this region indicates that clones arising early in embryogenesis are likely to be shared between these regions, while the opposite sides of the cotyledons, C1R and C2L, tend not to share early clones. This suggests that the fate specification event that establishes the left side of cotyledon 1 and, shortly after, the right side of cotyledon 2 occurs earlier in development than the fate specification event giving rise to the respective opposite marginal regions of each cotyledon. Thus, it is likely that the C1L: C2R region represents the earliest establishment of distinct ‘leaf’ identity in the embryo. If this is the case, the asymmetric accumulation of *STM* may be driven by the prior regional acquisition of adaxial leaf identity in the C1L: C2R marginal region. As C1R and C2L gain distinct leaf identity, they too might be expected to induce adjacent, axial expression of *STM*, an expectation born out by the later spread of *STM* expression throughout the central region of the presumptive meristem. The fact that cotyledon petioles are fused in the absence of STM expression further suggests that leaf identity, expressed first in C1L, represents a developmental ground state required for apical meristem induction. The model presented in Fig. 4 is suggested by the fate map generated in this study and by the currently understood expression pattern of *STM*. If this model is correct, spiral phyllotaxis begins with the inhibition of close primordiagenesis by C2R, displacing L1 toward the oldest leaf marginal region in the embryo, C1L. This model also suggests that mutations in master regulatory genes involved in the initial fate specification of the apical region of the plant embryo will cause loss of cotyledon fate specification.

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