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A Mutational Analysis of Leaf Morphogenesis in *Arabidopsis thaliana*

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ABSTRACT

As a contribution to a better understanding of the developmental processes that are specific to plants, we have begun a genetic analysis of leaf ontogeny in the model system *Arabidopsis thaliana* by performing a large-scale screening for mutants with abnormal leaves. After screening 46,159 M_2 individuals, arising from 5770 M_1 parental seeds exposed to EMS, we isolated 1926 M_2 putative leaf mutants, 853 of which yielded viable M_3 inbred progeny. Mutant phenotypes were transmitted with complete penetrance and small variations in expressivity in 255 lines. Most of them were inherited as recessive monogenic traits, belonging to 94 complementation groups, which suggests that we did not reach saturation of the genome. We discuss the nature of the processes presumably perturbed in the phenotypic classes defined among our mutants.

THE development of dicotyledonous plant leaves has received little attention in the way of causal analysis (reviewed in Telfer and Poethig 1994; Tsukaya 1995; Hall and Langdale 1996; Poethig 1997; Brutnell and Langdale 1998; Van Lijsebettens and Clarke 1998). In fact, the identification of genes that act as developmental controls in leaf ontogeny is problematic for two reasons. On the one hand, the inexistence of analogous phenomena in animals forces us to discard strategies based on gene cloning by homology. On the other hand, two important processes that take place in the leaf, photosynthesis and the exchange of gases with the environment, both require the participation of a large number of gene products, many of them presumably absent or poorly represented in other plant organs. Hence, a search for genes involved in the control of leaf morphogenesis by attempting to isolate gene products spatially restricted to this organ would identify those related to the execution of leaf functions rather than to the developmental mechanisms responsible for leaf architecture. As a result of the above, the spectrum of possible experimental approaches that can be used to identify the genes that control leaf morphogenesis is almost inevitably restricted to the identification and study of mutants.

Saturation mutagenesis is the term given to experiments intended to isolate mutant alleles in every gene affecting a particular developmental phenomenon (Wilkins 1993). Such a genetic approach to the study of ontogeny was pioneered by Waddington (1940) to analyze wing morphogenesis in *Drosophila melanogaster* and later allowed the understanding of the process of segmentation in this insect (Nüsslein-Volhard and Wieschaus 1980). The method continues to show its usefulness, since it has been successfully applied to animals and plants, in analyzing embryonic development of both the fish *Danio rerio* (Haffter *et al.* 1996) and the crucifer *Arabidopsis thaliana* (Jürgens *et al.* 1991).

We are interested in the study of leaf ontogeny as a model for answering general questions on pattern formation. To this end, we have isolated and analyzed *A. thaliana* variants showing abnormal venation pattern, marginal configuration, shape, or size of their vegetative leaves. Such alternatives in leaf architecture have been identified among wild-type strains, usually termed ecotypes (Candela *et al.* 1999; J. Serrano-Cartagena, J. M. Pérez-Pérez and J. L. Micol, unpublished results), mutants from existing collections (Serrano-Cartagena *et al.* 1999; J. Serrano-Cartagena, H. Candela, P. Robles and J. L. Micol, unpublished results), and new mutants (this work; P. Robles and J. L. Micol, unpublished results). As a contribution to the causal analysis of plant leaf development, we first performed a large-scale screen for EMS-induced mutants with aberrantly shaped or sized leaves. Our intention was to isolate as many mutants as possible showing abnormal leaves (1) to define the spectrum of possible perturbations in leaf morphogenesis, (2) to determine the number of genes whose mutations disturb the process, and (3) to choose for further studies those mutants whose phenotype and/or genetic interactions with others would suggest they are involved in the control of leaf development.

MATERIALS AND METHODS

Plant materials and growth: The M_2 *A. thaliana* (L.) Heyhn. seeds used for mutant screens were derived from mutagenesis of the ecotype Landsberg *erecta* with ethyl methanesulfonate

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TABLE 1

Quantitative profile of the search for EMS-induced mutants with abnormal rosette leaves

Screening experiment	Parental group	M_2 seeds sown	Early lethals		Putative mutants with abnormalities in leaf form and/or rosette structure				Phenotypic classes found in each sowing	
			No germination	Albino	Total	Fertile	Sterile	Lethal	Total	New
1	P1	5,255	904	ND	132	33	40	59	30	30
2	P2	5,955	1,480	177	261	95	83	83	40	17
3	P7	6,141	898	136	324	100	127	97	52	15
4	P8	5,998	1,398	122	323	122	140	61	52	9
5	P9	5,556	1,507	108	268	131	98	39	54	9
6	P10	5,637	1,263	125	164	75	42	47	45	4
7	P12	5,591	1,641	116	234	131	48	55	49	1
8	P14	6,026	1,781	101	220	166	30	24	42	0
Total		46,159	10,872	885	1,926	853	608	465		85

ND, not determined.

(EMS; to the 0.2% v/v during 12 hr at 23°), carried out by a commercial supplier, Lehle Seeds (catalog number M2E-4-2). Wild-type seeds were also provided by Lehle Seeds. Both sterile (in Petri dishes) and nonsterile (in pots containing a 1:1:1 mixture of perlite, vermiculite, and sphagnum moss) cultures were performed at 20 ± 1° under continuous illumination of 7000 lux as described in Ponce *et al.* (1998).

Conditions of the mutant screening: Seeds were sown by Pasteur pipette in a water suspension, in 150-mm Petri dishes containing 100 ml of solid culture medium, at a density of 100 regularly spaced seeds per plate. Growth was at 20 ± 1° and 60–70% relative humidity in Conviron (Winnipeg, Canada) TC16 tissue culture chambers under constant fluorescent light (7000 lux). These growth conditions allowed us to observe, 15 days after sowing, the architecture of the rosette and of its leaves as well as to identify putative mutants on the basis of their morphological differences from the wild-type individuals. The selection of mutants was carried out after observation of the M_2 plants, eliminating, 20 days after sowing, all those plantlets showing no clear differences from the ecotype L*er*. All putative mutants were transplanted to soil between 3 and 5 wk after sowing.

RESULTS

Isolation of putative mutants: M_2 seeds were sown and mutants sought that showed macroscopic alterations in the architecture of vegetative leaves and/or in their arrangement on the stem. Putative mutants were assigned to phenotypic classes, the number of new classes being determined after each screening experiment (Table 1). The two right-hand columns of Table 1 show that the number of phenotypic classes from each of the sowings varied from 30 to 54 and that the number of new phenotypic classes dropped from 30 in the first screening experiment to 0 in the eighth, suggesting that we were near saturation.

We sowed a total of 46,159 M_2 seeds, which originated from 5770 M_1 parental lines mutagenized with EMS. Of the M_2 seeds sown, 23.5% did not germinate or presented abortive germination against a 2.7% rate of wild-type L*er* control sowings. Together with this high percentage of early lethals, the appearance of a high number of albino plants (fifth column from the left in Table 1) indicated the efficiency of the mutagenesis.

Phenotypic classification of putative mutants: The strategy followed for the isolation and characterization of mutants is summarized in Figure 1. First, we phenotypically classified the 1926 isolated putative leaf mutants (4.2% of M_2 plants) that survived at least 3 wk after germination into three broad categories, corresponding

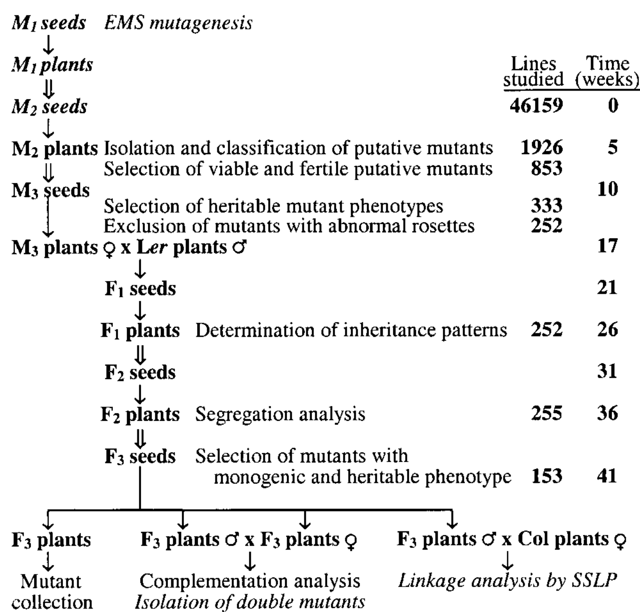

Figure 1.—Steps followed for the isolation and genetic analysis of mutants. The symbols → and ⇒ indicate, respectively, growth and selfing. The time scale refers to each single mutant line. Tasks indicated in italics were not performed by the authors (EMS mutagenesis accomplished by Lehle Seeds) or were initiated in this work but remain to be completed (isolation of double mutants and linkage analysis).

