

# Lessons from a search for leaf mutants in *Arabidopsis thaliana*

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**ABSTRACT** Large-scale exploratory approaches to understanding gene function laid the foundations for the “-omics era”. Based on modern technologies for the structural and functional characterization of genomes, these curiosity-driven approaches allow systematic accumulation of vast amounts of data, enabling subsequent hypothesis-driven research. Some years before the dawn of genomics, exploratory approaches were already furthering our understanding of gene function in the form of saturation mutagenesis experiments aimed at the identification of all genes that mutate to a given phenotype. Forward genetic approaches, conducted on experimental organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*, have led to the isolation of mutants affected in specific developmental processes, whose cellular and molecular characterization has unraveled the underlying genetic mechanisms of animal development. To shed light on the making of plant leaves, in 1993 we initiated an attempt to identify as many viable and fertile mutants with abnormal leaf morphology as possible, using the *Arabidopsis thaliana* model organism. We identified 25 fast-neutron- and 153 ethyl-methane sulfonate-induced mutations, which fell into eight and 94 complementation groups, respectively. We also studied 115 publicly available mutant lines isolated by previous authors, which fell into 37 complementation groups. Although we did not reach saturation of the *Arabidopsis thaliana* genome, the broad spectrum of leaf morphological alterations identified is facilitating the dissection of specific leaf developmental processes. In a complementary approach, we also analyzed leaf architecture in natural accessions and two populations of recombinant inbred lines. Using a high-throughput gene mapping method, we have already cloned 25 of the genes identified by mutation, in some cases in collaboration with other groups. The products of these genes participate in various developmental processes, such as polar cell expansion, transduction of hormonal signals, gene regulation, plastid biogenesis, and chromatin remodeling, among others. The range of phenotypes and processes identified reveal the complexity of leaf ontogeny and will help explain the diversity of leaf morphology in nature.

**KEY WORDS:** *Arabidopsis thaliana*, forward genetics, leaf development, mutant screen

*While walking in the Public Gardens of Palermo, it came to me in a flash that in the organ of the plant which we are accustomed to call the leaf lies the true Proteus who can hide or reveal himself in all vegetal forms. From first to last, the plant is nothing but leaf, which is so inseparable from the future germ that one cannot think of one without the other.*

Goethe (1816).

## Curiosity-driven versus hypothesis-driven research

The advent of high-throughput, cost-effective techniques for the structural and functional characterization of genomes has led to a revolution in biology. In an unprecedented approach, the emphasis of scientific research is not on the generation and experimental testing of hypotheses, but on the systematic gathering of vast amounts of data (i.e., genome sequences) and the generation of large collections of resources (i.e., tagged mutants, gene libraries), often accessible through publicly available databases. These initial observations provide the starting information

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that enables subsequent hypothesis-driven research, which would be impossible in the absence of any prior knowledge of the process under study. Underlining the fact that no guiding hypothesis is required, this type of research is referred to in various ways, including terms such as exploratory, inductive, observational, data-driven, question-driven, or curiosity-driven. Curiosity-driven approaches are sometimes neglected, even though they have the potential to yield substantial, rather than incremental, advances in knowledge simply because they are not constrained by the limits and expectations imposed by current paradigms. As some authors have highlighted (Kell and Oliver, 2004; Franklin, 2005), curiosity-driven research contributes to the advancement of science more than is usually recognized and is complementary in nature to hypothesis-driven research, since both act as iterative partners in the cycle of knowledge.

Some years before the “-omics era”, exploratory approaches were already furthering our understanding of gene function, in the form of large-scale mutagenesis experiments aimed at the identification of mutant alleles for all genes that contribute to a given phenotype (Murray, 2000). The classical example was pioneered by Nüsslein-Volhard and Wieschaus (1980), who devised the methodology for saturation mutagenesis and applied it to the dissection of embryonic development in the model organism *Drosophila melanogaster*, with no assumption other than that development is under genetic control. Our current understanding of many other important biological phenomena derives from applying the same rationale to many experimental systems. More recently, genomics has provided researchers with a wealth of data from high-throughput array-based experiments (Lockhart and Winzler, 2000), including global analyses of gene expression under multiple experimental conditions, and from novel massively parallel sequencing techniques (Margulies *et al.*, 2005; Rogers and Venter, 2005), which can be used to obtain direct estimates of the abundance of molecules being sequenced. These techniques are expected to assist in the design of hypotheses that will lead to a mechanistic understanding of the regulatory networks that underlie complex processes, such as the development of multicellular organisms. In this context, the generation of collections of mutants induced by random mutagenesis fulfills the dual objective of providing a survey of the range of phenotypes that can arise after perturbation of the wild-type condition and of being an invaluable resource for further hypothesis-driven research.

### Mutant genetic screens in model organisms: a historical perspective

Multicellular organisms develop from a unicellular zygote by highly regulated processes that result in progressively increased structural complexity. During embryonic development, a hierarchy of gene products participates in cell specification and morphogenetic pathways that cooperatively build the architecture of the adult body. Understanding how genetic information is integrated and used for the elaboration of complex biological patterns is among the main challenges of developmental biology.

Soon after the rediscovery of Mendel's laws in 1900, pioneering genetic studies conducted on the fruit fly (*Drosophila melanogaster*) in the laboratory of Thomas H. Morgan led to the discovery that genes are physically located on chromosomes and exist in

many allelic forms (reviewed by Sturtevant, 1965). Morphological variation was soon recognized as being caused by spontaneous mutations at very low rates, which could be increased experimentally (Müller, 1927). It is generally agreed that such mutations are a powerful tool for unraveling the normal function of individual genes during development.

Forward genetic approaches begin with the induction and isolation of mutants affected in a given process and then infer the biological role of mutated genes based on their associated phenotypes. In this way, high-throughput isolation of morphological mutants and the cloning of the corresponding genes have increased our knowledge of the events that take place during development in organisms such as fungi, nematodes, insects, vertebrates, and plants. One limitation of mutant screens is that mutations that produce subtle phenotypes are likely to remain undetected. A combination of reverse genetics, clonal analysis, and cell biology techniques provides the toolbox to overcome this problem and will help identify all genetically detectable functions at work during the development of complex organisms.

The fruit fly offers many advantages as an experimental organism for developmental genetic studies, including its small size, short life cycle, high fertility, and relatively small genome (Rubin and Lewis, 2000). Because even subtle wing deformations can easily be detected, mutations altering wing shape or wing venation were among the first to be studied at a large scale (Waddington, 1940). Four decades later, embryonic development of the fruit fly was examined with a saturation mutagenesis screen (Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard *et al.*, 1984). From approximately 27,000 lines, 580 mutants with defects in the larval segmentation pattern were identified, affecting 139 different genes (Table 1). Many of these genes are now known to encode transcription factors necessary for cell fate specification along the anteroposterior and dorsoventral embryonic axes (reviewed by Nüsslein-Volhard, 1991). These studies highlighted for the first time how a complex morphogenetic pattern emerges from a few local signals or morphogens that regulate hierarchies of downstream transcription factors, which interact to delimit cellular territories in the adult body (Nüsslein-Volhard, 1995). More recently, the use of sophisticated imaging techniques to visualize subtle patterning defects has allowed detailed screens aimed at the genetic dissection of other complex processes, such as neuronal morphogenesis (Gao *et al.*, 1999; Table 1).

