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Venation Pattern Formation in *Arabidopsis thaliana* Vegetative Leaves

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Branching net-like structures are a trait common to most multicellular organisms. However, our knowledge is still poor when it comes to the genetic operations at work in pattern formation of complex network structures such as the vasculature of plants and animals. In order to initiate a causal analysis of venation pattern formation in dicotyledonous plant leaves, we have first studied its developmental profile in vegetative leaves of a wild-type strain of the model organism *Arabidopsis thaliana*. As landmarks of the complexity of the venation pattern, we have defined three main developmental parameters, which have been quantitatively followed in time: the ratios of (a) the length and (b) the number of branchpoints of the vein network with the surface of the lamina, which decrease in parallel as the leaf grows, only small differences existing between successive leaves, and (c) the number of hydathodes per leaf, which increases both during leaf expansion and from juvenile to adult rosette leaves. We next searched for natural variations in the first vegetative leaves of 266 ecotypes, finding only 2 which showed a venation pattern unequivocally different from that of the rest, Ba-1 and Ei-5, the latter displaying an extremely simple pattern that we have called Hemivenata. This phenotype, which is inherited as a monogenic recessive trait, is visible both in leaves and in cotyledons and seems to arise from a perturbation in an early acting patterning mechanism. Finally, we have screened for mutants with abnormal venation pattern but normally shaped leaves, concluding that such a phenotype is rare, since only one recessive mutation was obtained, *extrahydathodes*, characterized by the presence of an increased number of hydathodes per leaf.

Key Words: ***Arabidopsis;*** **venation pattern; pattern formation; leaf morphogenesis; plant vegetative development.**

INTRODUCTION

Pattern formation is usually defined as the generation of regular differences in space as a consequence of mechanisms by which genetic information is translated into specific spatial patterns of cellular differentiation (Wolpert, 1969, 1971, 1989; Meinhardt, 1984). In recent decades, the vast majority of studies on pattern formation have been focused on developmental processes in animals, our current knowledge being derived mostly from genetic and molecular analyses performed in the embryos of model organisms such as *Drosophila melanogaster* (reviewed in Davidson, 1994). However, despite the extensive information available on how the basic body plan is laid down during animal embryogenesis, little is known about the causal agents of pattern formation in plants.

Complex branching networks of linear structures, organized in a species-specific pattern, are very common to the multicellular anatomy of plants and animals. Examples are the animal nervous system and the vasculature of higher animals, insect wings, and plant leaves (Meinhardt, 1976). However, although there are detailed descriptions and consistent theoretical models to account for the ontogeny of plant vascular patterns (Meinhardt, 1976, 1984; Mitchison, 1980, 1981), the mechanism by which such structures are built remains to be dissected at the genetic level.

Proposals concerning the principles that rule plant body patterning have traditionally been founded on morphological descriptions and surgical experiments performed on a wide variety of species (reviewed in Steeves and Sussex, 1989; Lyndon, 1990; Sachs, 1991b). Such studies have provided an essential basis for recent studies, characterized by the concentration of effort on a restricted number of model systems and by the identification by mutation of genes acting as developmental controls (Koornneef, 1991). Some remarkable examples of the usefulness of the genetic approach to the dissection of plant developmental phenomena in *Arabidopsis thaliana* are the studies on flower development (Weigel and Meyerowitz, 1994), trichome morphogenesis (Hülskamp *et al.*, 1994), root development

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(Benfey and Schiefelbein, 1994), and embryo patterning (Jürgens *et al.*, 1991).

Plant leaves are determinate structures responsible for primary productivity which arise as swellings on the flanks of the shoot apex in accordance with a specific phyllotactic pattern (Fosket, 1994). All the main functions of the leaf (light harvesting, gas exchange, water transport, and distribution of photosynthate) depend upon its architecture, which is defined as the position and form of all the elements which constitute the outward expression of the structure of the organ (Hickey, 1988). One such architectural element is the arrangement of the veins in the lamina, which is referred to as venation pattern (for a recent review, see Nelson and Dengler, 1997). Although there are numerous studies on the leaf vasculature of higher plants, very little is known about venation pattern formation. In fact, there is a rich diversity of venation patterns in both monocotyledonous (Inamdar *et al.*, 1983) and dicotyledonous (Hickey, 1973) plants, although most of the available information has been obtained in systems showing a simple pattern consisting of multiple longitudinal strands interconnected by transverse veins, as maize leaves (Inamdar *et al.*, 1983; Russell and Evert, 1985; Bosabalidis *et al.*, 1994), or which are poorly amenable to genetic and molecular analyses, as barley (Dannenhoffer and Evert, 1994), some other monocotyledonous species (Inamdar *et al.*, 1983), and the crucifer *Moricandia arvensis* (Beebe and Evert, 1990).

Leaf venation follows a complex branching net-like pattern in the dicotyledonous *A. thaliana*. Previous studies on the structural features, pattern, or development of the *Arabidopsis* wild-type leaf venation are limited to qualitative descriptions of the spatial sequence by which the lignification of tracheary elements proceeds in the leaves (Dharmawardhana *et al.*, 1992) and the increase in complexity of the reticulate venation pattern in the expanding first rosette leaf (Telfer and Poethig, 1994). A few pleiotropic mutations which cause phenotypes including vascular abnormalities have been reported in *Arabidopsis: pin-formed1* (*pin1*) homozygous mutants show split midveins (Okada *et al.*, 1991), *lopped1* (*lop1*) mutations cause disoriented growth and bifurcation of the midvein (Carland and McHale, 1996), and *monopteros* (*mp*) mutants display missing and/or interrupted veins both in cotyledons and in leaves (Berleth and Jürgens, 1993; Przemeck *et al.*, 1996). In addition, a few mutants lacking the midvein or with altered interveinal distances have been reported in monocotyledonous species such as *Panicum maximum* (Fladung *et al.*, 1991; Fladung, 1994) and *Pennissetum americanum* (Rao *et al.*, 1988, 1989). And yet, despite this information, our knowledge of the process remains quite rudimentary.