TABLE 2

Assignment of putative M_2 mutants to phenotypic groups

	Phenotypic classes		M_2 putative mutants	
Morphological alteration	Total	Fertile	Total	Fertile
Proportions or size of lamina				
Rounded	6	6	105	54
Broad	1	1	8	6
Small	4	3	86	17
Deformed	4	1	37	2
Oval	2	2	22	14
Wrinkled or undulated	4	3	87	54
Narrow	12	9	201	66
Pointed or lanceolate	5	4	177	81
Leaf margin				
Incised	7	7	279	183
Serrated	7	5	149	97
Curled	7	7	143	66
Rosette structure				
Disorganized	7	5	231	67
Compact	15	14	375	128
Erect	3	2	24	17
Loose	1	1	2	1
Total	85	70	1926	853

to alterations (1) in the proportions or size of the lamina, (2) in the form of the margin, and (3) in the structure of the basal rosette, which is dependent upon the length of the petiole of the leaves and their phyllotaxis. Each group was subdivided into other more specific groups, as presented in Table 2, that were further subdivided into several phenotypic classes, whose total number reached 85. We provisionally assigned the putative mutants to only one class according to their most conspicuous trait, although many of them presented more than one alteration.

Study of the heritability of the mutant phenotypes: More than half of the putative mutants bore lethal-effect mutations, dying before completing their life cycle, or were sterile. They were only studied to obtain photographs of their phenotypes. About 30 M_3 inbred seeds were sown from each of 853 putative mutants yielding viable progeny to ascertain the heritability of their phenotypes. Only in 333 lines was transmission of the phenotype unequivocally Mendelian, without variations in expressivity or incomplete penetrance. Among the remaining 520 families, which received no further attention, 135 showed only subtle phenotypes that were considerably weaker than those of the M_2 parents, variable expressivity was observed in 109 cases, and no mutant phenotype was observed in the M_3 progeny of the remaining 276 lines.

Determination of the inheritance patterns of mutant phenotypes: Among the 333 M_2 mutant lines that presented a stable phenotype, 81 displayed alterations in the structure of the basal rosette. These strains were not studied further, since it was considered that their mutations disturbed leaf phyllotaxis, leaf primordium initiation, or petiole elongation, aspects of leaf development beyond the scope of this work. One or more M_3 individuals from each of the remaining 252 lines were testcrossed to wild type to determine the inheritance pattern in the F_1 progeny. About 30 F_1 individuals were analyzed from each testcross. We found 245 cases of recessive pattern of inheritance, 6 of semidominance (all F_1 individuals displayed a mutant phenotype weaker than their M_3 parental), and only 1 of complete dominance (all F_1 individuals were indistinguishable from their M_3 parental; Table 3).

Identification of lines displaying monogenic mutant phenotypes: About 100 seeds of each F_2 family were sown to study the segregation of the mutant phenotype (Table 3). Two or more different mutant phenotypes were found in some F_2 populations, which demonstrated the existence of several mutations in their M_2 ancestor. Thirteen new mutations affecting leaf morphology were found in this way, 3 of which were studied, thus increasing to 255 the number of lines under study. Finally, three F_2 individuals displaying a phenotype identical to that of their M_2 and M_3 ancestors were chosen from each mutant line, with the aim of studying 100 of their F_3 inbred descendants. We chose a total of 153 lines with a fully heritable mutant phenotype and similar M_2, M_3, and F_3 individuals for subsequent studies. The phenotypes of 122 of these mutant lines were monogenic and recessive, 6 being semidominant and 1 completely dominant (Table 3). Of the remaining lines, 23 displayed unequivocally recessive mutant phenotypes but had F_2 phenotypic proportions that deviated significantly from the 3:1 ratio. However, we decided to include these lines in further studies, because the underrepresentation of the putative homozygous recessive individuals in the F_2 progeny might have been due to certation (Koornneef 1994), to the poor viability of their embryos compared with the heterozygous and wild-type homozygous, or to an insufficiently large sample size. The 15:1 and 9:7 phenotypic segregations observed in some F_2 populations might also be explained as a consequence of epistasis: in the first case the concurrence of two mutations, with no phenotypic effect in themselves, being responsible for the mutant phenotype; in the second the presence of at least one wild-type allele at each of two genes being essential for wild-type leaf morphology.

Complementation analysis: To simplify allelism testing, we defined a series of phenotypic classes (Table 4 and Figure 2), limiting the crosses to lines within a class. This decision was based on the assumption that most mutant alleles of a gene cause similar phenotypes, which can sometimes be put into a series, especially when they correspond to hypomorphic mutations that cause different degrees of loss of function. Consequently, it is reasonable to assume that mutants with similar pheno-

TABLE 3

Phenotypic segregations found in the genetic analysis of mutants with abnormal leaves

Phenotypic classes	Phenotype of mutants	Phenotypic segregation (WT:other)	Number of cases
		In the F_1	
	Not determined		3
1	No mutant phenotype		241
1	Heterogeneous		1
1	Like their M_3 parentals		1
1	Weaker than their M_3 parentals		3
2	Like their M_3 parentals	1:1	3
Total			252
		In the F_2	
	Not determined		5
1	No mutant phenotype		41
1	Heterogeneous		17
2	All albino	Several	3
2	Like their M_3 ancestors	3:1	131[a]
2	Like their M_3 ancestors	1:3	1
2	Like their M_3 ancestors	9:7	1
2	Like their M_3 ancestors	15:1	9
2	Like their M_3 ancestors	Other	20
2	Different from their M_3 parentals	15:1	1
3	Like their M_3 ancestors	1:2:1	2
3	Like their M_3 ancestors	9:3:4	5
3	Like their M_3 ancestors	Other	13
4	Like their M_3 ancestors	9:3:3:1	1
4	Like their M_3 ancestors	Other	2
Total			252
		In the F_3	
	Previously discarded		62
	Not determined		15
1	No mutant phenotype		28
1	Homogeneous		147
1	Heterogeneous		3
Total			255

[a] Includes line P12 18.8 (*ven3-4*), in whose F_2 progeny it was not possible to distinguish wild-type individuals from heterozygotes, since the latter showed a very subtle Ven phenotype.

types are candidates to be damaged in the same gene. However, the possibility that two mutations in the same gene determine phenotypes that are so different as to be assigned to different phenotypic classes cannot be excluded.

Figure 2 shows representative examples of the phenotypes of the 19 classes, obtained by grouping several of the 70 fertile classes initially defined (Table 2). The names given to these phenotypic classes are new, with the exception of Asymmetric leaves, which was first used by Rédei and Hirono (1964). For the remaining names we elected Latin words, following Stearn (1995) as closely as possible. In cases involving dominant or semidominant mutations, the complementation criterion was the appearance of wild-type individuals in the F_2 generation.

The 153 mutant lines studied, grouped in 19 phenotypic classes, were assigned to 94 complementation groups (Table 5), 76 of which had a single allele, while 11, 6, and 1 groups had 2, 3, and 4 alleles, respectively. The number of genes affected in those mutants would be <94 if any class included different phenotypic manifestations of damages in a single gene. On the other hand, the possibility that the phenotype of some of the mutants subjected to complementation analysis was due to the concurrent action of mutations in at least two different genes cannot be excluded.

Allelism tests with existing mutants: Some previously described mutants were included in the complementation tests: two nonallelic *asymmetric leaves* mutants, *as1-1* (NASC NW146; Rédei and Hirono 1964) and *as2-1* (ABRC CS3117; Fabri and Schäffner 1994), and five nonallelic mutants displaying involute leaves, the phenotype that we have called Incurvata (Table 5; Goodrich *et al.* 1997; Kim *et al.* 1998a; Telfer and Poethig 1998; J. Serrano-Cartagena, H. Candela, P. Robles and J. L. Micol, unpublished results), three of them recessive, *clf-18*, *icu2*, and *hst-5*, one semidomi-

TABLE 4

Phenotypic classification of EMS-induced mutants with abnormal leaves

Phenotypic class		Most representative traits of each mutant class	Mutant lines		Complementation groups
Full name	Abbreviation		Studied	Subjected to complementation analysis	
Asymmetric leaves	As	Rounded lamina, with some degree of bilateral asymmetry and margins slightly revolute	4	3	1
Rotunda	Ron	Broad and rounded lamina	7	5	3
Rugosa	Rug	Wrinkled lamina	5	5	2
Ondulata	Ond	Undulated lamina	14	5	4
Exigua	Exi	Small and darkened leaves	18	12	8
Orbiculata	Orb	Small, rounded, and yellowish leaves	7	6	2
Elongata	Elo	Narrow and elongated lamina and long petiole	7	6	4
Angusta	Ang	Narrow lamina	7	5	4
Apiculata	Api	Pointed lamina, with slightly incised margins	9	8	7
Denticulata	Den	Pointed lamina, with dentate margins	58	19	17
Angulata	Anu	Yellowish leaves with dentate margins	20	18	12
Erosa	Ero	Rounded lamina, with dentate margins	18	5	3
Scabra	Sca	Rounded and protruded lamina	12	8	5
Venosa	Ven	Conspicuous venation; some lines displaying incised margins	18	14	6
Dentata	Dea	Serrated margins	7	1	1
Serrata	Sea	Small leaves with strongly serrated margins	17	11	4
Transcurvata	Tcu	Margin obliquely revolute	10	7	4
Ultracurvata	Ucu	Lamina spirally rolled downward	4	4	1
Incurvata	Icu	Involute margins	13	11	6
Total			255	153	94

nant, *icu4-2*, and one dominant, *icu5* (NASC N345, N329, N314, N401, and N379, respectively; Bürger 1971).