Despite its late adoption as a model organism (Brenner, 1974), genetic screens performed with the nematode *Caenorhabditis elegans* were also important for the characterization of cell differentiation pathways, such as the differentiation of the vulva from undifferentiated epidermal cells (Ferguson and Horvitz, 1985; Ferguson *et al.*, 1987). In these screens, 95 mutations defining 22 genes were identified and later found to participate in two highly conserved signaling pathways required for cell fate specification (reviewed by Wang and Sternberg, 2001). The possibility of silencing target genes by RNA interference (RNAi) has greatly accelerated the search for loss-of-function phenotypes in *C. elegans* (Kamath *et al.*, 2003). For instance, a genome-wide RNAi screen allowed researchers to identify 661 genes required for the first two rounds of cell division in the nematode embryo (Sönnichsen *et al.*, 2005; Table 1).

Many genes involved in axial specification in *D. melanogaster*

TABLE 1

## SOME GENETIC SCREENS FOR DEVELOPMENTAL MUTANTS IN MODEL ORGANISMS

Model organism	Screened for	Mutants recovered	Genetic loci involved	Degree of saturation reached <sup>a</sup>
<i>Drosophila melanogaster</i>	Embryo lethality and larval cuticle defects <sup>1,2</sup>	580	139	98.5
	Defects in dendritic outgrowth, branching, and routing <sup>3</sup>	25 <sup>b</sup>	12	n.a.
<i>Caenorhabditis elegans</i>	Altered cell lineages of the vulva <sup>4</sup>	95	22	98.7
	Defects in the first two rounds of cell division in the embryo <sup>5</sup>	661	661	n.a.
<i>Danio rerio</i>	Abnormal embryonic and early larval phenotypes <sup>6</sup>	331	220	77.8
	Visible phenotype in embryos or early larvae <sup>7</sup>	894	372	91.0
<i>Arabidopsis thaliana</i>	Defects in embryonic development <sup>8</sup>	90	n.a.	n.a.
	Mutations that disrupt the spatial organization of the embryo <sup>9</sup>	77	9	100.0
	Abnormal trichome development <sup>10</sup>	71	22	96.0
	Viable and fertile mutants with abnormal leaves <sup>11</sup>	120	94	72.2

<sup>a</sup>Assuming a Poisson distribution of frequencies (equal mutability for all genes). <sup>b</sup>Only mutations on genes in the second chromosome were scored. n.a., not applicable. <sup>1</sup>Nüsslein-Volhard and Wieschaus, 1980. <sup>2</sup>Nüsslein-Volhard *et al.*, 1984. <sup>3</sup>Gao *et al.*, 1999. <sup>4</sup>Ferguson and Horvitz, 1985. <sup>5</sup>Sönnichsen *et al.*, 2005. <sup>6</sup>Driever *et al.*, 1996. <sup>7</sup>Haffter *et al.*, 1996. <sup>8</sup>Meinke and Sussex, 1979. <sup>9</sup>Mayer *et al.*, 1991. <sup>10</sup>Hülkamp *et al.*, 1994. <sup>11</sup>Berná *et al.*, 1999.

and *C. elegans* have homologs in vertebrates. The zebrafish (*Danio rerio*) is the only vertebrate systematically used in forward genetic screens (reviewed by Patton and Zon, 2001). Its translucent embryo is ideal for the identification of mutants deficient in genes required for patterning and differentiation from fertilization to late embryogenesis. In two large-scale screens for visible embryonic phenotypes, more than 1,200 mutants were isolated, which defined at least 550 genes (Driever *et al.*, 1996; Haffter *et al.*, 1996; Table 1). Although these screens failed to saturate the genome with embryonic mutations, they allowed the discovery of mutants with defective blood and heart formation, musculature, and pigmentation. Functional redundancy due to duplicated genes is probably the most severe limitation for determining gene function through mutational approaches in zebrafish (Haffter *et al.*, 1996). New morpholino-based gene silencing protocols have allowed researchers to overcome this problem and systematically knock down selected target genes (Sumanas and Larson, 2002).

Since multicellularity evolved independently in plants and animals, they are expected to use different developmental mechanisms. Like in animals, the basic body plan of plants is laid down during embryogenesis, but, unlike in animals, plant morphogenesis is mostly postembryonic. Mutational approaches became routine in the model plant *Arabidopsis thaliana* in the early 1990s, after initial screens for embryo-lethal mutants (Meinke and Sussex, 1979). The primary reason for studying seedling lethal mutants with altered spatial embryo organization, initiated by Mayer *et al.* (1991; Table 1), was to learn about the logic of pattern formation by determining how the embryo responds to the removal of a single, genetically defined component. Although many genes that regulate these early patterning events have been identified, some of them involved in cytokinesis and either auxin signaling or auxin transport, the underlying mechanisms remain poorly understood (reviewed by Jenik *et al.*, 2007).

Genetic analysis has furthered our understanding of the mechanisms responsible for the differentiation of specialized cell types within plant tissues. The ontogeny of trichomes, large polarized cells that are regularly spaced on the leaf surface, has been

studied genetically in *Arabidopsis thaliana* (Hülkamp *et al.*, 1994). In total, 71 mutations that affect trichome morphogenesis and spatial patterning were classified based on the phenotypes produced. Complementation tests indicated that these mutations affect 22 loci (Hülkamp *et al.*, 1994; Table 1). Although additional loci involved in trichome development have been discovered since this early work, an analysis of the cellular phenotypes in single and double mutants has allowed researchers to study specific events in trichome development (reviewed by Schellmann and Hülkamp, 2005) and to propose an elegant model for the differentiation of a specialized cell type from a uniform cell layer. The spatial pattern of presumptive trichome cells is first laid down in the developing leaf, and the committed trichome cells then undergo morphogenesis and differentiation through a defined network of genetic interactions.