Arabidopsis leaves exhibit heteroblasty, with small but clear morphological differences existing between early and late leaves in a given plant (Röbbelen, 1957; Telfer and Poethig, 1994). Variations in leaf architecture are also found among *A. thaliana* ecotypes, corresponding in most cases to polygenic traits (Serrano-Cartagena, Pérez-Pérez, and Micol, in preparation). In the present study, we first analyze in detail the venation pattern of *A. thaliana* leaves, its variation with time and among successive leaves, and its differences among ecotypes. Second, we attempt to estimate the frequency of mutations which specifically affect venation pattern formation in *Arabidopsis*. Finally, we present several venation pattern variants, whose genetic and molecular analyses will help to understand the process of plant leaf vein patterning.

MATERIALS AND METHODS

Plant Materials

A. thaliana (L.) Heyhn. Landsberg *erecta* (L*er*) wild-type and ethyl methane sulfonate (EMS)-mutagenized M_2 seeds (EMS at 0.2% v/v for 12 h at 23°C; Cat. No. M2E-4-2) were purchased from Lehle Seeds. Seeds of T-DNA tagged lines (Feldmann and Marks, 1987) and ecotypes were supplied by the Nottingham *Arabidopsis* Stock Centre (NASC). The list of studied ecotypes includes the following: NW20, N902, N904, N906-N908, N910, N911, N914, N917, N923, N924, N929, N936, N938, N946, N948, N952, N954, N956, N958, N962, N964, N976, N978, N986, N994, N996, N998, N1000, N1006, N1012, N1020, N1028, N1030, N1032, N1034, N1036, N1038, N1044, N1046, N1050, N1052, N1054, N1064, N1066, N1068, N1070, N1074, N1076, N1080, N1082, N1086, N1088, N1090, N1092, N1094, N1100, N1104, N1110, N1114, N1118, N1124, N1126, N1128, N1130, N1140, N1142, N1144, N1148, N1150, N1152, N1154, N1158, N1160, N1168, N1170, N1172, N1176, N1178, N1180, N1186, N1196, N1198, N1204, N1206, N1208, N1210, N1212, N1214, N1216, N1220, N1226, N1230, N1232, N1236, N1238, N1240, N1242, N1244, N1248, N1250, N1252, N1256, N1258, N1260, N1262, N1268, N1270, N1272, N1274, N1278, N1280, N1284, N1286, N1288, N1298, N1300, N1302, N1304, N1306, N1308, N1310, N1312, N1314, N1316, N1318, N1320, N1322, N1324, N1326, N1328, N1334, N1338, N1342, N1348, N1350, N1352, N1362, N1364, N1366, N1368, N1370, N1372, N1374, N1376, N1378, N1380, N1384, N1388, N1390, N1394, N1396, N1398, N1400, N1402, N1404, N1408, N1410, N1412, N1414, N1416, N1418, N1420, N1422, N1424, N1426, N1428, N1430, N1432, N1434, N1436, N1438, N1440, N1442, N1444, N1448, N1450, N1452, N1454, N1456, N1458, N1460, N1462, N1464, N1466, N1468, N1470, N1472, N1474, N1476, N1478, N1480, N1482, N1484, N1488, N1490, N1492, N1494, N1496, N1500, N1502, N1504, N1506, N1512, N1514, N1516, N1518, N1520, N1522, N1524, N1530, N1534, N1536, N1538, N1540, N1548, N1550, N1552, N1554, N1556, N1558, N1560, N1562, N1564, N1566, N1568, N1570, N1572, N1574, N1576, N1578, N1580, N1582, N1584, N1586, N1588, N1590, N1594, N1596, N1598, N1601, N1602, N1604, N1606, N1608, N1610, N1612, N1614, N1616, N1618, N1620, N1622, N1626, N1628, N1630, N1636, N1637, N1638, N1639, N1641, N1642, N1643, N1644, N2223, N3110, and Ws-2.

Growth Conditions and Screening

Seeds were sown on 150-mm petri dishes (100 regularly spaced seeds per plate) in Conviron TC16 culture chambers at 20 ± 1°C and 60–70% relative humidity under constant fluorescent light (7000 lux), as described by Ponce *et al.* (1998). When required, kanamycin was added at a final concentration of 50 μg/ml. Ecotypes, T-DNA tagged lines, and EMS-mutagenized M_2 seeds

were used in screenings for abnormal patterns of leaf venation. Leaves of the first rosette node were excised for tissue clearing and observation 20 days after sowing.

Microscopy and Morphological Characterization

Excised leaves were immediately submerged and kept overnight in a clearing solution (80 g chloral hydrate in 30 ml water) until tissue became transparent. Whole leaves were mounted on slides in a solution of 80 g chloral hydrate, 20 ml glycerol, and 10 ml water. Transmitted-light dark-field and interference contrast pictures were drawn with the help of a Leica DMRB microscope equipped with a drawing tube. Image analysis was performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Scanned pictures were skeletonized before determining the number of branching points in the vasculature and leaf area. The latter was calculated by counting the number of black pixels after coloring in black the region corresponding to the leaf lamina. A macro was written to determine the length of the leaf venation pattern from the skeletonized image based on a nearest-neighbor algorithm, as described by Travis *et al.* (1993). This macro is available upon request from the authors (hcandela@umh.es).

RESULTS

The vegetative phase of development in *A. thaliana* is characterized by a rosette of vegetative leaves with short internodes between successive leaf primordia. Heteroblastic differences in leaf morphology, leaf trichome density, and phyllotaxy have been shown between early (juvenile) and late (adult) vegetative leaves as well as between vegetative (rosette) and cauline (inflorescence) leaves (Röbbelen, 1957; Martínez-Zapater *et al.*, 1994; Telfer and Poethig, 1994). In order to ascertain any variations in venation pattern between juvenile and adult vegetative leaves, we chose leaves corresponding to the first, third, and eighth rosette nodes in the L*er* ecotype for quantitative studies. After the observation of leaves from the first to the ninth nodes of several plants, those three were considered to be representative of the whole spectrum of developmental stages in the vegetative phase of the *Arabidopsis* life cycle.

Venation Pattern in Landsberg erecta Rosette Leaves

Vein orders within a leaf are usually defined on the basis of vein thickness (Hickey, 1988). According to this criterion, the reticulate venation pattern of *Arabidopsis* mature vegetative leaves is pinnate, with a single primary vein (the midvein), which is the thickest vein and which serves as the origin of narrower secondary veins. Secondary veins branch off each side of the midvein toward the margin and acropetally toward the tip. At the branching points, secondaries are markedly finer than the continuation of their source (Figs. 1A and 1B). However, as Figs. 1A, 1B, and 1C show, the width of the midvein diminishes acropetally as new secondaries extend out from it until, in the apical region of the lamina, branches are originated which are indistinguishable in width from their source, making them difficult to classify as secondary veins on the criterion of thickness alone. Following the classification nomenclature of Hickey (1988), the venation of *A. thaliana* leaves is brochidodromous since secondary veins are joined together in a series of prominent arches. Secondary and higher order veins form an intricate pattern of loops which are irregular in shape, size, and orientation, some of them being incompletely closed (see Figs. 2D and 2E).

