Genetic Analysis of *incurvata* Mutants Reveals Three Independent Genetic Operations at Work in Arabidopsis Leaf Morphogenesis

José Serrano-Cartagena, Héctor Candela, Pedro Robles, María Rosa Ponce, José Manuel Pérez-Pérez, Pedro Piqueras and José Luis Micol

División de Genética, Universidad Miguel Hernández, Campus de San Juan, 03550 Alicante, Spain

ABSTRACT

In an attempt to identify genes involved in the control of leaf morphogenesis, we have studied 13 *Arabidopsis thaliana* mutants with curled, involute leaves, a phenotype herein referred to as Incurvata (Icu), which were isolated by G. Röbbelen and belong to the Arabidopsis Information Service Form Mutants collection. The Icu phenotype was inherited as a single recessive trait in 10 mutants, with semidominance in 2 mutants and with complete dominance in the remaining 1. Complementation analyses indicated that the studied mutations correspond to five genes, representative alleles of which were mapped relative to polymorphic microsatellites. Although most double-mutant combinations displayed additivity of the Icu phenotypes, those of *icu1 icu2* and *icu3 icu4* double mutants were interpreted as synergistic, which suggests that the five genes studied represent three independent genetic operations that are at work for the leaf to acquire its final form at full expansion. We have shown that *icu1* mutations are alleles of the Polycomb group gene *CURLY LEAF* (*CLF*) and that the leaf phenotype of the *icu2* mutant is suppressed in an *agamous* background, as is known for *clf* mutants. In addition, we have tested by means of multiplex RT-PCR the transcription of several floral genes in Icu leaves. Ectopic expression of *AGAMOUS* and *APETALA3* was observed in *clf* and *icu2*, but not in *icu3*, *icu4*, and *icu5* mutants. Taken together, these results suggest that *CLF* and *ICU2* play related roles, the latter being a candidate to belong to the Polycomb group of regulatory genes. We propose that, as flowers evolved, a new major class of genes, including *CLF* and *ICU2*, may have been recruited to prevent the expression of floral homeotic genes in the leaves.

THE question of how plant leaves develop is far from being answered at the genetic level even though Bateson realized the existence of inherited leaf shape variants as early as 1913 (Bateson 1913). During the ensuing 60 years, genetic investigations did not provide major insights into the dissection of leaf morphogenesis. In more recent decades, however, a large number of mutants with abnormally shaped leaves, most of them yet to be characterized, have been isolated in model systems such as *Arabidopsis thaliana* (Bürger 1971; Relichova 1976; Kranz 1978; Kranz and Kirchheim 1987). Nowadays, an expanding number of studies on leaf variants, including analyses of genetic interactions between mutations, are being published for several plant species, some examples being those of McHale (1993), Tsuge *et al.* (1996), Villani and Demason (1997), and Telfer and Poethig (1998).

In spite of the fact that most plant leaves are simple structures, many developmental processes are involved in leaf ontogeny. They include, among others, the positioning and initiation of leaf primordia at the flanks of the shoot meristem; the specification of leaf identity as opposed to that of other organs that are assumed to be modified leaves; the establishment of dorsal and ventral identities within the organ; the definition of domains such as ligule, sheath, and blade in some monocotyledonous plants, as well as petiole and lamina in dicots; the control of cell division and expansion; the formation of patterns such as those of venation, trichomes, or stomata; and the mechanisms responsible for the diversity of compound and simple leaves and those that specify heteroblastic differences among different leaves within a plant. A large body of detailed information on what actually happens at a morphological level is available for most, if not all, such processes but only a few studies have focused on the nature, action, and interactions of the genes driving the sequence of developmental events that contribute to the making of a leaf (reviewed in Hake and Sinha 1991; Smith and Hake 1992; Sinha *et al.* 1993; Telfer and Poethig 1994; Tsukaya 1995; Hall and Langdale 1996; Poethig 1997; Brutnell and Langdale 1998; Tsiantis and Langdale 1998; Van Lijsebettens and Clarke 1998; Pozzi *et al.* 1999).