Homeotic mutants (i.e., mutants with normal organs in positions typically occupied by another organ type) have facilitated the

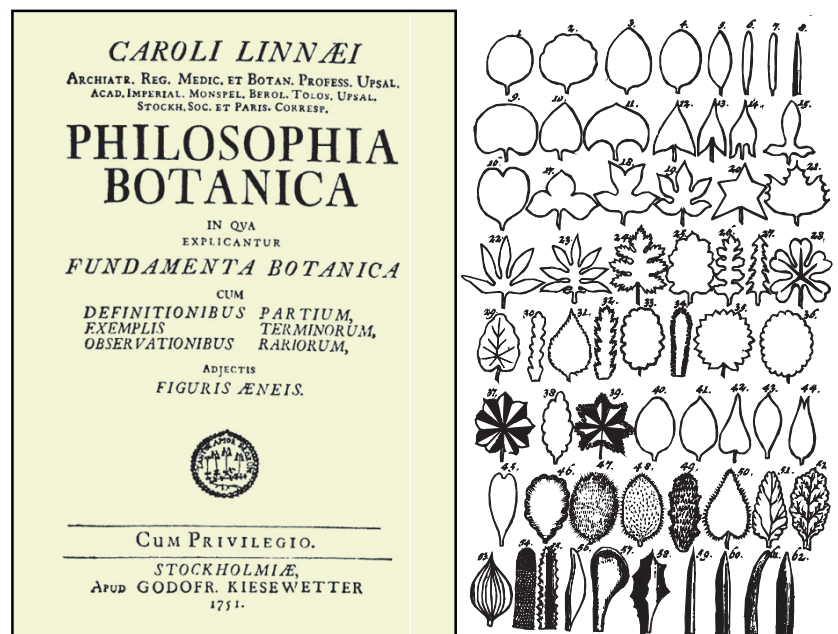


Fig. 1. Leaf diversity as seen by Carolus Linnaeus in a page of his *Philosophia Botanica* (1751).

identification of organ identity genes responsible for the whorled structure of *Arabidopsis thaliana* and *Antirrhinum majus* flowers (reviewed by Meyerowitz *et al.*, 1991). Studies of single and double homeotic mutants in these species led to the ABC model, which states that the identity of each whorl in a wild-type flower is specified by a unique combination of organ identity genes, all of which encode plant transcription factors (reviewed by Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994; Ng and Yanofsky, 2000; Coen, 2001).

### A search for leaf mutants in *Arabidopsis thaliana*

The leaf, one of the most conspicuous organs of higher plants, is the main site of primary productivity and a basic anatomical unit. Plant leaves have determinate growth and develop from a small population of founder cells at the flanks of the shoot apical meristem (Byrne, 2005; Tsukaya, 2005; 2006). In addition to their main role in light harvesting, leaves are also important for nutrient storage, defense, and stress responses. Leaves were considered crucial for taxonomic purposes by Linnaeus (1751) (Fig. 1) and seen as the basic units of plant body architecture by Goethe (1816). It is therefore surprising that in 1993, when the laboratory of José Luis Micol was established, a Medline search using the keywords "*Arabidopsis* AND leaf AND mutant AND development" yielded only 12 articles. Obviously, plant vegetative development was not receiving much attention from the scientific community at that time. We considered answering questions on how many genes control leaf development and how these genes interact essential to understanding how postembryonic lateral organs are built in plants.

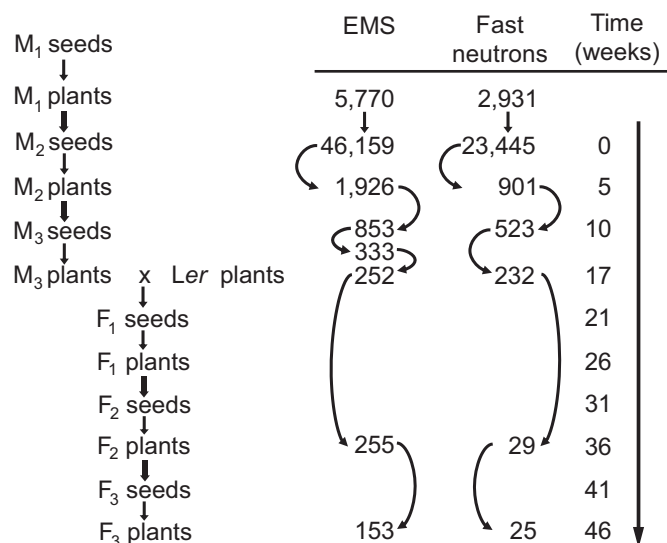
To dissect leaf organogenesis, we followed a forward genetic approach using *Arabidopsis thaliana* as a model organism. We aimed to identify as many viable and fertile mutants with abnormal leaves as possible, in an attempt to isolate mutant alleles of most genes involved in leaf development. The Landsberg *erecta* (*Ler*; Fig. 3A) accession was selected as the genetic background for our mutagenesis because it is easily grown at high density in the laboratory, has an upright inflorescence that facilitates crossing and seed harvesting, and because the polymorphism between the *Ler* and Columbia-0 (*Col-0*) genomes makes genetic mapping and map-based cloning straightforward. The alkylating agent ethyl-methane sulfonate (EMS), by far the most efficient and commonly used mutagen, was chosen because it induces G-to-A transitions and has no known bias for particular genomic regions (Greene *et al.*, 2003). Based on codon usage in the *Arabidopsis thaliana* genome, the frequencies of EMS-induced stop codons and missense mutations are thought to be around 5 and 65%, respectively (McCallum *et al.*, 2000).

In a first screen for leaf mutants (Fig. 2; Berná *et al.*, 1999), we analyzed 46,159  $M_2$  seeds derived from 5,770  $M_1$  parental lines mutagenized with EMS at the seed stage by a commercial supplier (Lehle seeds; <http://www.arabidopsis.com/>). In the  $M_2$  generation, we found 23.5% non-germinating seeds and 1.9% albino plants, as expected for an efficient mutagenesis. After visual inspection of individual  $M_2$  plants, we selected 1,926 putative mutants (4.2% of the  $M_2$  population) with leaves clearly different from those of *Ler* or that were abnormally arranged in the basal rosette. These mutants were initially assigned to three broad categories, corresponding to alterations in: (1) shape or

size of the lamina, (2) margin configuration, or (3) structure of the basal rosette. Many putative  $M_2$  mutants (853) yielded viable  $M_3$  progeny but only 333 transmitted their mutant phenotype to the next generation in a Mendelian manner, with complete penetrance and only subtle variation in expressivity. Eighty-one mutants with altered rosette structure were discarded because they were not necessarily affected in the aspects of leaf development that were of interest to us. The inheritance patterns of the remaining 252 mutant lines were determined in backcrosses to *Ler*. The segregation ratios of the leaf mutant phenotypes were studied in their corresponding  $F_2$  families, and the phenotypes of  $F_3$  inbred descendants from the  $F_2$  mutant plants were recorded. For subsequent genetic analyses, we selected 153 mutants whose phenotype remained unchanged in the  $M_2$ ,  $M_3$ , and  $F_3$  generations and was inherited as a monogenic trait.