Several other already described lines, which resembled some of our mutants, were tested for allelism: *revoluta* (*rev*; Talbert *et al.* 1995), displaying large rosette and cauline leaves; *cab underexpressed1* (*cue1*; Li *et al.* 1995), *differential development of vascular associated cells* (*dov1*; NASC N557; Kinsman and Pyke 1998), and *reticulata-1* (*re-1*; NASC NW129; Rédei and Hirono 1964) showing reticulate leaves with veins greener than their interveinal regions; *angustifolia* (*an*; Tsuge *et al.* 1996) with narrow leaves; and *rotundifolia3* (*rot3*; Tsuge *et al.* 1996), with rounded leaves. Crosses were performed involving *rev* and *elo1-elo4* mutants, while *cue1-5*, *dov1*, and *re-1* were crossed by *ven1-ven6.* In addition, *an* was crossed by *ang1-ang4* and *elo1-elo4*, as well as *rot3-1* by *ron1-ron3.* Complementation was found in 32 of these 33 crosses, the only exception being the cross involving *ven2* and *re-1*, which were found to be allelic.

DISCUSSION

To answer the question of how plant leaves are constructed, the most obvious approach involves the identification, characterization, and manipulation of genes that are candidates for controlling leaf development in a model system such as *A. thaliana.* Previous authors have isolated mutants with abnormal leaves in Arabidopsis as well as in other plant species, but only some have subjected their findings to detailed study, leading in a few cases to the molecular characterization of the genes involved in leaf development. This is shown in Table 6, where Arabidopsis genes known to affect leaf morphology are listed, most of them still to be cloned. The situation is similar in other plant systems, such as pea, tobacco, and tomato (see Marx 1987; McHale 1993; Keddie *et al.* 1996; Hofer *et al.* 1997).

The monocot *Zea mays* is, alongside Arabidopsis, the plant species that has received most attention regarding the genetic and molecular analyses of leaf development. The *KNOTTED1* (*KN1*) gene, the first homeobox gene

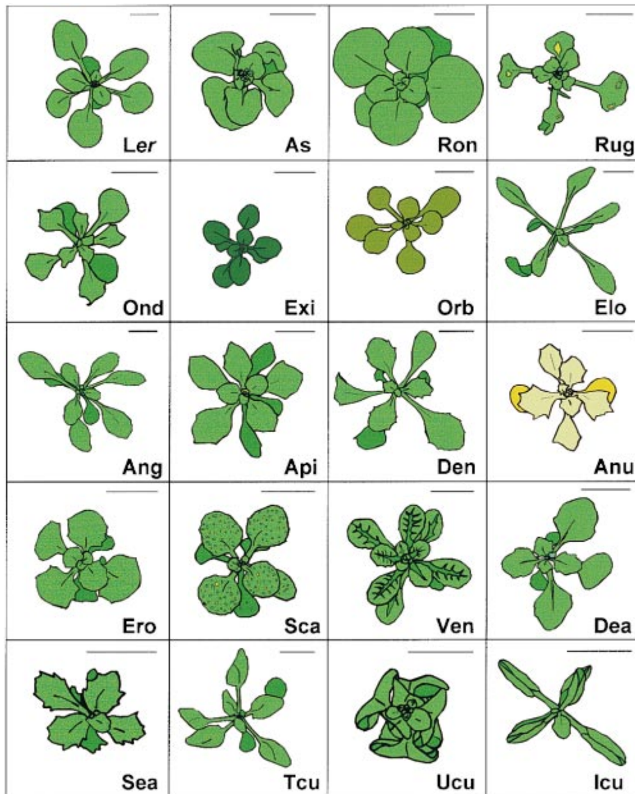

Figure 2.—Diagram showing representative individuals of the phenotypic classes defined for the complementation analysis of mutants with abnormal leaves. Images were obtained from pictures taken 21 days after sowing. Bars, 5 mm.

found in plants, plays an indirect role in leaf development, maintaining the undifferentiated meristematic state (Vollbrecht *et al.* 1991; Sinha *et al.* 1993). The *LIGULELESS* (*LG*) genes code for nuclear proteins involved in the differentiation of the ligule, a concrete region of the leaf that appears between the blade and the sheath (Moreno *et al.* 1997; Muehlbauer *et al.* 1997; Walsh *et al.* 1997). A gene specifically affecting epidermal differentiation is *CRINKLY4* (*CR4*), whose coded protein, a TNFR-like receptor kinase, might be part of a signal transduction chain (Becraft *et al.* 1996).

Usefulness of a screen for Arabidopsis mutants with abnormal leaves: Mutations in genes that control leaf development might have phenotypic effects that vary from lethality to the absence of visible alterations. The lethal alleles would include the most hypomorphic and null mutations, affecting genes with housekeeping functions or those required for initiating the leaf, an essential organ for the plant, or those with products shared by other developmental pathways, such as embryogenesis. A second class would be represented by mutations in genes of redundant functions, which would not cause phenotypes distinguishable from the wild type. After considering these two extreme cases, it is reasonable to assume that mutations in the genes involved in the control of leaf ontogeny might determine viable phenotypes, characterized by alterations in leaf shape or size. Such mutants might express morphological abnormalities in the leaves alone or in other parts of the plant, too, depending on whether or not the damaged morphogenetic controls were leaf specific. If mutations in some of the genes crucial to leaf development cause viable phenotypes, distinguishable from the wild type by leaf architectural traits, a search for such mutants merely based on the observation of plants derived from mutagenesis might be considered worth attempting. This is what we have done in this work. Such mutant screening has the advantage of not necessitating manipulation of the individuals under study, although it is a very slow process, since plants should be studied one by one, under conditions that do not limit or prevent the growth of wild-type individuals. Another disadvantage is that lethal mutations and those mutations affecting genes of redundant functions would be lost in the search for viable and visible mutants.

The criterion used in our search was to distinguish between mutant and wild-type plants simply by observation of structural alterations. Mutants obtained through this procedure should show disturbances in the structure, spatial arrangement and/or division or expansion patterns of their leaf cells. One objection that could be made to this screen procedure is that it might lose all those mutations that, while altering some of the parameters of cell proliferation or differentiation, do not determine any obvious modification in leaf structure. In fact, mutations modifying cell division patterns without disturbing morphogenesis are known. Such is the case of the mutant alleles of *TANGLED-1* (*TAN-1*) in maize (Smith *et al.* 1996), whose deviations in the direction of cell division do not affect leaf shape, and the *ton* (*tonneau*) mutants, in Arabidopsis, in which the planes of cell division are altered while the correct position of all the plant organs is maintained (Traas *et al.* 1995). Therefore, it is reasonable to suppose that leaf morphology does not depend exclusively on cell division patterns and that intercellular communication is one of the processes responsible for the final shape of the organ.

Degree of saturation of the Arabidopsis genome reached in the mutant screening: In a saturation mutagenesis experiment, the number of mutants obtained should be high enough to render negligible the probability of having found no representative alleles for any of the genes involved in the process under study. Assuming that all the genes are equally mutable, the occurrence of mutations in a mutagenesis should follow Poisson's distribution. Consequently, the proportion of genes undetected in a mutant search will be smaller than 5% when the average number of allelic mutations is ≥3 (Jürgens *et al.* 1991). To achieve a proportion of undetected genes of less than 1%, the average number of alleles obtained must be ≥5, which can be possible only in organisms that allow screening of large progenies of

mutagenized individuals, such as Drosophila, Arabidopsis, or the zebra fish.