As a consequence of differential balances between anticlinal and periclinal division patterns of the cells that contribute to their final architecture, most leaves are characteristically flattened, displaying bilateral symmetry and dorsoventrality, while other plant organs have

radial symmetry. A coordination mechanism has to be invoked to account for the matching areas of the adaxial (dorsal) and abaxial (ventral) leaf sides, notwithstanding that they display dorsoventral asymmetry, being unlike in the number, size, spatial arrangement, and differentiation state of their cells. Under the above hypothesis, mutants displaying deviations from the planar blades that characterize wild-type leaves can be regarded as defective in putative mechanisms coordinating the growth of the dorsal and ventral tissues of the leaf. We decided to study *A. thaliana* mutants displaying leaf curling to identify genes that coordinate growth of the abaxial and adaxial sides of the leaf. To this end, we have followed two complementary approaches: the isolation and study of new mutants, induced by ethyl methanesulfonate (Berná *et al.* 1999) and fast neutrons (P. Robles and J. L. Micol, unpublished results), and the study of mutants obtained by previous authors (this work; Serrano-Cartagena *et al.* 1999).

In this work, we took advantage of the availability of the large collection of *A. thaliana* variants stored at the Nottingham Arabidopsis Stock Centre (NASC), which we have found to be instrumental for developmental studies such as the identification of lines displaying perturbations in leaf venation pattern formation (Candela *et al.* 1999). We grew 152 mutant lines already known to display abnormally shaped leaves, finding 22 that exhibited involute (curled up) vegetative leaves, a phenotype that we have named Incurvata (Icu). Our genetic analyses of 13 of such *icu* strains showed that most of them carried recessive mutations and that they corresponded to five genes, whose map positions were determined. Comparison of double mutants involving representative alleles at the *ICU* loci was also made, allowing the classification of these genes in three different groups depending on the genetic interactions detected. On the basis of these interactions and those observed in double-mutant combinations, including a mutant allele of the *AGAMOUS* (*AG*) gene, a model is proposed for the role of the studied genes in leaf morphogenesis.

MATERIALS AND METHODS

Plant culture: *A. thaliana* (L.) Heyhn. seeds from mutant and wild-type strains were obtained from the NASC. Their stock numbers are the following: N313, N314, N328, N329, N345, N346, N347, N348, N350, N351, N379, N400, N401, and N419. All these mutant strains were isolated by G. Röbbelen from the Enkheim-2 (En-2) ecotype (Bürger 1971). Plants were grown as described by Ponce *et al.* (1998) on petri dishes in Conviron TC16 culture chambers at $20° \pm 1°$ and 60–70% relative humidity under continuous fluorescent light (7000 lux).

Genetic analysis: We chose the Latin word *incurvata* to designate the mutants studied in this work. In accordance with the nomenclature of Meinke and Koornneef (1997), the mutant phenotype is referred to as Incurvata (Icu), the wild-type allele as *INCURVATA* (*ICU*), and the mutant alleles as *incurvata* (*icu*).

Dominance relationships were established in the F_1 progeny of crosses between mutant and En-2 wild-type plants and further confirmed in the inbred F_2 generation.

Complementation groups were defined after the observation of the F_1 progeny of crosses between homozygous recessive mutant lines. In those cases where one of the mutant lines crossed was homozygous for a dominant or semidominant mutation, allelism was discarded when phenotypically wild-type individuals were found in the inbred F_2 generation. As a general rule, recessive mutants were used as female parents to easily distinguish any self-pollination.

At least one mutant line from each complementation group was outcrossed to the Landsberg *erecta* (L*er*) ecotype so that linkage to polymorphic simple sequence length polymorphism (SSLP) markers could be assessed. DNA of F_2 plants showing the recessive phenotype was isolated by the method of Edwards *et al.* (1991). PCR amplification products were obtained by utilizing Arabidopsis SSLP specific primers (Mappairs; Research Genetics, Huntsville, AL) and scored for polymorphisms. Oligonucleotide sequences were as described by Bell and Ecker (1994), with the sole exception of the primers used to amplify the T27K12-Sp6 and MBK5 markers, whose sequences were taken from http://genome.bio.upenn.edu/ SSLP_info/coming-soon.html. Amplifications and gel electrophoresis were undertaken as described in Bell and Ecker (1994) although modified as follows: regular agarose (4 to 6%) was used instead of acrylamide or low-melting-point agarose to visualize all polymorphisms, each PCR amplification included only 0.025 units/μl of Bio*Taq* polymerase (BioLine), and no hot start was performed. Unlike other genes, the mapping of *ICU4* was carried out using the multiplex PCR fluorescence-based procedure of Ponce *et al.* (1999). Map distances were calculated using the map function of Kosambi (1944).

We obtained all the possible double-mutant combinations involving representative alleles from each of the five complementation groups studied in this work. Some of them were unequivocally identified by their conspicuous phenotypes in the F_2 progeny of crosses between homozygous single mutants. In most cases, however, doubly homozygous individuals for two recessive mutations had to be identified as those plants displaying a mutant phenotype present only in one-fourth of the F_3 siblings obtained by selfing F_2 mutant plants.