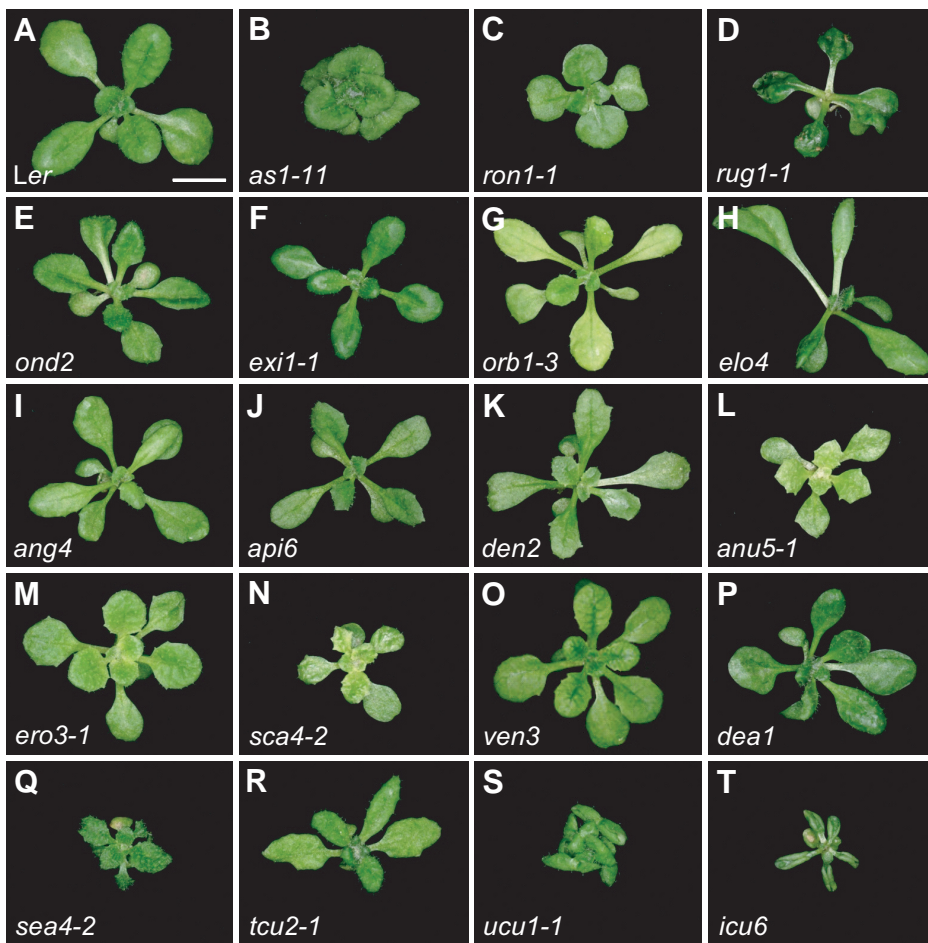
A complementation test (Lewis, 1939) makes the assessment of allelism possible by crossing any two recessive mutants and analyzing the phenotype of their  $F_1$  progeny. Despite the simplicity of this test, the number of crosses required to assign a large collection of mutants to a discrete number of complementation groups becomes enormous and time-consuming even for a moderate number of mutants. For instance, testing the allelism of 153 mutants requires  $153!/(2 \times (153-2)!) = 11,628$  crosses. To reduce this number, we defined 19 phenotypic classes (Fig. 3) by grouping mutants with similar characteristics and limited the crosses to mutants within the same class. This seemed reasonable because mutant alleles of the same gene are likely to yield similar phenotypes. The phenotypic classes and the corresponding genes were given Latin names descriptive of their most conspicuous leaf characteristics, inspired by Stearn's *Botanical Latin* (1995). One exception was the Asymmetric leaves phenotypic class, whose name was already in use for mutants with leaf bilateral asymmetry and a slightly revolute leaf margin (Rédei and Hirono, 1964).

After completing 525 crosses, we assigned our 153 leaf mutants to 94 complementation groups. Since each  $M_1$  parental was



**Fig. 2. Flow-chart for the isolation and genetic analysis of leaf mutants.** The symbols  $\downarrow$  and  $\Downarrow$  indicate growth and selfing, respectively. The time scale refers to each mutant. Tasks indicated in italics were outsourced to a company.





**Fig. 3.** Pictures showing representative individuals of the phenotypic classes defined for the complementation analysis of mutants with abnormal leaves. The wild-type strain (A) *Landsberg erecta* (*Ler*) and the mutants (B) asymmetric leaves1-11 (*as1-11*), (C) *rotunda1-1* (*ron1-1*), (D) *rugosa1-1* (*rug1-1*), (E) *ondulata2* (*ond2*), (F) *exigua1-1* (*exi1-1*), (G) *orbiculata1-3* (*orb1-3*), (H) *elongata4* (*elo4*), (I) *angusta4* (*ang4*), (J) *apiculata6* (*api6*), (K) *denticulata2* (*den2*), (L) *angulata5-1* (*anu5-1*), (M) *erosa3-1* (*ero3-1*), (N) *scabra4-2* (*sca4-2*), (O) *venosa3-3* (*ven3-3*), (P) *dentata1* (*dea1*), (Q) *serrata4-2* (*sea4-2*), (R) *transcurvata2-1* (*tcu2-1*), (S) *ultracurvata1-1* (*ucu1-1*), and (T) *incurvata6* (*icu6*). Pictures were taken 19 days after sowing. Scale bar is 5 mm.

creates deletions and chromosomal rearrangements (Shirley *et al.*, 1992). In a second screen, we sowed 23,445  $M_2$  seeds derived from 2,931  $M_1$  parents irradiated with fast neutrons. We found 1% albino plants in the  $M_2$  population and 901 putative leaf mutants, 523 of which produced  $M_3$  seeds (Robles, 1999). In 232 of these mutants, the phenotype was transmitted to the  $M_3$  generation with complete penetrance and only subtle variation in expressivity. We selected 25 mutant lines with stable phenotypes and grouped them into five phenotypic classes. Complementation tests allowed us to conclude that they represented eight damaged genes (Robles and Micol, unpublished results).

represented by eight  $M_2$  seeds (46,159/5,770), it is possible that allelic mutants isolated from the same parental group represented repeated isolations of the same mutation. Thus, after correcting for this fact, we conservatively estimated the number of different mutations to be 120. When classified according to their mode of inheritance, 113 of the mutations were recessive, six were semi-dominant, and only one was completely dominant. According to the number of alleles per gene, the 94 complementation groups were defined by one (76 complementation groups), two (11), three (six), or four (one) mutant alleles.

Assuming equal mutability of all genes, the number of mutant alleles per gene in a mutagenesis experiment is often thought to follow a Poisson probability distribution (Jürgens *et al.*, 1991). To reach saturation, the number of mutants obtained should be high enough to render negligible the probability of a gene not being represented by any mutant allele. With an average of 1.27 (120/94) alleles per gene, the fraction of genes that mutate to viable leaf phenotypes but were missed by our screen can be estimated from the zero term of a Poisson distribution to be  $e^{-1.27} = 27.8\%$ . Although relatively far from saturation, we identified a broad spectrum of leaf morphological alterations that appear in *Arabidopsis thaliana* after chemical mutagenesis, and they provided us with a precious collection of viable, fertile mutants that has helped us and other groups to dissect specific aspects of leaf development.

Physical mutagenesis with X-rays or fast neutrons normally

The *Arabidopsis* Information Service (AIS) Form Mutants collection, which includes mutants isolated by G. Röbbelen and A. R. Kranz, was the largest morphological mutant collection available at that time. We selected 115 AIS form mutants for genetic analysis (Serrano-Cartagena *et al.*, 1999). The leaf mutant phenotypes were inherited as recessive monogenic traits and were classified into 11 previously defined phenotypic classes (Berná *et al.*, 1999), plus three new classes. These AIS mutants fell into 37 complementation groups. The most abundant class, *Incurvata* (*icu*), comprised 22 mutants with involute (curled upward) vegetative leaves, falling into ten different complementation groups. The study of double mutants involving alleles of five *ICU* genes allowed us to classify them into three groups based on the genetic interactions detected among them (Serrano-Cartagena *et al.*, 2000).