We subjected a total of 153 mutant lines to complementation analysis, assigning them to 94 complementation groups. Hence, the average number of alleles obtained per gene is $153/94 = 1.6276$. It follows that the class of genes not represented among our mutants would represent almost one-fifth (19.64%) of those that can yield phenotypically abnormal leaves, since $m = 1.6276$, $f(0) = e^{-m} = e^{-1.6276} = 0.1964 = 19.64\%$. On the other hand, we obtained in several cases 2 or more noncomplementing mutant lines with quite similar phenotypes from the same parental group. If such lines represented the isolation of individuals with identical genotypes, the number of different mutations would decrease from 153 to 120, so the average number of alleles per gene would be 1.275, and we would not have identified 27.94% of the genes that we were looking for. In the previous calculations we have assumed that all the studied mutant lines display monogenic phenotypes, including those whose phenotypic segregation in F_2 is significantly different from 3:1 (at least 23 of the 153) and in some instances (7 lines) is very acceptably close to the 15:1 ratio. Nonetheless, we believe that we have covered a substantial part of the Arabidopsis genome with our collection of mutants. The results obtained indicate that the proportion of genes not represented among our mutants might be up to one-third of those that give rise to phenotypes characterized by an abnormal leaf morphology. A more accurate approach to a saturation state of the genome would probably require additional sowings, although the relation between benefit and effort would be slight.

Speculations regarding the function of the genes identified by isolating mutants: The genetic operations at work in organogenesis remain poorly understood in most biological systems. Such an assertion is particularly true when it comes to the study of leaf organogenesis, a field where many questions still await answers. Up to now, leaf development has not been analyzed following a mutational approach involving a large-scale screen as starting point, aimed to define as far as possible the spectrum of genes participating in the process. On the other hand, molecular analyses, until now, have not identified genes controlling the process, with only three possible exceptions: the MYB superfamily member *PHANTASTICA* (*PHAN*; Waites *et al.* 1998), in Antirrhinum, whose participation in the establishment of the dorsoventral asymmetry seems to be required mostly in the leaves, bracts, and petals (Waites and Hudson 1995); *ARGONAUTE1* (*AGO1*; Bohmert *et al.* 1998) in Arabidopsis, whose product, a protein without homologs encoded by unicellular organisms, such as yeasts or bacteria, is required in the entire plant body for correct cell proliferation, its mutation resulting in a pleiotropic phenotype mainly characterized by the absence of lateral expansion in leaves and floral organs; and *ROTUNDIFOLIA3* (*ROT3*; Kim *et al.* 1998b), which encodes for a cytochrome P-450 required for the polar elongation of cells in the longitudinal axis, its mutant alleles determining short petioles and rounded leaves.

When trying to genetically dissect leaf ontogeny in Arabidopsis, we have employed logic similar to that applied by Jürgens *et al.* (1991) with respect to pattern formation in the embryo. These authors attempted to identify most of the genes generating the body plan of the Arabidopsis embryo by means of a large-scale screening for seedlings with an abnormal morphology. Such an approach was based on the supposition that, as occurs in *D. melanogaster* (see, for example, Nüsslein-Volhard and Wieschaus 1980), most of the mutations that cause embryonic lethality would affect genes with housekeeping cell functions, while mutations in many of the genes responsible for embryonic morphogenesis would not prevent completion of embryogenesis, despite causing strong morphological alterations that would be expressed in the seedling. The assumption of Jürgens *et al.* with respect to the embryo can also be applied to the leaf, since mutants in ubiquitous and essential metabolic processes should determine lethality. Viable mutants with aberrant leaves should correspond to dysfunctions in morphogenetic controls or in the synthesis, spatial distribution, or assembly of the structural or metabolic elements required for the proliferation, spatial arrangement, and differentiation of the cells of the organ.

A large number of new mutants with abnormal leaves are presented in this work. It cannot be established, *a priori*, how many of them are affected in genes that control the morphogenesis of this organ and how many represent genes that participate in the process by merely providing, locating, or assembling cell structural elements. It cannot be ruled out that some, or even most, of our mutants correspond to partial loss-of-function damage in genes participating in metabolic pathways whose alteration causes the modification of leaf form. The most obvious candidates to be included in this group are the mutants that show an anomalous pigmentation, which are probably associated with a generalized (in the mutants of the Exigua, Orbiculata, and Angulata classes) or localized (Venosa) abnormal chloroplast development as occurs in the *cue1* (Li *et al.* 1995), *dov1* (Kinsman and Pyke 1998), and *pac* (*pale cress*; Reiter *et al.* 1994) mutants. Other mutants, the most extreme examples of which are those of the Exigua class, show anomalies in size, which is inferior to that of the wild type. This might reflect disorders in the general processes that control cell division or expansion, as occurs in the *diminuto* (*dim*; Takahashi *et al.* 1995) mutant and its allele *dwarf1* (*dwf1*; Kauschmann *et al.* 1996) or in the *constitutive triple response 1* (*ctr1*; Kieber *et al.* 1993) mutant, which displays constitutive ethylene response. The phenotypic classes Rugosa and Scabra include members that display irregularities on the surface of the

TABLE 5

Complementation testing of mutants

Asymmetric leaves	P14 28.1	*as1-1*	*as2-1*
P7 28.1 (*as1-11*)		–	+
P12 25.1 (*as1-12*)		–	+
P14 28.1 (*as1-13*)		–	
as2-1 (ABRC CS3117)	+		

Rotunda	P10 23.2	P12 42.6F2Ron	P12 49.1 (*ron2-2*)	P14 7.3
P9 24.4 (*ron1*)	+	+		
P10 23.2 (*ron2-1*)				–
P12 42.6F2Ron (*ron3*)	+			
P14 7.3 (*ron2-3*)	–		–	

Rugosa	P7 38.3 (*rug2*)	P14 1.4	P14 2.4	P14 39.1	P14 42.3
P14 1.4 (*rug1-1a*)					–
P14 2.4 (*rug1-1c*)	+				
P14 39.1 (*rug1-1d*)		–	–		
P14 42.3 (*rug1-1b*)				–	

Ondulata	P918.3	P10 2.3	P14 9.4	P14 18.7
P9 18.3 (*ond1*)		+		
P10 2.3 (*ond2*)			+	
P14 9.4 (*ond3*)	+			
P14 18.7 (*ond4-1a*)	+		+	
P14 63.3 (*ond4-1b*)		+		–

Exigua	P8 44.4	P8 45.4	P9 44.6	P9 50.8	P9 58.2	P10 26.2	P10 29.4	P10 37.5	P10 41.1	P12 41.6	P14 59.3
P8 44.4 (*exi1-1*)						+		+		–	+
P8 45.4 (*exi3*)						+					+
P9 44.6 (*exi1-2*)	–	+					+	+		–	
P9 50.8 (*exi4*)		+	+		+	+	+		+	+	+
P9 58.2 (*exi5*)	+	+	+				+				+
P10 26.2 (*exi8-1*)					+						
P10 29.4 (*exi6*)		+		+		+				+	+
P10 37.5 (*exi2-1a*)		+							–		
P10 41.1 (*exi2-1b*)			+		+	+	+				
P12 5.1 (*exi8-2*)						–					
P12 41.6 (*exi1-3*)			–					+			
P14 59.3 (*exi7*)						+			+		

Orbiculata	P8 37.6	P8 63.5 (*orb1-1a*)	P12 43.5	P12 46.4	P14 1.5	P14 41.1
P8 37.6 (*orb1-1b*)		–		–		
P12 43.5 (*orb1-2b*)				–		
P12 46.4 (*orb1-2a*)					+	–
P14 1.5 (*orb2*)	+		+			
P14 41.1 (*orb1-3*)				–		

Elongata	P7 3.8	P7 4.4	P7 30.1	P7 40.11	P12 57.5
P7 3.8 (*elo1-1a*)			+		
P7 4.4 (*elo1-1b*)	–				
P7 30.1 (*elo2*)		+			+
P7 40.11 (*elo1-1c*)		–			
P12 42.6F2Elo (*elo4*)	+		+		+
P12 57.5 (*elo3*)				+	

Apiculata and Angusta	P9 36.5	P9 38.2	P10 7.2	P10 32.3	P14 8.3	P14 11.1	P14 34.2	P14 44.2	P7 51.2	P9 10.5	P9 45.1	P14 3.6	P14 23.1
P9 36.5 (*api2*)				+	+						+		
P9 38.2 (*api1*)	+			+	+			+					
P10 7.2 (*api3*)	+	+		+		+		+	+		+		+
P10 32.3 (*api4*)					+				+		+		+
P14 8.3 (*api5*)			+			+		+			+	ND	
P14 11.1 (*api6*)	+	+		+			+	+	+		+		
P14 34.2 (*api7-1b*)				+		+		–					
P14 44.2 (*api7-1a*)	+			+			–				+	+	
P7 51.2 (*ang1-1*)	+	+			+					–	+	+	
P9 10.5 (*ang1-2*)		+						+				+	
P9 45.1 (*ang2*)		+							+	+			
P14 3.6 (*ang3*)	+	+	+	+		+					+		
P14 23.1 (*ang4*)	NG	+			+	+		+		+	+	+	