Drawings in Fig. 2 show the venation of a series of leaves from the third rosette node in different stages of expansion. Leaves from the first and eighth nodes were also studied, showing similar variations with time in the complexity of the pattern (data not shown). Third-node leaf primordia with a length of around 200 μm contain small mesophyll cells and show immature provascular elements which give rise to the developing midvein. This connects at its base with the vascular system of the plant. The presence of tracheary elements becomes evident in the midvein when the organ reaches ca. 500 μm in length (Fig. 2A). Leaves at this stage also show two secondary provascular strands which branch off the midvein and bend up toward the leaf tip, where they connect to form two loops that do not always appear to be completely lignified. These two secondary strands connect with secondary and tertiary provascular elements, giving rise to some few immature loops. By the time the leaves have attained a size of 700 to 1000 μm (Fig. 2B), both the midvein and the two apical loops contain lignified tracheary elements that are clearly distinguishable. A few secondary strands begin to differentiate vascular elements, while the rest of the secondary and tertiary provascular strands form an intricate network of immature loops. Third-node leaves slightly longer than 1 mm show an increasing number of lignified veins (Fig. 2C), mainly in the area near the tip where there are extensive intercellular spaces between enlarged mesophyll cells. Immature provascular strands are abundant in the rest of the leaf, particularly at its basal region, proximal to the petiole, where there are small and tightly packed cells. Leaves that have reached a length of 2 mm contain veins that are sufficiently lignified to be clearly visible (Fig. 2D). They show most features of the vein pattern, although they still lack some vascular elements which will be observed in fully expanded leaves. At this and immediately later (Fig. 2E) stages, the formation of new provascular strands is mainly confined to the base of the lamina. Finally, the leaf lamina attains a length of 6 to 8 mm, with no further formation of new vascular elements (data not shown).

Complexity of the Venation Pattern

Figure 3A shows the increases in lamina area during the expansion of the first, third, and eighth rosette leaves. This area increases exponentially during the initial stages of leaf expansion. For instance, from the 11th to the 16th day after

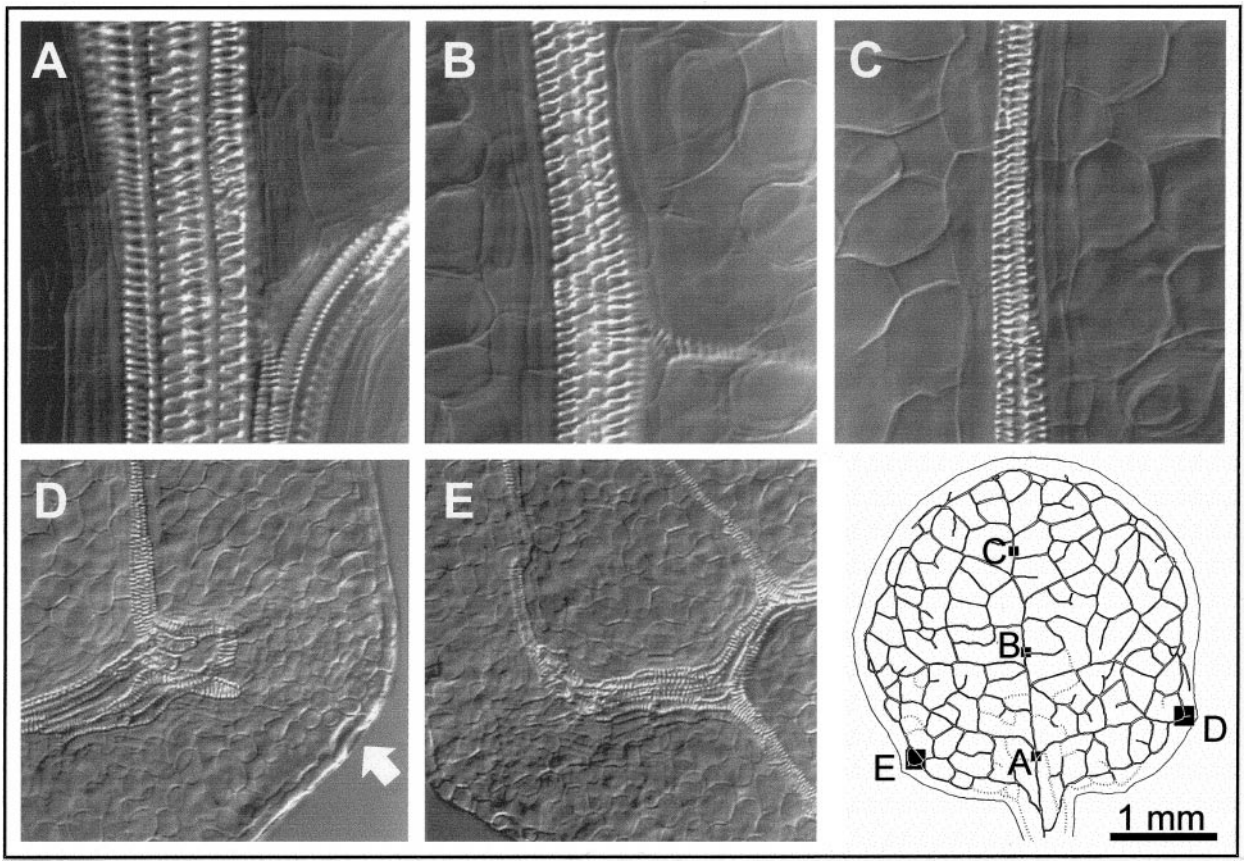

FIG. 1. Interference contrast micrographs of three midvein segments (A, B, and C) and two lateral hydathodes (D and E) in a third rosette leaf of the L*er* ecotype of *A. thaliana*, 18 days after sowing. Black squares and letters in the drawing indicate correspondences between pictures and positions in the leaf. The images show the progressive decrease, from the petiole to the leaf apex, in the number of tracheary elements integrating the midvein as well as in its width. The proximal part of the midvein (A) includes more tracheary elements than the medial (B), which in turn includes more than the distal (C). A and B micrographs also show thickness differences in the branching point between the primary and a secondary vein. Hydathodes (D, E) can be seen as a group of tracheary elements (at the center of both images), next to the epithem, a group of cells which are smaller in size than those typical of the mesophyll (see cells located in the upper part of D). The epithem intercellular space is continuous with the external atmosphere through stomata (white arrow in D). Note that the magnification for A, B, and C micrographs is twofold that of D and E, as indicated by the size of the black squares in the drawing.

sowing, the area of the first leaf increases daily by an average factor of 1.62. The lamina area for the mature eighth leaf (ca. 38 mm^2) was higher than those of the first (ca. 24 mm^2) and the third (ca. 27 mm^2).