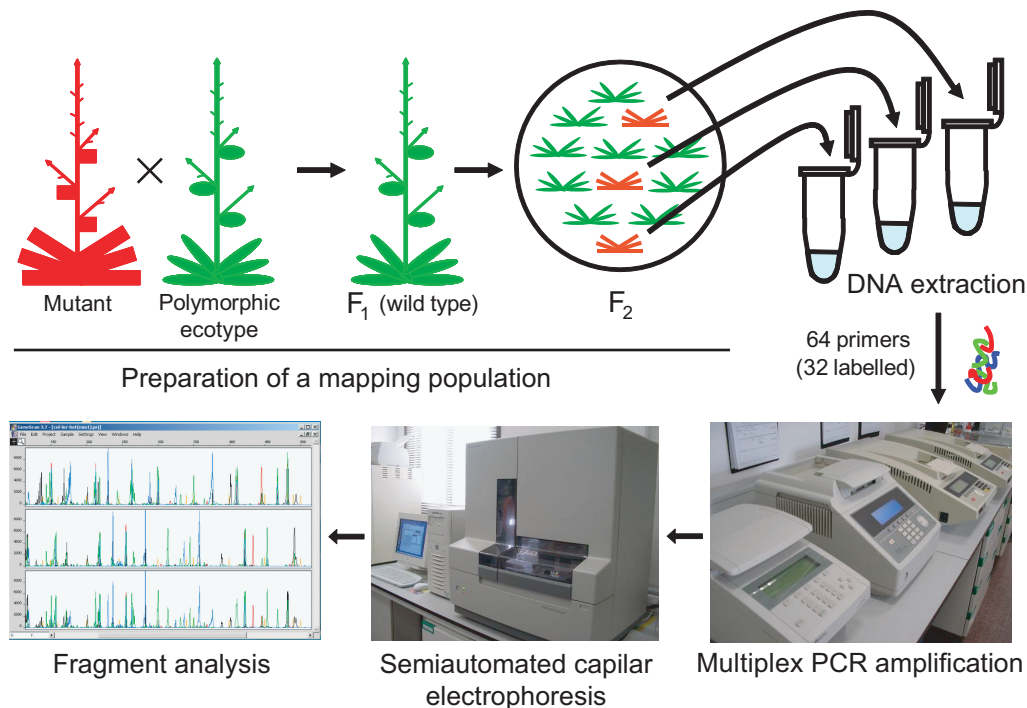
In addition to experimentally induced mutants, we also used natural allelic variation to study the genetic basis of leaf morphogenesis. We screened 188 natural accessions for variations in leaf architecture. These lines were grown under strictly controlled environmental conditions and assigned to 14 phenotypic categories based on petiole length, marginal serration, and overall lamina shape (Pérez-Pérez *et al.*, 2002a). Accessions showing extreme and opposite variations were crossed and their progeny studied. Continuous variation was present in the  $F_2$  progeny, as expected for polygenic traits. Interestingly, leaf shape and size were among the characters initially studied by Mendel but were

finally discarded by him due to difficulties in assigning the observed variation to discrete classes (Lewis, 2001). Our results suggested that diverse leaf morphology in *Arabidopsis thaliana* results from natural allelic variation at quantitative trait loci (QTL). These observations prompted us to characterize leaf morphology in two recombinant inbred line (RIL) populations, derived from *Ler-0* x *Col-4* and *Ler* x *Cvi* crosses, in an attempt to detect, map, and estimate the effects of the QTL responsible for the differences between natural accessions (Pérez-Pérez *et al.*, 2002a; Juenger *et al.*, 2005). We detected 16 and 13 QTL affecting highly correlated leaf morphological traits in juvenile and adult leaves, respectively, using the *Ler* x *Col-4* RIL population, and three additional QTL for juvenile leaf traits using the *Ler* x *Cvi* RIL population. Finding QTL confirmed the multifactorial nature of the observed variation in leaf architecture among *Arabidopsis thaliana* natural accessions.

Leaf venation follows a complex net-like pattern in *Arabidopsis thaliana*. We also screened for natural variants in the venation pattern of the first vegetative leaves in 266 *Arabidopsis thaliana* accessions, finding two, Ba-1 and Ei-5, with abnormal vascular patterns. Ei-5 has an extremely simple pattern that we named Hemivenata and is inherited as a monogenic recessive trait (Candela *et al.*, 1999). Finally, to test if vascular patterning is regulated independently of whole leaf morphogenesis, we screened for mutants with abnormal vein patterns and normal leaf shape and size. More than 6,000 EMS-mutagenized  $M_2$  seeds and 17,313  $T_4$  plants derived from 1,800 independent T-DNA transformants were screened but only one mutant was found, *extrahydathodes* (*ehy*), characterized by the presence of an increased number of hydathodes per leaf (Candela *et al.*, 1999). These results suggest a direct relationship between leaf venation patterning and leaf morphogenesis in *Arabidopsis thaliana*.

### High-throughput gene mapping as an alternative to massive complementation analysis

From our screens for leaf mutants using either EMS or fast neutrons as mutagens, and those mutants obtained by other authors, we ended up with 94, 8, and 37 genes, respectively (see above). As an alternative to massive crossing for testing the allelism of mutations affecting leaf development, we developed a high-throughput mapping procedure based on simultaneous PCR coamplification, fluorescent semiautomated detection, and sizing of several microsatellite markers (Ponce *et al.*, 1999), which



**Fig. 4. Schematic representation of our high-throughput gene mapping method.** To assess the linkage of a given phenotype to polymorphic molecular markers, the mutant line was first outcrossed to the Columbia-0 accession and the  $F_2$  plants displaying the recessive phenotype were harvested for their DNA extraction. From each individual  $F_2$  plant, DNA was amplified in a single-tube multiplex PCR as described in Ponce *et al.* (1999, 2006). The amplification products were electrophoresed in an ABI PRISM 3100 and sized with fragment analysis software.

allowed us to assign map positions to 97 of these genes (Robles and Micol, 2001). Allelism tests were subsequently performed only for closely linked mutations.

A major step in the genetic characterization of a mutant is the molecular identification of the corresponding gene affected by the mutation. Mutations tagged by insertional mutagenesis often facilitate the recovery of the genomic sequences flanking the insertional element. A drawback of this approach is that insertional mutagenesis usually yields null alleles that might be lethal if homozygous. In contrast, chemical mutagenesis is more efficient and usually produces viable hypomorphic and antimorphic alleles (Noveroske *et al.*, 2000).