Denticulata	P1 1.3	P1 5.1	P2 14.2	P2 20.2	P2 33.3 (*den5-1*)	P2 62.4	P7 19.2	P7 34.1	P7 41.1	P8 9.2	P8 26.1	P8 30.3	P8 49.2	P9 54.3	P9 58.3	P9 60.1	P14 8.8	P14 21.1	P14 50.8
P1 1.3 (*den1*)		+	+	+		+				+	+		+					+	+
P1 5.1 (*den2*)			+			+				+	+		+	+	+	+	+	+	
P2 14.2 (*den3*)				+	+		+	+	+	+	+	+	+	+	+	+	+	+	+
P2 20.2 (*den4*)		+			+			+			+		+		+	+	+		+
P2 62.4 (*den6-1*)			+	+	+			+				–					+	+	+
P7 19.2 (*den7*)	+	+		+	+	+			+			+	+	ND	+		+	+	
P7 34.1 (*den5-2*)	+	+			–		+		+		+	+	+		+			+	
P7 41.1 (*den8*)	+	+		+	+	+					+		+			+	+	+	
P8 9.2 (*den11*)				+		+	+	+	+		+			+		+	+	+	+
P8 26.1 (*den9*)							+					+		+	+	+		+	
P8 30.3 (*den6-2*)											+		+						
P8 49.2 (*den10*)										+	+	+					+	+	+
P9 54.3 (*den14*)	+			+		+		+	+		+		+					+	+
P9 58.3 (*den12*)	+					+			+	+			+	+		+	+	+	
P9 60.1 (*den13*)	+					+	+	+					+	+	+		+	+	
P14 8.8 (*den16*)	+							+			+			+				+	+
P14 21.1 (*den17*)				+			+												
P14 50.8 (*den15*)		+					+	+	+		+				+	+		+	

Angulata	P1 13.3	P2 12.5	P2 34.2	P2 38.2	P7 5.7	P7 17.5	P7 24.2	P7 41.3	P7 45.4	P8 2.5	P8 48.2	P9 3.4	P9 9.2	P9 50.7	P12 9.4	P12 40.1	P12 51.1	P12 53.1
P1 13.3 (*anu1-1*)		+		+		+			+						+		+	
P2 12.5 (*anu2*)				+	+	+				+		+					+	
P2 34.2 (*anu4*)	+	+			+				NG	+				+			+	
P2 38.2 (*anu3*)			+		+					+			+		+		+	
P7 5.7 (*anu5-1a*)	+		+			+		–				+					+	
P7 17.5 (*anu6*)			NG	+											+		+	
P7 24.2 (*anu7*)	+	+	+	+	+	+				+				+	+		+	
P7 41.3 (*anu5-1b*)											+							
P7 45.4 (*anu12*)		+		+	+	+	+				+	+			+		+	
P8 2.5 (*anu8-1a*)					+						–						+	
P8 48.2 (*anu8-1b*)						+									+			
P9 3.4 (*anu9-1a*)	+			+		+								–				
P9 9.2 (*anu1-2*)	–		+		+	+	+		+	+				+	+		+	
P9 50.7 (*anu9-1b*)										+								
P12 9.4 (*anu10-1a*)		+	+		+						+			+			+	
P12 40.1 (*anu10-1b*)																	+	
P12 51.1 (*anu11*)		+												+				
P12 53.1 (*anu10-1c*)															–	–		

Erosa	P7 54.2	P8 14.3	P8 23.2 (*ero2-1b*)	P9 38.1	P9 52.3
P7 54.2 (*ero1*)				+	+
P8 14.3 (*ero2-1a*)	+		–		
P9 38.1 (*ero3-1a*)	+	+			NG
P9 52.3 (*ero3-1b*)		+			

Scabra	P2 67.5	P7 29.3	P7 62.2	P9 51.3	P9 53.1	P12 4.6	P14 28.5
P2 67.5 (*sca1*)							+
P7 29.3 (*sca4-1*)	+				+	–	–
P7 62.2 (*sca2*)	+	+			+		
P9 25.1 (*sca3-1a*)	+	+		–			
P9 51.3 (*sca3-1b*)			+		+		
P9 53.1 (*sca5*)	+						
P12 4.6 (*sca4-2*)	+						
P14 28.5 (*sca4-3*)						–	

Venosa	P2 13.3	P7 10.1	P8 5.10	P10 23.1	P10 37.2	P12 10.8	P12 18.8	P12 55.1 (*ven5-2*)	P14 28.6
P2 13.3 (*ven1*)								+	+
P2 13.7 (*ven3-1*)				–					+
P7 10.1 (*ven2-1a*)	+		+		+			+	+
P7 37.2 (*ven2-1b*)		–				+		+	
P8 5.10 (*ven3-2*[a])	+							+	+
P9 5.6 (*ven2-2*)		–							
P10 23.1 (*ven3-3*)							–		+
P10 26.1 (*ven5-1a*)		+			–	+		–	
P10 37.2 (*ven5-1b*)			+						+
P10 54.1 (*ven5-1c*)	+	+			–				
P12 10.8 (*ven4*)	+		+					+	
P12 18.8 (*ven3-4*[a])			–	–					
P14 28.6 (*ven6*[a])			+	+		+	+		

(*continued*)

TABLE 5

(Continued)

Serrata	P2 20.4	P2 22.2 (*sea2-1a*)	P2 39.3	P9 10.1	P9 60.4	P14 18.2	P14 47.1	P14 52.1	P14 58.3
P2 20.4 (*sea1*)			+						
P2 39.3 (*sea2-1b*)		−			+				
P9 10.1 (*sea4-1a*)						+			
P9 60.4 (*sea4-1b*)						+			
P14 10.2 (*sea4-2a*)			+					−	+
P14 18.2 (*sea3-1a*)	+		+						−
P14 21.2 (*sea4-2b*)				−					
P14 47.1 (*sea4-2c*)						+			
P14 52.1 (*sea4-2d*)	+			−	−	+	−		
P14 58.3 (*sea3-1b*)								+	

Transcurvata	P10 37.1	P10 51.5	P12 32.1	P14 2.2	P14 20.1	P14 26.2	*as1-1* (N3374)	*as2-1*
P10 37.1 (*tcu1-1a*)		−						
P10 51.5 (*tcu1-1b*)	−							
P12 32.1 (*as2-11*)	+						+	−
P14 2.2 (*tcu3-1a*)	+	+	+					
P14 6.3 (*tcu2-1a*)					−			
P14 20.1 (*tcu2-1b*)	+		+	+		+	+	
P14 26.2 (*tcu3-1b*)	+		+	−				
as2-1 (ABRC CS3117)					+			

Ultracurvata	P1 4.5 (*ucu1-1*[a])	P9 42.1	P14 12.1 (*ucu1-3*)
P9 36.1 (*ucu1-2a*[a])	−	−	−
P9 42.1 (*ucu1-2b*[a])	−		

Incurvata	P1 7.4	P7 40.1	P8 49.6	P10 16.4	P10 22.2	P12 1.1	P12 24.1	P12 41.2	P14 8.1	P14 8.5	*clf-18* (NASC N345)	*icu2*	*hst-5*	*icu4-2*	*icu5*
P1 7.4 (*icu1-9a*)						−									
P1 32.1 (*icu1-9b*)	−					−					−				
P7 40.1 (*icu3-2*)										+	+	+	−	+	+
P8 49.6 (*icu9-1*)										−					
P10 16.4 (*icu7-1*)							+	−					+	+	+
P10 22.2 (*icu6*[b])	+	+					+	+							
P12 1.1 (*icu1-10*)		+					+		+						
P12 24.1 (*icu8*)		+										+	+	+	+
P12 41.2 (*icu7-2*)	+												+	+	+
P14 8.1 (*icu9-2a*)				+						−					
P14 8.5 (*icu9-2b*)					+		+		−						
icu2 (NASC N329)					+			+							
hst-5 (NASC N314)					+										
icu4-2[a] (NASC N401)					+										
icu5[b] (NASC N379)					+										

Mutant lines are noted according to their protocol number, as PN X.Y, where PN indicates the corresponding parental group, provided by Lehle Seeds (see second column of Table 1), X refers to the number of the plate where the mutant was isolated, and Y is an ordinal assigned to each of the mutants found in a given plate. NASC or ABRC stock numbers or allele names given after the complementation analysis are indicated in brackets. Since noncomplementing mutations isolated from the same parental group are candidates to be identical, they are given the same allele number and provisionally distinguished by a letter (a–d). NG, not germinated; ND, not determined.

[a] Semidominant mutation.

[b] Dominant mutation. All the remaining mutations are recessive.

lamina that might be due to uncoordinated division and/or expansion of epidermal and/or mesophyll cells, as in the *crinkly-4* mutant of maize (Becraft *et al.* 1996). Another explanation for the rough surface of these mutants could be the existence of local processes of cell death affecting the epidermis or mesophyll. In any case, it is difficult to determine on only a gross phenotype basis if these mutants are affected in tissue patterning or in morphogenesis.