One criterion to estimate the complexity of the venation pattern could be total venation length, defined as the sum of the length of all the veins in a leaf. This parameter increases along with lamina area until it reaches a value close to 83, 135, and 187 mm for the first, third, and eighth rosette leaves, respectively. Nevertheless, a better estimate of the complexity would consider the length of the vascular bundles related to the lamina area. We collected data on venation density (defined as the ratio between total venation length and lamina area) from young to fully expanded leaves. As Fig. 3C shows, we found that the density of vascular elements diminished as the leaf expanded. At their latest stage studied, leaves attained venation densities of 3.49 ± 0.20 mm/mm^2 for the first, 4.48 ± 0.60 for the third, and 4.82 ± 0.18 for the eighth node. As observed, adult rosette leaves have a slightly more complex (dense) venation pattern than the juvenile ones.

We also thought that the number of vein branching points per leaf area unit might be another good way of measuring the complexity of the venation pattern. Our measurements of this parameter yielded a result very similar to that seen for venation density (Fig. 3B). As their growth progressed, this ratio decreased in leaves from the three nodes considered. The first leaf evolved from 57.89 ± 15.30 branching points/mm^2 at day 11 after sowing to 5.40 ± 0.27 when fully expanded, the third leaf from 48.85 ± 3.47 at day 14 to 8.57 ± 2.13, and the eighth leaf from 82.08 ± 19.18 at day 22 to 9.84 ± 0.43. Using this different

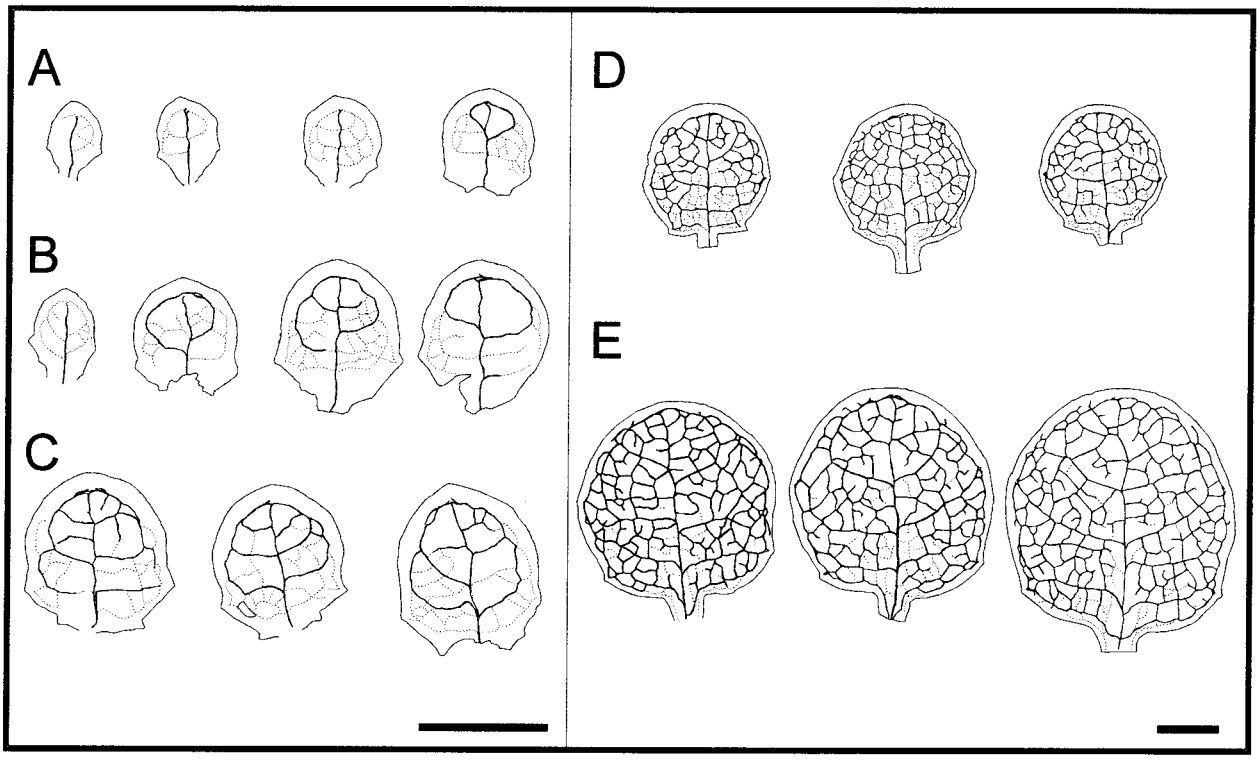

FIG. 2. Camera lucida drawings of venation pattern in leaves from the third rosette node of a wild-type *A. thaliana* ecotype L*er*. Each group of diagrams includes leaves from different plants grown in petri dishes and harvested 12 (A), 13 (B), 14 (C), 16 (D), and 18 (E) days after sowing. Discontinuous lines indicate differentiating, partially lignified tracheary elements. Continuous lines, other than those representing leaf margins, indicate well-lignified xylem strands. Note that magnification for drawings A, B, and C is different from that of D and E. Scale bars indicate 1 mm.

parameter, adult rosette leaves show themselves to have a more complex venation pattern than the juvenile leaves at the latest stage studied. The similarity between the plots in Figs. 3B and 3C is of note since it indicates that both parameters, venation density and number of branching points per surface unit, develop in parallel, suggesting a direct relationship between vein length and vein branching.