As expected, double mutants involving dominant or semidominant mutations appeared in the corresponding F_2 progenies more frequently than double mutants bearing two recessive mutations. Furthermore, the genotype of such F_2 putative double mutants was tested by isolating several of them from each cross and studying their F_3 inbred progenies separately. The absence of phenotypic segregation in an F_3 family was considered evidence of its double-mutant nature. Exceptions were those few double-mutant combinations that resulted in sterility.

Since plants homozygous for the *ag-1* allele are sterile, heterozygous *AG/ag-1;er/er* individuals from the NW25 line were used to pollinate plants homozygous for a given *icu* mutation. One-half of the resulting wild-type F_1 individuals carried the *ag-1* allele, so that the Ag phenotype reappeared in their F_2 progenies. Again, F_3 families were derived from individual F_2 plants displaying either wild-type or Icu phenotype to identify segregating double mutants.

Multiplex RT-PCR amplification and fluorescence-based semiautomated detection of gene expression: Transcription of floral genes in leaf tissues was tested as described in Ponce *et al.* (2000). Total RNA was isolated from excised rosette leaves or flowers, reverse transcribed, and multiplex RT-PCR amplified. Fluorescence-based semiautomated detection of the amplification products was carried out in a Perkin-Elmer (Norwalk, CT) ABI PRISM 377 DNA sequencer using the

GENESCAN 2.1 DNA fragment analysis software (Applied Biosystems, Foster City, CA). Each pair of primers was intron spanning, to differentiate between cDNA and contaminating genomic DNA amplifications, and included one oligonucleotide labeled with 6-hexachlorofluorescein phosphoramidite.

Light and scanning electron microscopy: For scanning electron microscopy observation, plants were fixed overnight with 3% glutaraldehyde in 25 mm sodium phosphate buffer (pH 6.8). After being washed twice with phosphate buffer for 30 min, plant material was postfixed with a 1% OsO_4 solution in 25 mm phosphate buffer (pH 6.8) for 2 hr and washed again with phosphate buffer before being dehydrated through an ethanol series of increasing concentration (70, 80, 90, 95, and 100%). Plant tissue was then critical point dried with CO_2 and covered with gold using a Balzers SCD 004 sputter coater. Micrographs of samples were taken in a JSM-840 Jeol (Tokyo) scanning electron microscope.

For light microscopy, plant material was fixed with FAA/Triton (1.85% formaldehyde, 45% ethanol, 5% acetic acid, and 1% Triton X-100). Samples were fixed overnight at room temperature after having had a vacuum treatment (400 mbar) applied for 30 min when in the fixative. Dehydration was carried out through a graded ethanol series (70, 80, 90, and 95%) at room temperature. After dehydration, the tissue was preincubated overnight at 4° in a solution containing 50% (v/v) JB-4 resin (Polysciences, Inc., Niles, IL) and 50% ethanol. The samples were then dipped at least twice in 100% JB-4 resin at 4° for 2 hr each time. Polymerization and embedding of the samples in 100% JB-4 resin were carried out following the instructions of the manufacturer. Sections of 4-μm thickness were cut using histoknives (Heraeus Kulzer GmbH) on a 2050 Supercut microtome (Reichert-Jung, Cambridge Instruments GmbH) and stained with 0.1% (w/v) toluidine blue. Photographs were taken in a Leica DMR microscope under bright-field illumination.

RESULTS

Determination of inheritance patterns of *icu* mutants: We began our study by requesting 152 *A. thaliana* mutant lines from the NASC, all of them belonging to an already existing collection, the Arabidopsis Information Service (AIS) collection of Form Mutants, which was initially maintained by Kranz and includes mutants obtained by either Röbbelen or Kranz (Kranz and Kirchheim 1987). These 152 mutant lines were first classified into several phenotypic classes in accordance with their leaf morphology as observed under our growth conditions. One such phenotypic class included 22 mutants that consistently presented leaves curling toward their adaxial side, a phenotype that we named Incurvata (Icu). Fourteen *icu* mutants have been considered for this study (see materials and methods), whose leaf and rosette phenotypes are shown in Figure 1, B–F, and Figure 2, B–F, compared to their wild-type ancestor, the ecotype Enkheim-2 (En-2; Figures 1A and 2A). Phenotypic and genetic analyses of AIS Form Mutants belonging to other phenotypic classes have been presented in Serrano-Cartagena *et al.* (1999). A study of the remaining 8 *icu* mutants will be presented elsewhere.