It has been proposed that at least two independent controls contribute to the final size of the cells of the expanding Arabidopsis leaf (Tsuge *et al.* 1996), which would determine either cell width or cell length. Evidence for the existence of such polar controls is provided by some mutant phenotypes: Rotundifolia, with short rounded leaves (Tsuge *et al.* 1996), and Angustifolia, with narrow thick leaves (Rédei 1962; Tsuge *et al.* 1996). Such a model is applicable to some of our mutants, which might be affected in the polar controls of cell expansion: the lateral (expansion in width; the Angusta, Asymmetric leaves, Rotunda and Erosa classes) and the longitudinal or proximo-distal (in length; Elongata). The final morphology of all these mutants, however, might also be due to perturbations in the patterns of cell division.

The phenotypic classes whose members are characterized by different types of indentations in the margin constitute a sizeable part of the obtained mutants, which may be due to localized cell death or to local disturbances of the division and expansion patterns. On the other hand, it has been demonstrated that hydathodes, guttation organs connected to the vasculature, are associated with prominences in the leaf margin (Tsukaya and Uchimiya 1997; Van Lijsebettens and Clarke 1998). A direct correlation exists between the number of hydathodes and the number of prominences in the leaf margin, both increasing in parallel in successive vegetative leaves of the ecotype L*er.* In fact, a mutant, *extrahydathodes*, that presents an increased number of hydathodes, and, consequently, of prominences in the leaf margin was obtained in our laboratory (Candela *et al.* 1999). In accordance with the above hypothesis, we may assume that our mutants that show alterations in leaf marginal configuration represent disturbances in the distribution of such elements of the vascular pattern, which are externally expressed in the prominences appearing in the leaf margin, or in the differentiation of adjacent tissues or the expansion or division of their cells. It is striking that the three phenotypic classes that are characterized by alterations in the marginal config-

TABLE 6

Genes affecting leaf morphology in *Arabidopsis thaliana*

Gene	Molecular nature of product[a]	Mutant phenotype	Proposed function	Reference[b]
ACAULIS1 (*ACL1*)		Small and twisted cauline and rosette leaves	Involved in cell maturation (elongation) in both leaf cells and internodal cells	Tsukaya *et al.* (1993)
ACAULIS2 (*ACL2*)		Leaves with short petiole	Elongation of internodal cells	Tsukaya *et al.* (1995)
ANGUSTIFOLIA (*AN*)		Narrow and thick leaves	Regulation of lateral cell expansion	Rédei (1962); Tsuge *et al.* (1996)
ARGONAUTE1 (*AGO1*)	Cytoplasmic protein	Narrow and succulent rosette leaves	Generation of positional information in proliferation processes	Bohmert *et al.* (1998)
ASYMMETRIC LEAVES1 (*AS1*)		Lobed and slightly asymmetric leaves		Rédei and Hirono (1964); Tsukaya and Uchimiya (1997)
CUE1		Reticulate leaves	Control of transcription from light-regulated promoters	Li *et al.* (1995)
CURLY LEAF (*CLF*)	Polycomb-group protein	Rosette and cauline leaves curled up	Repression of homeotic gene expression	Goodrich *et al.* (1997); Kim *et al.* (1998a)
DDM1		Rounded rosette leaves; increased number of cauline leaves	DNA methylation	Vongs *et al.* (1993)
DIMINUTO (*DIM*)	Protein with putative nuclear localization signals	Rounded and curly leaves with short petioles	General process of plant cell elongation	Takahashi *et al.* (1995); Kauschmann *et al.* (1996)
DIFFERENTIAL DEVELOPMENT OF VASCULAR ASSOCIATED CELLS (*DOV*)		Reticulate leaves	Control of cell-specific plastid development	Kinsman and Pyke (1998)
HASTY (*HST*)		Juvenile leaves with adaxial and abaxial trichomes	Regulation of shoot maturation timing	Telfer and Poethig (1998)
PALE CRESS (*PAC*)	Acidic α-helical protein	Pale leaves with thin blade	Regulation of light-induced chloroplast and leaf development	Reiter *et al.* (1994)
POINTED FIRST LEAVES (*PFL*)	Ribosomal protein S18	Narrow blade in first leaves	Translation in meristematic tissues	Van Lijsebettens *et al.* (1994)
REVOLUTA (*REV*)		Long rosette and cauline leaves	Regulation of the relative growth of apical and nonapical meristems	Talbert *et al.* (1995)
ROTUNDIFOLIA3 (*ROT3*)	Cytochrome P-450	Rounded leaves	Regulation of longitudinal cell expansion	Tsuge *et al.* (1996); Kim *et al.* (1998b)
TOUSLED (*TSL*)	Putative serine/threonine protein kinase	Serrated rosette leaves; curled cauline leaves	Element of a signaling or regulatory pathway	Roe *et al.* (1993)

[a] Unknown, unless otherwise indicated.
[b] Although in some cases several references are relevant, only one or two are quoted for simplicity.

uration of the leaf are among those showing a higher number of complementation groups: The first is Denticulata with 17, the second is Angulata with 12, and the fourth is Apiculata with 7.

Since cell migration does not play any role in plant morphogenesis, the body pattern of an adult plant is a consequence of mechanisms that control the number and direction of cell divisions, the direction of cell expansion, and the local differentiation of the cells (Meyerowitz 1994) and, eventually, of the existence of programmed cell death (Van Lijsebettens and Clarke 1998). On the other hand, it is generally assumed that developmental genetic decisions of individual cells are subordinated to coordination systems among groups of cells, which are mediated by intercellular communication mechanisms. The leaf of Arabidopsis is a fundamentally flat structure, which consists of only a few cell layers (Pyke *et al.* 1991; Tsuge *et al.* 1996), its final form resulting from cell division and expansion. The growth of the adaxial and abaxial surfaces, which are equivalent in area, must be coordinated by some unknown mechanism. It is reasonable to suppose that perturbations in such a coordination mechanism would lead to differences between the dorsal and ventral cell division and/or expansion patterns. This would determine differences between the adaxial and abaxial surfaces, which, in turn, would cause alterations in the morphology of the leaf reflected by its curling up or downward. We obtained some mutants whose leaves display different kinds of deviation from planarity, their lamina being curled upward (the Incurvata phenotype), spirally rolled downward (Ultracurvata), or obliquely folded (Transcurvata). Since all these mutants are candidates to be affected in genes involved in coordinating the growth of the dorsal and ventral surfaces of the leaf, their characterization might provide information on the coordination mechanisms between proliferating cells during development. Alternatively, the above-mentioned phenotypes might arise from hormonal imbalances. In fact, it is likely that some of our mutants have suffered damage in the synthesis, transport, or perception of phytohormones as well as in the related responses. Abnormal size and/or morphology have been observed in mutants impaired in such processes with regard to auxin, gibberellins, brassinosteroids, and ethylene (see, for reviews, Hobbie 1998; McGrath and Ecker 1998; Phillips 1998; Szekeres and Koncz 1998).

Pleiotropy of the mutant phenotypes: Some of our mutants seem to be affected in processes mainly or exclusively required in leaf ontogeny, whereas others seem to have suffered damage in functions that participate in the construction of several organs. We have verified that 60 of the 153 mutants studied show anomalies visible only in the vegetative leaves, while another 15 also express alterations in the cauline leaves although not in the rest of the plant. Not infrequently (a total of 78 mutant lines) morphological abnormalities were observed in other organs, mainly the flowers. This is not surprising, since leaves represent the basal state of floral organs, which they preceded in the evolutionary history of flowering plants. In fact, it is generally assumed that floral organs are modified leaves, which have acquired specific functions throughout evolution. Furthermore, the elimination by mutation of all major A, B, and C morphogenetic functions has led to the homeotic transformation of floral organs into leaves (Coen and Meyerowitz 1991). All of the above lends plausibility to the fact that mutations that disturb the morphogenetic controls shared by leaves and floral organs might perturb the morphology of both flowers and leaves.