Hydathodes

Hydathodes are glands connected to the leaf vascular system which secrete water in the process known as guttation (Wilkinson, 1988). In *Arabidopsis* vegetative leaves, the midvein terminates in a fan-shaped group of tracheary elements which is a part of the apical hydathode present in all rosette leaves. The lateral hydathodes (Figs. 1D and 1E) are located along the leaf margin in positions related to the presence of the lateral teeth that are visible during early stages of leaf expansion. Following the classification of de Bary (quoted in Wilkinson, 1988), the hydathodes of *A. thaliana* are passive, since they present multicellular structures directly connected to the vascular system, opening to the exterior via stomata (Fig. 1D). *Arabidopsis* hydathodes show an epithem (Wilkinson, 1988), a structure formed by colorless isodiametric cells smaller than the mesophyll cells (Figs. 1D and 1E).

We found that the average number of hydathodes varied in leaves from the three nodes studied in the L*er* ecotype. Considering all leaves at maturity, when they are fully expanded, it is a general rule that the later a leaf originates, the more hydathodes it contains. This is in accordance with the observed higher number of marginal teeth in adult vegetative leaves than in juvenile ones. First rosette leaves usually showed three hydathodes, one apical and two laterals, although some lacked one or both lateral hydathodes. The average number of these glands in the third leaf was about 5 (one apical and four laterals) and about 7 in the eighth leaf (one apical and six laterals). The number of studied leaves was 50 for the first node, 48 for the third, and 29 for the eighth.

Natural Variability in A. thaliana Venation Pattern

We looked for natural variants in the venation pattern of rosette leaves from 266 ecotypes. The venation pattern of

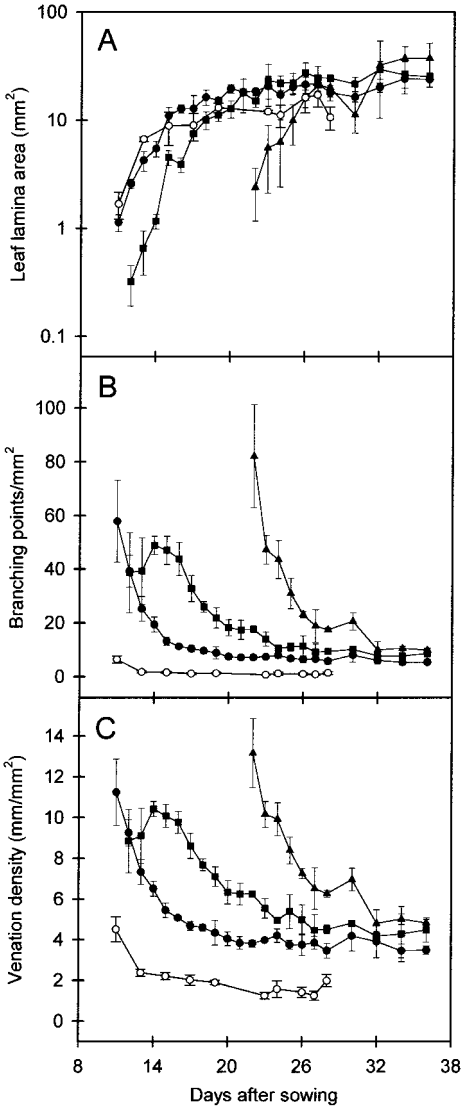

FIG. 3. Variation with time of (A) leaf lamina area, (B) venation branching points per leaf lamina surface unit (mm^2), and (C) leaf venation density (ratio of total venation length, in mm, to lamina area, in mm^2) for the first (●), third (■), and eighth (▲) nodes of the ecotype L*er*, and the first node (○) of the *hemivenata* mutant. Each point is the mean value of four independent measurements. Error bars indicate standard errors. Representing data in A on a logarithmic axis allows an estimate of the leaf expansion rate by determining the slope of each curve in the early stages.

the first leaf was studied in all of them and no major differences with respect to the L*er* ecotype were found, the only exceptions being two late flowering ecotypes (Fig. 4). The Ei-5 ecotype (N1128; isolated in Eifel, Germany) showed a venation pattern simpler than that of L*er* both in leaves and in cotyledons, the latter displaying only two loops in Ei-5 instead of the four loops usually found in L*er*. This extremely simple venation pattern was consistently present in all Ei-5 cotyledons studied and completely absent from the F_1 progeny of outcrosses of Ei-5 by wild-type Ws-2 individuals. The simple venation pattern of cotyledons and leaves reappeared together in a quarter of the F_2 progeny [77 F_2 plants scored; χ^2 (3:1) = 0.2; P = 0.5–0.7]. Such a monogenic trait has been called Hemivenata (Hve). The density of venation and the number of branching points per lamina area were recorded for the first vegetative leaf of this variant and compared to the same parameters obtained in the wild-type (Figs. 3B and 3C). As expected, these parameters pointed to the considerably reduced venation pattern of the Hve phenotype. The highest values for *hve*, obtained in young leaves, were 4.51 ± 0.61 mm/mm^2 for venation density and 6.43± 1.37 branching points/mm^2 of lamina area while the lowest values in mature leaves corresponded to 1.26 ± 0.23 mm/mm^2 and 0.81 ± 0.18 branching points/ mm^2.

The second ecotype displaying an atypical leaf venation pattern was Ba-1 (N952; isolated in Blackmount, UK). Free-ending vascular strands were found with unusual frequency at the apical region of their leaves (Fig. 4), although the trait was not shown by every plant of this extremely late flowering ecotype. Distinct from Ei-5, Ba-1 cotyledons did not display any obvious difference with those of L*er*. Work is in progress to genetically characterize the basis of the leaf venation phenotype of Ba-1, which we have called Inconexa (Ixa).