To determine the mode of inheritance of their phenotypes, mutants were crossed to the wild type. Differences

Figure 1.—Leaf phenotypes of *incurvata* mutants. The gross morphology of nine rosette leaves from wild-type En-2 and *icu* mutant plants is shown. From left to right, leaves in each row are ordered from early to late appearance. Pictures were taken immediately after the excision of leaves 32 days after sowing. Bar, 1 cm.

were not found either between reciprocal crosses or between crosses involving a given mutant strain and L*er* or En-2 ecotypes. The analysis of the corresponding F_1 and inbred F_2 progenies revealed that the Icu phenotype is caused by two semidominant (in lines N400 and N401) and one completely dominant (in N379) mutation, the remaining 10 being recessive (data not shown).

Complementation and linkage analyses: Crosses to assess allelism were performed as described in materials and methods. The results of such a complementation analysis, shown in Table 1, indicated that five genes are represented among the *icu* mutants studied. The map positions of the *ICU* loci were determined after testing for linkage polymorphic SSLP markers in a population consisting of phenotypically recessive F_2 individuals (see materials and methods). The complementation groups were defined as follows.

INCURVATA1 (ICU1): This group consists of eight recessive alleles, carried by the N313, N328, N345, N346, N347, N350, N351, and N419 strains, respectively. Their mutant phenotype included involute leaves, small rosette size, early flowering (∼15 days after sowing in mutant plants compared to 30 days after sowing in En-2 wild-type individuals), and a short, thin flowering stem (Figure 2B). In addition, the flowers displayed a phenotype reminiscent of some *apetala2* (*ap2*) mutants (Bowman *et al.* 1989; Figure 3B), and homeotic transformations of sepals into carpels and petals into stamens were observed mainly in the latest flowers. As three of these strains (N313, N328, and N350) had been reported to be the *clf-1*, *clf-17*, and *clf-19* alleles of the *CURLY LEAF* (*CLF*) gene (Goodrich *et al.* 1997), we concluded that *icu1* mutations are alleles of the *CLF* gene, five of which
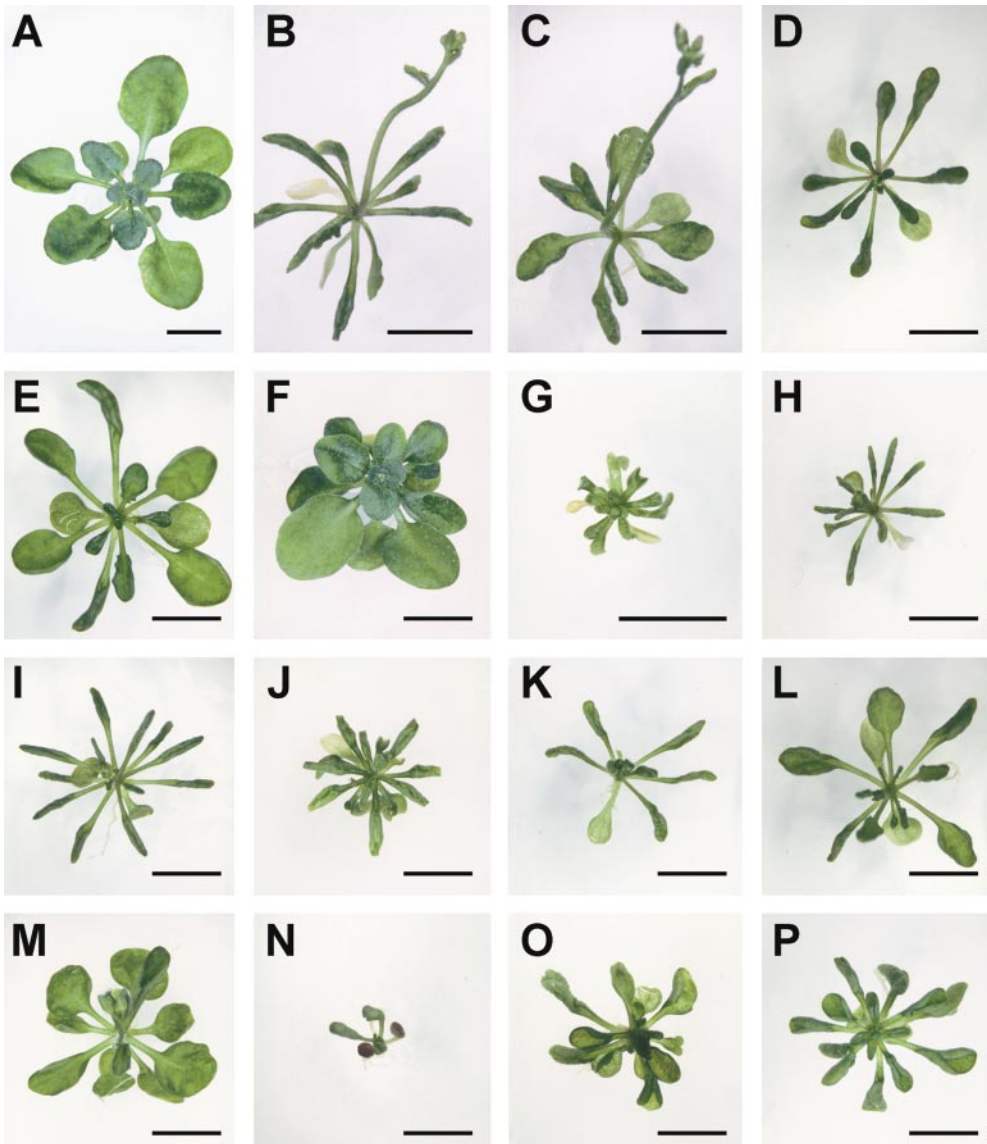
FIGURE 2.—Rosette phenotypes of single and double *incurvata* mutants. (A) Wild-type En-2 rosette. (B) *clf-18/clf-18* mutant showing a flowering stem only 20 days after sowing, as well as Incurvata leaves. (C) *icu2/icu2* mutant as early flowering as the *clf-18/clf-18* mutant in Figure 1B, although its leaves curl up to a lesser extent. (D) *hst-5/hst-5* mutant displaying strong curling in all its leaves. (E) *icu4-1/icu4-1* mutant showing the strongest effects of the mutation in the first pair of leaves. (F) *icu5/icu5* mutant rosette showing its characteristic weak Incurvata leaf phenotype. (G) *clf-18/clf-18;icu2/icu2* double mutant displaying strongly involute leaves and a very small rosette as compared to each single mutant. (H) *clf-18/clf-18;hst-5/hst-5*, (I) *clf-18/clf-18;icu4-1/icu4-1*, and (J) *clf-18/clf-18;icu5/icu5* double-mutant plants showing additive phenotypes consisting of early flowering and leaf curling slightly stronger than that of the single mutants. (K) *icu2/icu2;hst-5/hst-5* double mutant. (L) *icu2/icu2;icu4/icu4* and (M) *icu2/icu2;icu5/icu5* double-mutant plants showing additive phenotypes consisting of uneven leaf surface and early flowering. (N) *hst-5/hst-5;icu4-1/icu4-1* double mutant showing an extremely reduced rosette and strong curling both in the leaves and cotyledons. (O) *hst-5/hst-5;icu5/icu5* and (P) *icu4-1icu5/icu4-1 icu5* plants displaying similar phenotypes with a rosette similar to that of *icu5/icu5* individuals but with stronger Incurvata leaf phenotype. Bars, 5 mm. Pictures were taken 20 days after sowing.