Map-based cloning is a classic approach for the molecular identification of a gene when a molecular tag is not available. It involves genotyping the individuals of an  $F_2$  mapping population to identify recombination events that occurred in  $F_1$  hybrids derived from an outcross involving the mutant of interest (Fig. 4). With the completion of the genome sequence and the availability of enough molecular markers to cover the entire genome, map-based cloning emerged as a powerful approach to identifying genes altered by mutation in many experimental organisms, including plant species other than *Arabidopsis thaliana* (Bortiri *et al.*, 2006). The number of polymorphic markers and  $F_2$  individuals needed to detect unambiguous linkage between the mutation under study and a linked genetic marker is proportional to genome size. In *Arabidopsis thaliana*, with a genome of 500 cM (125 Mb) distributed on five chromosomes, a minimum of 17 markers regularly spaced every 30 cM and a mapping population of 15-20

F<sub>2</sub> plants are required to significantly detect linkage between a mutation under study and at least one marker (Ponce *et al.*, 1999). Several high-throughput but low-resolution mapping approaches have been developed in *Arabidopsis thaliana*: PCR-based mapping with simple sequence length polymorphism (SSLP) markers, either alone (Ponce *et al.*, 1999) or combined with small insertions and deletions (Jander *et al.*, 2002; Ponce *et al.*, 2006), with amplified fragment length polymorphism (AFLP) markers (Peters *et al.*, 2004), or with high-density oligonucleotide array hybridizations (Spiegelman *et al.*, 2000; Gong *et al.*, 2004). The last approach has been useful for mapping moderately large deletions such as those induced by physical mutagens.

Based on our experience, successful positional cloning of *Arabidopsis thaliana* genes starts with low resolution mapping of a mutation using about 50 F<sub>2</sub> mutant homozygotes (Ponce *et al.*, 1999; Robles and Micol, 2001). Subsequently, if no obvious candidate gene is found in the genomic region, the mutation is fine mapped using 400-1000 additional F<sub>2</sub> individuals. Once the F<sub>2</sub> mapping population is ready, less than 2 weeks are required for low-resolution mapping and less than 2 months for delimiting a 100-200-kb candidate interval (Ponce *et al.*, 2006).

### Genes cloned, molecular nature of their products, and processes in which they are involved

The map positions obtained with the high-throughput method outlined above (Ponce *et al.*, 1999; 2006) have led to the positional cloning of genes identified in our screens (Berná *et al.*, 1999; Serrano-Cartagena *et al.*, 1999; Robles and Micol, unpublished). This is the case for 25 of the 97 mutations we initially mapped, including 86 induced by EMS, nine taken from the AIS collection, one induced by fast neutrons, and one of spontaneous origin (Robles and Micol, 2001). The cloning and functional characterization six of these genes [*ICU6*, *ICU8*, *ICU9*, *ICU15*, *RON1*, and *RUGOSA2* (*RUG2*)] is described in manuscripts currently in preparation. We recently cloned another six genes [*TRANSCURVATA1* (*TCU1*), *ONDULATA3* (*OND3*), *RUG1*, *VENOSA1* (*VEN1*), *VEN4*, and *VEN5*], which are under study in our laboratory.

We published, in some cases in collaboration with other

groups, the cloning and functional characterization of 13 genes that affect leaf development (Table 2). The first two genes that we cloned, *UCU1* and *UCU2*, encode proteins involved in the signal transduction of brassinosteroids (BR), steroid hormones that control growth processes such as cell expansion, vascular differentiation, and reproductive development. The *ucu1* and *ucu2* mutants resemble BR-biosynthesis or BR-perception mutants (Fig. 3S): dwarf plants with roots, hypocotyls, stems, and fruits shorter than in the wild type (Pérez-Pérez *et al.*, 2002b; 2004). *UCU1* [also called BRASSINOSTEROID INSENSITIVE2 (*BIN2*); Li and Nam, 2002] is a member of the SHAGGY/GSK3 family of intracellular protein kinases, which negatively regulate several signal transduction pathways in metazoans, such as those triggered by insulin or the Wingless/Wnt signal (Cohen and Frame, 2001). In *Arabidopsis thaliana*, *UCU1* may act as a negative modulator of the BR transduction pathway and likely in the crosstalk of the BR and auxin signaling pathways (Pérez-Pérez *et al.*, 2002b). *UCU2* encodes a peptidyl-prolyl cis/trans-isomerase of the FK506-binding protein family involved in protein activation. In addition to BR signaling, *UCU2* might also be required for the transport of auxin and, like *UCU1*, *UCU2* plays an important role in the overlap between BR and auxin signaling pathways during leaf expansion (Pérez-Pérez *et al.*, 2004).

In collaboration with Mieke van Lijsebettens' group, *RON2* was identified as the same gene as *LEUNIG* (*LUG*; Conner and Liu, 2000), which encodes a WD40 repeat protein similar to transcriptional co-repressors in yeast, *Drosophila*, and humans (Cnops *et al.*, 2004). *RON2* seems to act at the late stages of leaf development and may control leaf growth through restriction of cell expansion. Perturbation of *RON2* activity causes an increase in leaf cell size, as observed in the *ron2* mutants, which display wider laminae and longer petioles than the wild type (Cnops *et al.*, 2004). *ELO1*, *ELO2*, and *ELO3* are orthologs of the yeast genes *ELP4*, *ELP1*, and *ELP3*, respectively, whose products form part of the Elongator complex (Nelissen *et al.*, 2005), a histone acetyl transferase that co-purifies with RNA polymerase II and facilitates the passage of the polymerase along the template during transcription elongation (Otero *et al.*, 1999; Hawkes *et al.*, 2002). The *ELO4* gene is the same gene as *DEFORMED ROOTS AND LEAVES1* (*DRL1*), an ortholog of the yeast *TOT4/KTI12* gene

TABLE 2

#### GENES IDENTIFIED BY POSITIONAL CLONING USING MUTANTS ISOLATED IN OUR LABORATORY

Mutants	Genes affected	Alternative gene names	Function of the gene cloned	Developmental process
<i>ultracurvata1</i> ( <i>ucu1</i> )	<i>UCU1</i> <sup>1</sup>	<i>BIN2</i> <sup>2</sup>	Signal transduction of brassinosteroids and auxin	Polar cell expansion
<i>ultracurvata2</i> ( <i>ucu2</i> )	<i>UCU2</i> <sup>3</sup>	<i>TWD1</i> <sup>4</sup>	Signal transduction of brassinosteroids and auxin	Polar cell expansion
<i>rotunda2</i> ( <i>ron2</i> )	<i>RON2</i> <sup>5</sup>	<i>LUG</i> <sup>6</sup>	Transcriptional co-repressor	Restriction of leaf cell expansion
<i>elongata</i> ( <i>elo1-elo4</i> )	<i>ELO1-ELO3</i> <sup>7</sup>		Elongation of nuclear gene transcription	Leaf lateral expansion
	<i>ELO4</i> <sup>8</sup>	<i>DRL1</i> <sup>8</sup>		
<i>reticulata</i> ( <i>re</i> )	<i>RE</i> <sup>9</sup>	<i>LCD1</i> <sup>10</sup> , <i>VEN2</i> <sup>11</sup>	Unknown function	Differential contribution of the epidermal and internal tissues to leaf organogenesis
<i>incurvata4</i> ( <i>icu4</i> )	<i>ICU4</i> <sup>12</sup>	<i>CNA</i> <sup>13</sup>	Transcription factor	Meristem activity
<i>scabra3</i> ( <i>sca3</i> )	<i>SCA3</i> <sup>14</sup>		Chloroplast gene transcription	Relationship between plastid biogenesis and leaf organogenesis
<i>hemivenata</i> ( <i>hve</i> )	<i>HVE</i> <sup>15</sup>	<i>CAND1</i> <sup>16</sup> , <i>ETA2</i> <sup>17</sup>	Ubiquitination	Venation pattern formation
<i>angusta4</i> ( <i>ang4</i> )	<i>ANG4</i> <sup>18</sup>	<i>HUB1</i> <sup>19</sup>	Histone ubiquitination	Cell cycle regulation
<i>incurvata2</i> ( <i>icu2</i> )	<i>ICU2</i> <sup>20</sup>		DNA replication	Chromatin-mediated cellular memory