Search for Mutants with Normally Shaped Leaves But Abnormal Venation Pattern

To assay the existence of eventual morphogenetic controls operating independently on whole leaf shape and leaf

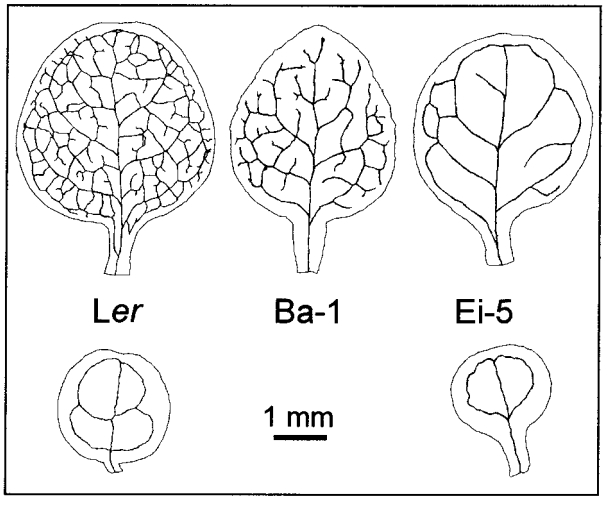

FIG. 4. Camera lucida drawings of divergent venation patterns in representative cotyledons (bottom) and first node rosette leaves (top) of different *A. thaliana* ecotypes. The cotyledon of the ecotype Ba-1 is not represented because it does not show significant differences from that of L*er*.

venation pattern, we screened for mutants with abnormal vein pattern and relatively normal leaf shape and size. Over 6000 M_2 seeds from EMS-mutagenized populations were sown to screen for alterations in the venation pattern. No such mutants were found, despite the fact that several hundred plants with morphological abnormalities were identified and discarded. We studied also 17,313 T_4 plants derived from 1800 independent transformants obtained from T-DNA insertional mutagenesis. About 57% (9875) of the seedlings were resistant to kanamycin, and so their first rosette leaves were excised, cleared, and observed under a microscope. Only 1 plant presented a clearly distinguishable abnormality, consisting of an excess of hydathodes in the first leaf compared with Ws-2. This mutation, which appeared to be associated to clearly marked lateral teeth in young first leaves has been named *extrahydathodes* (*ehy;* Fig. 5). Although the Extrahydathodes phenotype appeared with incomplete penetrance, it was shown to be monogenic and recessive after studying F_1 individuals of an *ehy*/*ehy* × Ws-2 cross and their inbred F_2 progeny. Additional studies on the T_4 mutant initially isolated showed that it carried two unlinked T-DNA insertions, neither of which cosegregated with the Ehy phenotype.

DISCUSSION

The first question that a developmental genetic analysis is expected to answer relates to the differences between mutant and wild-type alternatives for a given biological process. In this respect, a study of the wild-type system provides the information required for the isolation and characterization of mutants. To facilitate the study of the venation pattern in wild-type *A. thaliana* vegetative leaves, we used a simple procedure to clear leaf tissues and visualize veins (see Materials and Methods). This single-step method is particularly useful when searching for mutants in screenings that require the manipulation of thousands of leaves.

We found that the wild-type venation of *Arabidopsis* fully expanded vegetative leaves is brochidodromous, with a single primary vein (midvein) and a series of loops formed by secondary veins which are connected by a variable number of other secondary and higher order veins. As previously reported for the first leaf of the Columbia ecotype (Telfer and Poethig, 1994), we found that the two secondary veins closer to the base of the leaf in all L*er* rosette leaves branch off the midvein in the distal region of the petiole and join the rest of the vasculature to form a continuous vascular structure along the margin of the lamina, enclosing other venation elements. Secondary and higher order veins interconnect and originate polygonal areolas, where some higher order veins terminate blindly without connecting with other veins. As inferred from progressive visualization of the veins, lignification proceeds basipetally during leaf development, from the apex to the petiole, in agreement with the previous results of Dharmawardhana *et al.* (1992).

The midvein divides the leaf lamina into two regions of loose bilateral symmetry. Although the same generative rules clearly emerge in both regions and the midvein is an obvious reference axis, they are far from being perfect mirror images. The midvein reaches its maximum width at the basal region of the lamina and gradually diminishes in size acropetally as new secondaries ramify from it, the width of the midvein in the apical region being similar to that of the tertiaries and quaternaries, as has previously been described in *M. arvensis* (Beebe and Evert, 1990).

We established in this work two main criteria to quantitatively define the complexity of the venation pattern in *Arabidopsis* vegetative leaves and to follow its time profile: the density of venation and the number of branching points per lamina surface unit (mm^2). Both parameters indicate the presence of pattern elements related to the extension of the lamina, whose area shows very different rates of expansion depending on the developmental stage of the leaf. As previously described (Pyke *et al.*, 1991), our data show that the lamina area increases exponentially during the early stages of leaf development. As leaf area increases, the rate of leaf expansion diminishes to reach a maximum size at maturity. Although the length of vascular bundles and the number of branching points increase throughout development, the ratios between both of them and the lamina area decrease as leaf expansion progresses. For instance, in the case of venation density, the length of the vascular bundles per square millimeter of lamina diminishes until it reaches a value of about 3.5 mm/mm^2 in the mature first leaf, a result quite close to the value previously reported by Rüffer-Turner and Napp-Zinn (1979).

Our results quantitatively support the qualitative conclusions of Telfer and Poethig (1994), who stated that the density of vascular bundles diminishes during first leaf expansion, and agree with those of Pyke *et al.* (1991), who showed that the proportion of leaf volume occupied by the vasculature in transverse sections decreases as development progresses. A question which arises from these observations is whether and how the venation pattern is related to cell proliferation and cell expansion during leaf growth. The whole spectrum of results might be interpreted as the vascular tissue growing at a different rate from other leaf tissues (Telfer and Poethig, 1994), which might suggest that the development of veins and that of other leaf tissues are not completely coupled. Alternatively, differential frequency and orientation of cell divisions and/or direction of cell enlargement can be assumed. Nevertheless, we think that the results might be better explained as a mere consequence of comparing a one-dimensional (venation length) with a two-dimensional (lamina surface) variable.