Speculations regarding the molecular nature of the genes identified by isolating mutants: Little can be said about the molecular nature of the genes affected in our mutants. Nevertheless, those termed *incurvata1* deserve commentary, since we have found them to be allelic to the *CURLY LEAF* (*CLF*) gene. The phenotype associated with such mutant alleles consists of curvature of the leaf margin and disturbances in the size and the spatial arrangement of some floral organs. The cloning of *CLF* (Goodrich *et al.* 1997) has revealed that it is structurally related to members of the Polycomb and trithorax groups of *D. melanogaster* (Kennison 1993; Simon 1995), which include regulatory genes required for the maintenance of the expression of homeotic genes such as those belonging to the Bithorax and Antennapedia complexes. It has been demonstrated that the leaf phenotype of *clf* mutants is due to the ectopic derepression of some genes required for flower morphogenesis, but inactive in wild-type leaves, such as *AGAMOUS* (*AG*). Since *CLF* represses *AG* in leaves as well as in some floral organs, its loss of function leads to the ectopic derepression of *AG*, which causes leaf curling. On the other hand, mutant phenotypes such as those of our Incurvata class have been observed in Arabidopsis plants transformed with an antisense construction of the *METI* gene, whose product is a methyltransferase (Finnegan *et al.* 1996). These plants suffer severe hypomethylation of their DNA, one of the consequences of which is the ectopic expression of the *AGAMOUS* and *APETALA3* genes in the leaf. According to the above data, some of our *incurvata*, *transcurvata*, or *ultracurvata* mutants might be regarded as suffering the ectopic derepression in the leaf of genes whose normal realm of action is the flower. We hypothesize that some of such mutants owe their phenotype to mutations in genes whose function is to repress floral genes in the leaf. Such genes would have appeared in order to safeguard the identity of leaves as organs, distinguishing them from floral organs. The Incurvata phenotype can be explained as a consequence of the ectopic expression in the leaf of some floral organ identity gene that participates in the control of cell division patterns and probably contributes to building partially concave organs in the flower, such as sepals

and petals, or fundamentally cylindrical ones, such as the stamens and carpels. In the leaf, however, a flat structure is desirable and the curvature observed in some *incurvata* mutants would be a consequence of the imposition on this organ of the typical division patterns of floral organs.

Given the nature of the chemical mutagen that we have employed, EMS, it is likely that most of the alleles obtained are hypomorphic. However, the semidominance of six of them and the complete dominance of another constitute singular cases that could correspond to hypermorphic or antimorphic mutations or null alleles of haploinsufficient loci. The semidominant *ven3-2* and *ven3-4* mutations probably deserve attention, since they are members of an allelic series including a further two alleles that are recessive. The same can be said of the *ucu1* alleles, two of which are semidominant (*ucu1-1* and *ucu1-2*), and the third recessive. Finally, the *icu6* mutation is the only dominant one that we have found, its mutant phenotype in *icu6/ICU6* heterozygous being more extreme than that of most of the homozygotes for recessive mutations in the remaining *ICU* loci.

Perspectives for the genetic and molecular analyses of mutants obtained: Histological analyses of the mutants presented in this work will be necessary for establishing the nature of the underlying perturbations in their phenotypes at the cellular level. Other information to be determined is their eventual allelism with mutants already isolated by previous authors. We have performed some of such allelism tests, finding that at least four of the genes that we have identified are alleles of already known Arabidopsis genes: *AS1*, *AS2*, *CLF*, and *RE.* Although additional intercrosses with old mutations could be made to test for allelism, we decided to restrict this approach to existing mutants with phenotypes very similar to those obtained in this work. The reason such a choice was made is that the sheer volume of both old and new mutations affecting leaf morphology is quite large. Take, as an example, the AIS collection of form mutants (Bürger 1971; Kranz and Kirchheim 1987, 1990; Anderson 1993), which includes >150 old mutant strains, all of which are candidates to be intercrossed with representative individuals of our 94 complementation groups. The genetic analysis of 77 lines of the AIS collection of form mutants revealed that they represent 47 different genes (J. Serrano-Cartagena, H. Candela, P. Robles and J. L. Micol, unpublished results; Serrano-Cartagena *et al.* 1999). To choose further candidates for allelism tests, proximity between genetic map positions might be a better criterion than a mere resemblance between phenotypes. Hence, another aspect in the study of our mutants that would constitute a natural continuation of the work presented here is their genetic mapping. Crosses to reach this objective to obtain the F_2 plants that will be employed for simple sequence length polymorphism (SSLP) linkage analysis have been accomplished (Bell and Ecker 1994; Ponce *et al.* 1999).

An obvious avenue that opens up with the availability of the mutants described in this work is an analysis of their interactions in double mutant combinations. A study of their phenotypes will help to discriminate mutations that disturb morphogenetic controls from those affecting other processes. By analyzing the interactions that take place between the genes that we have identified, we will be able to propose models for the genetic control of leaf development. Advantage will be taken of the progeny obtained from the crosses made for the complementation analysis. Since some of the mutants that we have obtained display flower abnormalities, it is possible that they may correspond to any of the numerous mutations already known in genes affecting floral morphogenesis in Arabidopsis. Finally, we emphasize that the collection of mutants that we have obtained provides the scientific community with new tools for studying leaf morphogenesis in *A. thaliana.*

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LITERATURE CITED

Anderson, M., 1993 *The Nottingham Arabidopsis Stock Centre: Seed List.* The University of Nottingham, Nottingham, UK.

Becraft, P. W., P. S. Stinard and D. McCarty, 1996 CRINKLY4: a TNFR-like receptor kinase involved in maize epidermal differentiation. Science **273:** 1406–1409.

Bell, C. J., and J. R. Ecker, 1994 Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis.* Genomics **19:** 137–144.

Bohmert, K., I. Camus, C. Bellini, D. Bouchez, M. Caboche *et al.* 1998 *AGO1* defines a novel locus of *Arabidopsis* controlling leaf development. EMBO J. **17:** 170–180.

Brutnell, T. P., and J. A. Langdale, 1998 Signals in leaf development. Adv. Bot. Res. **28:** 36–42.

Bürger, D., 1971 Die morphologischen mutanten des Göttinger *Arabidopsis*-sortiments, einschließlich der mutanten mit abweichender samenfarbe. Arabidopsis Inf. Serv. **8:** 36–42.

Candela, H., A. Martínez-Laborda and J. L. Micol, 1999 Venation pattern formation in *Arabidopsis thaliana* vegetative leaves. Dev. Biol. **205:** 205–216.

Coen, S. C., and E. M. Meyerowitz, 1991 The war of the whorls: genetics interactions controlling flower development. Nature **353:** 31–37.

Fabri, C. O., and A. R. Schäffner, 1994 An *Arabidopsis thaliana* RFLP mapping set to localize mutations to chromosomal regions. Plant J. **5:** 149–156.

Finnegan, E. J., W. J. Peacock and E. Dennis, 1996 Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development. Proc. Natl. Acad. Sci. USA **93:** 8449–8454.