<sup>1</sup>Pérez-Pérez *et al.*, 2002b. <sup>2</sup>Li and Nam, 2002. <sup>3</sup>Pérez-Pérez *et al.*, 2004. <sup>4</sup>Geisler *et al.*, 2003. <sup>5</sup>Cnops *et al.*, 2004. <sup>6</sup>Conner and Liu, 2000. <sup>7</sup>Nelissen *et al.*, 2005. <sup>8</sup>Nelissen *et al.*, 2003. <sup>9</sup>González-Bayón *et al.*, 2006. <sup>10</sup>Barth and Conklin, 2003. <sup>11</sup>Berná *et al.*, 1999. <sup>12</sup>Ochando *et al.*, 2007. <sup>13</sup>Green *et al.*, 2005. <sup>14</sup>Hricová *et al.*, 2006. <sup>15</sup>Alonso-Peral *et al.*, 2006. <sup>16</sup>Cheng *et al.*, 2004. <sup>17</sup>Chuang *et al.*, 2004. <sup>18</sup>Fleury *et al.*, 2007. <sup>19</sup>Liu *et al.*, 2007. <sup>20</sup>Barrero *et al.*, 2007.

putatively involved in the regulation of the Elongator complex (Nelissen *et al.*, 2003). Perturbation of the *ELO* genes causes a reduction in overall cell division rate that explains the delay in germination and the retarded seedling and primary root growth observed in *elo* mutants (Fig. 3H). The product of *HISTONE MONOUBIQUITINATION1* (*HUB1*; initially named *ANGUSTA4* [*ANG4*]), is a functional homolog of yeast and human histone H2B-monoubiquitinating BRE1 E3 ligases (Fleury *et al.*, 2007; Liu *et al.*, 2007), which are associated with chromatin modifications and transcriptional activation. Mutations in *HUB1* affect plant growth and cause reduced plant size, narrow leaves (Fig. 3I), and a reduction in root growth rate, as a consequence of a block in the G2-to-M transition and a prolonged duration of the cell cycle, as also revealed by microarray analyses (Fleury *et al.*, 2007). *HUB1* controls gene activation and cell cycle regulation in leaf cells through chromatin modifications.

Together with Antonio Martínez-Laborda's group, we found that the *ICU4* gene, also known as *CORONA* (*CNA*; Green *et al.*, 2005), encodes the HD-ZIPIII transcription factor ATHB15 (Ochando *et al.*, 2006). The transcripts of *ICU4* and other HD-ZIPIII genes have a microRNA (miRNA) complementary site and are under post-transcriptional regulation. The semidominant *icu4-1* and *icu4-2* alleles carry identical nucleotide substitutions at the miRNA165/166 complementary site of *ICU4*. These mutations lead to defective cleavage and accumulation of *ICU4* transcripts in semidominant *icu4* mutants. Phenotypic studies revealed that *ICU4* plays an adaxializing role in leaves, and is required for shoot meristem function and for the patterning of shoot vascular bundles (Ochando *et al.*, 2006).

The *scabra3* (*sca3*) mutant, with characteristic pale green leaves, shares with *re* mutants a marked decrease in mesophyll cell number. The SCA3 protein corresponds to the nuclear encoded plastid-targeted RNA polymerase RpoTp, which controls the expression of a subset of plastid genes (Hricová *et al.*, 2006). Strong loss-of-function *sca3* alleles affect the number, size and morphology of chloroplasts, and perturb the expression of both plastid and nuclear genes, many encoding chloroplast-targeted proteins (Hricová *et al.*, 2006). Our analyses of *sca3* mutants demonstrate the intertwining of plastid biogenesis and leaf organogenesis.

The *hemivenata-1* (*hve-1*) recessive allele was isolated in a search for natural variants of the *Arabidopsis thaliana* leaf venation pattern (Candela *et al.*, 1999). *hve-1* reduces the complexity of the venation pattern in leaves and cotyledons. The *HVE1* gene encodes the *Arabidopsis thaliana* ortholog of the mammalian CAND1 protein (Alonso-Peral *et al.*, 2006), which binds to unneddylated CULLIN1 (CUL1), a protein that forms part of the SCF ubiquitin ligase complexes that regulate target protein degradation through the 26S proteasome. Similar venation patterns in *auxin resistant1* (*axr1*), a mutant insensitive to auxin, and *hve* mutants indicate that auxin signaling mediated by ubiquitination is required for venation patterning in *Arabidopsis thaliana* leaves (Alonso-Peral *et al.*, 2006).

*INCURVATA2* (*ICU2*) encodes the putative catalytic subunit of the DNA polymerase  $\alpha$  of *Arabidopsis thaliana* (Barrero *et al.*, 2007). Genetic and molecular interactions between chromatin remodeling factors and components of the DNA replication machinery have been identified in yeast. The weak *icu2-1* allele causes early flowering, leaf incurvature, and homeotic transfor-

mations in flowers, traits that can be attributed to derepression of flowering time, floral meristem, and floral organ identity genes, respectively. Our genetic analyses indicate that *ICU2* interacts with *TERMINAL FLOWER2*, the ortholog of *HETEROCHROMATIN PROTEIN1* of animals and yeasts (Takada and Goto, 2003), and with the Polycomb group (PcG) gene *CURLY LEAF* (Goodrich *et al.*, 1997). Our results suggest the existence in plants of a cellular memory mechanism that involves DNA polymerase  $\alpha$ , as proposed by previous authors for the fission yeast.

Contrary to all genes discussed above, no clear function could be assigned to the *RETICULATA* (*RE*) gene (González-Bayón *et al.*, 2006), named *LOWER CELL DENSITY1* (*LCD1*) by Barth and Conklin (2003). The *RE* gene is ubiquitously expressed, and the RE protein has been identified in the chloroplast proteome (Dunkley *et al.*, 2006). The *re* mutant was initially isolated by Rédei and Hirono (1964), and used as a classic phenotypic marker for mapping for many years. Our histological characterization of *re* mutants shows a significant reduction in mesophyll cell density but little effects on leaf shape, indicating that *RE* is normally required for the development of internal leaf tissues (González-Bayón *et al.*, 2006). These results were obtained in collaboration with the group of Kevin Pyke and suggest that the epidermis plays a major role in controlling leaf shape in *Arabidopsis thaliana*, whereas the division and expansion of the internal tissues appear to be secondary in this process, as in tobacco leaves (Avery, 1933). Furthermore, *re* and wild-type leaves have similar shapes, suggesting that the epidermis plays a major role in controlling leaf shape.