Our observations indicate that leaf vascular development proceeds in two stages. In a first step, the main features of the venation pattern are determined in meristematic zones, which are distributed in the leaf according to a basipetal gradient. Indeed, cell division is known to cease first at the

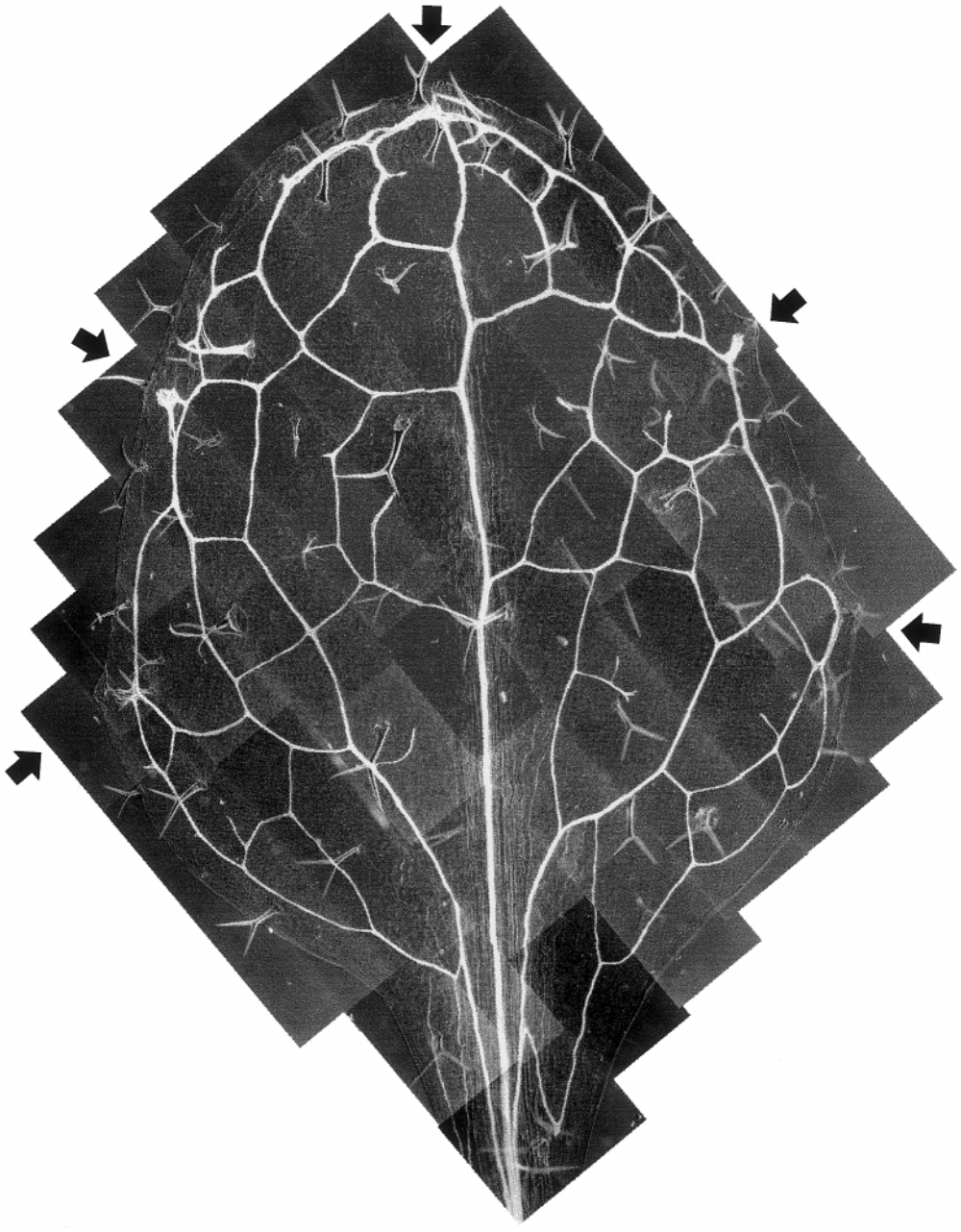

FIG. 5. Interference contrast micrograph of a first-node *extrahydathodes* leaf. Arrows indicate hydathodes.

distal regions of the developing lamina, while they continue in the leaf base (Dale, 1988). Second, the vasculature and other leaf tissues expand coordinately, differentiating earlier in the apical region and reaching their final size at maturity.

In *Arabidopsis,* cell axialization, the first evidence of vascular development, is first observed in early leaf primordia before mesophyll cells differentiate as either palisade or spongy. In fact, during this period, both venation length and the number of branching points show the highest rates of increase, the veins developing throughout the whole leaf and outlining the peculiarities of the vascular pattern. It is therefore to be expected that the genes which play an important role in venation pattern formation will already be active in these early stages of leaf development. Later, after cell differentiation has started at the leaf apex, new

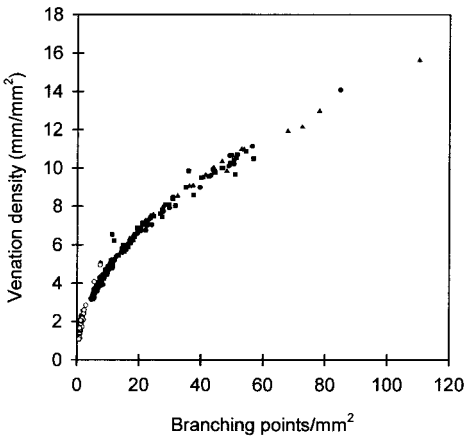

FIG. 6. Correlation between the variation with time of the venation density and that of the number of branching points in leaves of the first (●), third (■), and eighth (▲) nodes of the ecotype L*er* and of the first node (○) of the *hemivenata* mutant.

provascular strands continue developing mainly at the base of the leaf, where the cells differentiate later (Pyke *et al.*, 1991), and by a process of intercalary growth between existing veins. Subsequently, new vascular elements are formed exclusively in the leaf region close to the petiole until the leaf reaches maturity.

The *hemivenata* variant is affected at an early process, as suggested by the extremely low complexity of the venation pattern already seen in the youngest leaves studied. The complexity of the pattern in the Hemivenata leaf apparently diminishes at the same rate as in the wild-type, as can be inferred from a comparison of the venation density values for the first leaf of the mutant and the wild type. In the mutant, the venation density fell from 4.51 to 1.26 mm/mm^2, (a 3.58-fold reduction) and from 11.24 to 3.49 mm/mm^2 in the wild type (a 3.22-fold reduction). Further genetic and molecular analyses of the *hemivenata* gene are in progress in our laboratory and will allow a better understanding of the process of vein patterning in *A. thaliana.*

As shown in Figs. 3B and 3C, the length and the number of branching points in the vein network evolve in a similar way during leaf expansion. Figure 6 shows the high correlation, with minimum variation, between venation density and the number of branching points per lamina area. When plotted together, the values for *hemivenata* are lower than those of the wild type, as is to be expected. It is noteworthy that wild-type leaves from the three nodes studied show the same close relationship between both parameters, suggesting that a common patterning mechanism functions in all these leaves and excluding a role for such a mechanism in heteroblasty. It can be said therefore that there is no phase change for venation patterning in *Arabidopsis* vegetative leaves.