Goodrich, J., P. Puangsomlee, M. Martin, D. Long, E. M. Meyerowitz *et al.*, 1997 A polycomb-group gene regulates homeotic gene expression in *Arabidopsis.* Nature **386:** 44–51.
Haffter, P., M. Granato, M. Brand, M. C. Mullins, M. Hammerschmidt *et al.*, 1996 The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio.* Development **123:** 1–36.
Hall, L. N., and J. A. Langdale, 1996 Molecular genetics of cellular differentiation in leaves. New Phytol. **132:** 533–553.
Hobbie, L. J., 1998 Auxin: molecular genetic approaches in *Arabidopsis.* Plant Physiol. Biochem. **36:** 91–102.
Hofer, J., L. Turner, R. Hellens, M. Ambrose, P. Matthews *et al.* 1997 *UNIFOLIATA* regulates leaf and flower morphogenesis in pea. Curr. Biol. **7:** 581–587.
Jürgens, G., U. Mayer, R. A. Torres Ruiz, T. Berleth and S. Miséra, 1991 Genetic analysis of pattern formation in the *Arabidopsis* embryo. Dev. Suppl. **1:** 27–38.
Kauschmann, A., A. Jessop, C. Koncz, M. Szekeres, L. Willmitzer *et al.*, 1996 Genetic evidence for an essential role of brassinosteroids in plant development. Plant J. **9:** 701–713.
Keddie, J. S., B. Carroll, J. D. G. Jones and W. Gruissem, 1996 The *DCL* gene of tomato is required for chloroplast development and palisade cell morphogenesis in leaves. EMBO J. **16:** 4208–4217.
Kennison, J. A., 1993 Transcriptional activation of *Drosophila* homeotic genes from distant regulatory elements. Trends Genet. **9:** 75–79.
Kieber, J. J., M. Rothenberg, G. Roman, K. A. Feldmann and J. R. Ecker, 1993 *CTR1*, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the Raf family of protein kinases. Cell **72:** 427–441.
Kim, G., H. Tsukaya and H. Uchimiya, 1998a The *CURLY LEAF* gene controls both division and elongation of cells during the expansion of the leaf blade in *Arabidopsis thaliana.* Planta **206:** 175–183.
Kim, G., H. Tsukaya and H. Uchimiya, 1998b The *ROTUNDIFOLIA3* gene of *Arabidopsis thaliana* encodes a new member of the cytochrome P-450 family that is required for the regulated polar elongation of leaf cells. Genes Dev. **12:** 2381–2391.
Kinsman, E. A., and K. A. Pyke, 1998 Bundle sheath cells and and cell-specific plastid development in *Arabidopsis* leaves. Development **125:** 1815–1822.
Koornneef, M., 1994 *Arabidopsis* genetics, pp. 89–120 in *Arabidopsis*, edited by E. M. Meyerowitz and C. R. Somerville. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Kranz, A. R., and B. Kirchheim, 1987 Genetic resources in *Arabidopsis.* Arabidopsis Inf. Serv. **24**.
Kranz, A. R., and B. Kirchheim, 1990 Additions and corrections to the AIS-seed bank listing. Arabidopsis Inf. Serv. **27:** 89–200.
Li, H., K. Culligan, R. A. Dixon and J. Chory, 1995 *CUE1*: a mesophyll cell-specific positive regulator of light-controlled gene expression in *Arabidopsis.* Plant Cell **7:** 1599–1610.
Marx, G. A., 1987 A suite of mutants that modify pattern formation in pea leaves. Plant Mol. Biol. Rep. **5:** 311–335.
McGrath, R. B., and J. R. Ecker, 1998 Ethylene signaling in *Arabidopsis*: events from the membrane to the nucleus. Plant Physiol. Biochem. **36:** 103–113.
McHale, N. A., 1993 *LAM-1* and *FAT* genes control development of the leaf blade in *Nicotiana sylvestris.* Plant Cell **5:** 1029–1038.
Meyerowitz, E. M., 1994 Pattern formation in plant development: four vignettes. Curr. Opin. Genet. Dev. **4:** 602–608.
Moreno, M. A., L. C. Harper, R. W. Krueger, S. L. Dellaporta and M. Freeling, 1997 *liguleless1* encodes a nuclear-localized protein required for induction of ligules and auricles during maize leaf organogenesis. Genes Dev. **11:** 616–628.
Muehlbauer, G. J., J. E. Fowler and M. Freeling, 1997 Sectors expressing the homeobox gene *liguleless3* implicate a time-dependent mechanism for cell fate acquisition along the proximal-distal axis of the maize leaf. Development **124:** 5097–5106.
Nüsslein-Volhard, C., and E. Wieschaus, 1980 Mutations affecting segment number and polarity in *Drosophila.* Nature **287:** 795–801.
Phillips, A. L., 1998 Gibberellins in *Arabidopsis.* Plant Physiol. Biochem. **36:** 115–124.
Poethig, R. S., 1997 Leaf morphogenesis in flowering plants. Plant Cell **9:** 1077–1087.
Ponce, M. R., V. Quesada and J. L. Micol, 1998 Rapid discrimination of sequences flanking and within T-DNA insertions in the *Arabidopsis* genome. Plant J. **14:** 497–502.
Ponce, M. R., P. Robles and J. L. Micol, 1999 High-throughput genetic mapping in *Arabidopsis thaliana.* Mol. Gen. Genet. **261:** 408–415.
Pyke, K. A., J. L. Marrison and R. M. Leech, 1991 Temporal and spatial development of the cells of the expanding first leaf of *Arabidopsis thaliana* (L.) Heynh. J. Exp. Bot. **42:** 1407–1416.
Rédei, G. P., 1962 Single locus heterosis. Z. Vererbungsl. **93:** 164–170.
Rédei, G. P., and Y. Hirono, 1964 Linkage studies. Arabidopsis Inf. Serv. **1:** 9–10.
Reiter, R. S., S. A. Coomber, T. M. Bourett, G. E. Bartley and P. A. Scolnik, 1994 Control of leaf and chloroplast development by the *Arabidopsis* gene *pale cress.* Plant Cell **6:** 1253–1264.
Roe, J. L., C. J. Rivin, R. A. Sessions, K. A. Feldmann and P. C. Zambrisky, 1993 The *Tousled* gene in A. thaliana encodes a protein kinase homolog that is required for leaf and flower development. Cell **75:** 939–950.
Serrano-Cartagena, J., P. Robles, M. R. Ponce and J. L. Micol, 1999 Genetic analysis of leaf form mutants from the *Arabidopsis* Information Service collection. Mol. Gen. Genet. (in press).
Simon, J., 1995 Locking in stable states of gene expression: transcriptional control during *Drosophila* development. Curr. Opin. Cell Biol. **7:** 376–385.
Sinha, N. R., R. E. Williams and S. Hake, 1993 Overexpression of the maize homeobox gene, *KNOTTED-1*, causes a switch from determinate to indeterminate cell fates. Genes Dev. **7:** 787–795.
Smith, L. G., S. Hake and A. W. Sylvester, 1996 The *tangled-1* mutation alters cell division orientations throughout maize leaf development without altering leaf shape. Development **122:** 481–489.
Stearn, W. T., 1995 *Botanical Latin.* David and Charles, Devon, U.K.
Szekeres, M., and C. Koncz, 1998 Biochemical and genetic analysis of brassinosteroid metabolism and function in *Arabidopsis.* Plant Physiol. Biochem. **36:** 145–155.
Takahashi, T., A. Gasch, N. Nishizawa and N. Chua, 1995 The *DIMINUTO* gene of *Arabidopsis* is involved in regulating cell elongation. Genes Dev. **9:** 97–107.
Talbert, P. B., H. T. Adler, D. W. Parks and L. Comai, 1995 The *REVOLUTA* gene is necessary for apical meristem development and for limiting cell divisions in the leaves and stems of *Arabidopsis thaliana.* Development **121:** 2723–2735.
Telfer, A., and R. S. Poethig, 1994 Leaf development in *Arabidopsis*, pp. 379–401 in *Arabidopsis*, edited by E. M. Meyerowitz and C. R. Somerville. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Telfer, A., and R. S. Poethig, 1998 *HASTY*: a gene that regulates the timing of shoot maturation in *A. thaliana*. Development **125:** 1889–1898.
Traas, J., C. Bellini, P. Nacry, J. Kronenberger, D. Bouchez *et al.*, 1995 Normal differentiation patterns in plants lacking microtubular preprophase bands. Nature **375:** 676–677.
Tsuge, T., H. Tsukaya and H. Uchimiya, 1996 Two independent and polarized processes of cell elongation regulate leaf blade expansion in *Arabidopsis thaliana* (L.) Heynh. Development **122:** 1589–1600.
Tsukaya, H., 1995 Developmental genetics of leaf morphogenesis in Dicotyledonous plants. J. Plant Res. **108:** 407–416.
Tsukaya, H., and H. Uchimiya, 1997 Genetic analyses of the serrated margin of leaf blades in *Arabidopsis*: combination of a mutational analysis of leaf morphogenesis with the characterization of a specific marker gene expressed in hydathodes and stipules. Mol. Gen. Genet. **256:** 231–238.
Tsukaya, H., S. Naito, G. P. Rédei and Y. Komeda, 1993 A new class of mutations in *Arabidopsis thaliana*, *acaulis1*, affecting the development of both inflorescences and leaves. Development **118:** 751–764.
Tsukaya, H., K. Inaba-Higano and Y. Komeda, 1995 Phenotypic and molecular mapping of an *acaulis2* mutant of *Arabidopsis thaliana* with flower stalks of much reduced length. Plant Cell Physiol. **36:** 239–246.
Van Lijsebettens, M., and J. Clarke, 1998 Leaf development in *Arabidopsis.* Plant Physiol. Biochem. **36:** 47–60.
Van Lijsebettens, M., R. Vanderhaeghen, M. De Block, G. Bauw, R. Villaroel *et al.*, 1994 An S18 ribosomal protein gene copy at

the *Arabidopsis PFL* locus affects plant development by its specific expression in meristems. EMBO J. **13:** 3378–3388.
Vollbrecht, E., B. Veit, N. Sinha and S. Hake, 1991 The developmental gene *Knotted-1* is a member of a maize homeobox gene family. Nature **350:** 241–243.
Vongs, A., T. Kakutani, R. A. Martienssen and E. J. Richards, 1993 *Arabidopsis thaliana* DNA methylation mutants. Science **260:** 1926–1928.
Waddington, C. H., 1940 The genetic control of wing development in *Drosophila.* J. Genet. **41:** 75–139.
Waites, R., and A. Hudson, 1995 *Phantastica:* a gene required for dorsoventrality of leaves in *Antirrhinum majus.* Development **121:** 2143–2154.
Waites, R., H. R. N. Selvadurai, I. R. Oliver and A. Hudson, 1998 The *PHANTASTICA* gene encodes a MYB transcription factor involved in growth and dorsoventrality of lateral organs in *Antirrhinum.* Cell **93:** 779–789.
Walsh, J., C. A. Waters and M. Freeling, 1997 The maize gene *liguleless2* encodes a basic leucine zipper protein involved in the establishment of the leaf blade-sheath boundary. Genes Dev. **11:** 208–218.
Wilkins, A. S., 1993 *Genetic Analysis of Animal Development.* Wiley-Liss, New York.

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