## Concluding remarks

From our EMS screen in the crucifer *Arabidopsis thaliana*, we estimate that about 130 genes [ $94 \times 100 / (100 - 27.8)$ ] yield morphological leaf phenotypes and hence are required for normal leaf ontogeny. However, this value underestimates the real number of genes because it is based on an average number of alleles per gene (1.27) that did not consider genes not identified in the screen. Estimates that take them into account are expected to be more accurate. By applying the formulae of Rice (1973), Meneely and Herman (1979), and Engels (1981) to our data, we obtain values of 357, 279, and  $233 \pm 39$  genes, respectively. An immediate conclusion from these results is that a significant fraction of the genome must work cooperatively to produce a leaf. These values only refer to those genes that can mutate to viable leaf shape phenotypes, and represent a lower limit that does not take into account mutations with pleiotropic effects that prevent their transmission, such as those that cause sterility or are lethal. For example, genes essential for developmental decisions in the embryo may also act at later stages, but the study of their postembryonic functions will only be possible using special techniques, such as clonal analysis.

Mutations in genes required for the establishment of shoot meristem and leaf identities have been extensively studied in plants. In maize, ectopic expression of class I *KNOX* genes in dominant *Knotted1* and *Rough sheath1* mutants causes abnormal cell proliferation and differentiation patterns (Hake *et al.*, 1989; Schneeberger *et al.*, 1995). The *rough sheath2* gene of maize (Tsiantis *et al.*, 1999) and its orthologs *PHANTASTICA* (*PHAN*, Waites *et al.*, 1998) and *ASYMMETRIC LEAVES1* (*AS1*;



Byrne *et al.*, 2000) from *Antirrhinum majus* and *Arabidopsis thaliana*, respectively, encode transcription factors that downregulate class I *KNOX* genes in leaves (Hake *et al.*, 2004). Other genes such as *SERRATE* (*SE*; Grigg *et al.*, 2005) and *AS2* (Iwakawa *et al.*, 2002) also participate in the *KNOX* regulatory pathway in leaf primordia, helping to establish their identity as lateral organs. Our leaf mutant screens identified new alleles of *AS1* (Fig. 3B), *AS2*, and *SE*, whose molecular characterization may help to define functional domains in the corresponding proteins.

We deliberately excluded those mutations that cause normal leaf shape and altered phyllotaxy because they might affect genes that regulate aspects of leaf initiation but are not directly involved in leaf morphogenesis. However, other genes known to be important for leaf organogenesis are not represented in our collection of mutants. Indeed, our screens failed to identify alleles of some genes crucial for leaf polarity, such as members of the *YABBY* and *KANADI* families (reviewed by Byrne, 2005), probably because these families have functionally redundant members.

We failed to identify new alleles of the *ANGUSTIFOLIA* (*AM*) and *ROTUNDIFOLIA* (*ROT*) genes, whose mutations specifically affect cell expansion along the proximodistal and mediolateral axes of the leaf, respectively (Tsuge *et al.*, 1996). Despite previous authors have reported compensatory cell enlargement in the leaves of mutants affected in cell proliferation (reviewed by Tsukaya, 2003), we have not observed such a compensation in the mutants characterized so far at the cellular level in our laboratory. Further histological characterization of our collection of leaf mutants may identify some that undergo similar compensation.

The leaves of the *cincinnata* (*cin*) mutant of *Antirrhinum majus* are crinkly (instead of flat) due to excessive growth of marginal tissues. *CIN* encodes a transcription factor of the TCP family (Nath *et al.*, 2003). Analysis of the dominant *jaw* mutant of *Arabidopsis thaliana* revealed that expression of TCP genes in leaf margins is regulated by miRNAs, which are also required for leaf flatness (Palatnik *et al.*, 2003). No alleles of TCP genes or of the *JAW* miRNA gene have been identified among our mutants. However, we found alleles of genes that encode components of the miRNA pathway (Ponce and Micol, unpublished), including *HASTY* (Park *et al.*, 2005), *ARGONAUTE1* (Kidner and Martienssen, 2004), *HUA ENHANCER1* (*HEN1*; Park *et al.*, 2002), and *HYPONASTIC LEAVES1* (*HYL1*; Han *et al.*, 2004), as well as a miRNA target, *ICU4* (Ochando *et al.*, 2006).

The cloning of 25 genes identified by mutation in our laboratory shows that many unrelated processes converge in leaf morphogenesis. It is remarkable that, when classified according to function, 15 of the cloned genes encode regulators of gene expression. This enrichment suggests that mutations in genes necessary for basic metabolic functions, which are also expected to yield visible leaf phenotypes, are selected against at the haploid gametophytic phase or at early stages of the diploid phase of the life cycle, soon after fertilization. Furthermore, all the mutants that we have studied in detail display pleiotropic phenotypes in other organs, such as the flowers; in agreement with Goethe's classic hypothesis that all plant laminar organs are modified leaves.

As noted by Jürgens (1992), the identification of all genes that participate in a given biological process is necessary to under-

stand how the process works. A genetic dissection normally begins with the isolation of mutations perturbing the normal course of development, to identify the genes at work in the process under study. A high-throughput genetic screen for mutants with altered leaf size and shape aiming to understand the mechanisms of leaf-size control was recently published (Horiguchi *et al.*, 2006a; 2006b). A comparative study between these two large collections of mutants should help to identify most of the genes involved in leaf growth and development in *Arabidopsis thaliana*. The positional cloning of untagged mutations in model organisms such as *Arabidopsis thaliana* has proved a very fruitful approach even though it occasionally yields genes with very limited novelty or involved in totally unexpected functions. We have shown that the range of leaf phenotypes identified in our laboratory results from perturbation of a variety of unrelated gene functions. Combined with elegant work by other authors, our results have begun to reveal the outstanding complexity of leaf development. The molecular identification of additional genes uncovered by mutation will provide a better picture of how leaves develop and is expected to help explain the immense diversity in leaf shape and leaf size present in nature.

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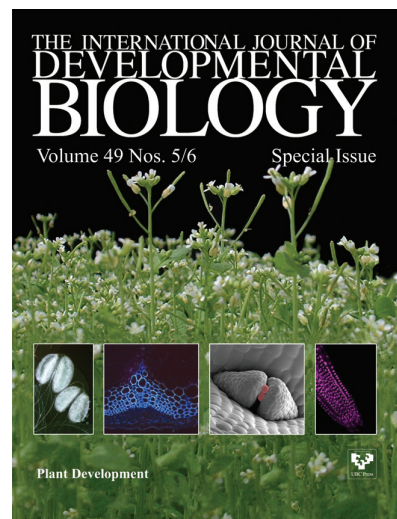
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