Although our approach to the search for mutants with abnormal venation pattern and wild-type shape was far from exhaustive, our results clearly indicate that venation mutants are not easily found in screens merely based on the clearing and observation of leaves from mutagenized populations. The rarity of viable mutants which are specifically affected in venation patterning suggests that the number of genes involved is small, that their functions are redundant, or that most if not all of their mutations are lethal, as it is the case with *monopteros* (Przemeck *et al.*, 1996). A specific reason to expect them to be lethal is that they might affect auxin transport, which might be necessary for tissue organization, not only vascular patterning, even at embryonic stages. Another reason could be that the developmental system is plastic and covers up defects that arise from any one mutant (T. Sachs, personal communication). Alternatively, it is possible that the same morphogenetic controls operate both in whole leaf expansion and in vein formation and patterning, vein patterning therefore being largely a consequence of leaf growth, as suggested by Goebel (1922) and Mitchison (1981). The study of vein patterns in mutants with aberrant leaf shape and size will undoubtedly clarify the relationship between vein formation and lamina growth.

Our results suggest that the relationship between leaf venation patterning and whole leaf morphogenesis in *Arabidopsis* and maize might be closer than expected. The analysis of the latter monocotyledonous model organism is considerably more advanced than in any dicot, the available results suggesting that epidermal patterns and venation pattern are related in maize leaves, primordial veins being likely to organize leaves symmetrically around themselves (Silvester *et al.*, 1996; Schichnes *et al.*, 1997). If leaf vein traces are the signal transmitters that organize the entire shape of the leaf, as has been proposed for maize leaves, then a morphology without underlying signal transmission sources would be expected to be impossible. Such assumption is satisfied by our failure to isolate viable *Arabidopsis* mutants displaying leaves that must remain wild type, whereas the underlying venation pattern should be altered.

Although *Arabidopsis* leaf venation pattern and *Drosophila* wing vein pattern are quite different biological entities, they both represent the same kind of developmental problem: the formation of a branching pattern (Meinhardt, 1976). However, we must assume that circumstances in *Arabidopsis* and *Drosophila* are totally different, since there are 81 genes with known mutant alleles which affect venation pattern in the wing of *Drosophila* (García-Bellido and de Celis, 1991). Furthermore, the number of *Arabidopsis* genes required to achieve normal leaf size and shape is considerable, and we have found 94 such loci in a screen covering 46,159 M_2 seeds obtained from 5770 M_1 parental lines exposed to EMS (Berná, Robles, and Micol, submitted for publication).

Another developmental parameter that we have studied is the number of hydathodes associated with the leaf vasculature. While cotyledons and all rosette leaves contain an apical hydathode, the first, third, and eighth leaves generally show in addition two, four, and six lateral hyda-

thodes, respectively. We have observed that the number of lateral hydathodes can be predicted from the number of lateral teeth in the leaf primordia, in agreement with previous observations of Tsukaya and Uchimiya (1997) and Van Lijsebettens and Clarke (1998). Supporting this relationship between teeth and hydathodes, we have found that the latest adult vegetative leaves on late-flowering ecotypes, which show a higher number of teeth than those of L*er*, also display an increased number of hydathodes (data not shown). Our observations on the number of hydathodes in wild-type leaves of *Arabidopsis* have allowed us to isolate an untagged T-DNA induced mutant, *extrahydathodes* (*ehy*), with a higher number of hydathodes than the wild-type in the first leaf, a phenotype that could easily be explained by an acceleration in the normal sequence of generation of hydathodes.

Very little is known about the morphogenetic controls involved in generating the venation pattern. The phytohormone auxin is thought to promote vascular tissue development (Hobbie and Estelle, 1994) and the canalization hypothesis accounts for the generation of complex patterns of vasculature in response to a polarized flow of auxin (Sachs, 1991a,b). An alternative point of view arises from the diffusion–reaction prepattern hypothesis (Meinhardt, 1984; Koch and Meinhardt, 1994), which explains the formation of net-like structures by the coupling of a short-range autocatalytic process with a long-range inhibitory process. Although there is no experimental evidence for the existence of such morphogenetic molecules (Nelson and Dengler, 1997), auxin could act as activator (Mitchison, 1980; Meinhardt, 1984; Nelson and Dengler, 1997). Indeed, several pieces of evidence point toward a role for auxin in vascular development. The fact that auxin can replace the effects of the leaves on the induction of vascular differentiation has been known since the early work of Snow (1935), Jost (1942), and Jacobs (1952). In addition, plants with the *iaaL* transgene from *Pseudomonas*, which encodes the enzyme IAA–lysine synthase which converts IAA to the inactive conjugate IAA–lysine (Roberto *et al.*, 1990), are deficient in auxin and show a reduced vascular development (Romano *et al.*, 1991). Increased levels of vascular development are shown in petunia and tobacco plants with high levels of auxin, as a consequence of the expression of the *iaaM* transgene from *Agrobacterium tumefaciens*, which is involved in IAA synthesis from tryptophan (Klee *et al.*, 1987; Sitbon *et al.*, 1992). The *pin1-1*, *lop1*, and *mp* mutants of *Arabidopsis*, which are thought to be affected in auxin transport, have leaves with an abnormal venation pattern (Okada *et al.*, 1991; Carland and McHale, 1996; Przemeck *et al.*, 1996). Two homeobox genes which are expressed in provascular tissue and are possibly involved in vascular development have been isolated: the *Arabidopsis Athb-8* gene, whose expression is modulated by auxin (Baima *et al.*, 1995), and the tomato *VAHOX1* gene (Tornero *et al.*, 1996).

In contrast to other *Arabidopsis* organs or tissues, the leaf has received little attention from a developmental point of view. Recent reviews agree that our understanding of leaf development is far inferior to that of root and flower development (Telfer and Poethig, 1994; Tsukaya, 1995; Hall and Langdale, 1996; Poethig, 1997). In this context, the combination of qualitative description and use of quantitative parameters to describe leaf venation development will be useful for later studies of mutants. Our work provides the basis for genetic analyses which will allow a more thorough study of the process of venation pattern formation, one of the most intriguing elements of leaf architecture.

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