were first reported here. Additional support for this finding came from the allelism tests performed with *pif-1* and *pif-2* (*photoperiod insensitive flowering*; Table 1), mutations that were already known to be alleles of *CLF* (J. M. MARTÍNEZ-ZAPATER, personal communication). We were not able to get seeds from crosses involving N348, a line that was considered a putative allele of the *ICU1* (*CLF*) gene solely on the basis of its phenotype. The N313, N328, N345, and N419 lines showed the most extreme Icu leaf phenotype, while N351 displayed the weakest (data not shown). Since our results coincided with those of other laboratories, we agreed to use the same allele numbering scheme (J. GOODRICH, personal communication), N345, N346, N347, N351, and N419 becoming, respectively, *clf-18*, *clf-21*, *clf-22*, *clf-24*, and *clf-25*.

INCURVATA2 (ICU2): Only one recessive allele, carried by N329, was identified, which maps near the lower telomere of chromosome 5, 30.8 ± 7.0 cM away from nga129, and is 8.6 ± 3.2 cM distal to MBK5, with no linkage to nga76 detected. Involute leaves (Figures 1C and 2C), early flowering (∼15 days after sowing), and Apetala flowers (Figure 3C) were pleiotropic traits that *icu2* individuals shared with *clf* mutants. However, not all the leaves of a given *icu2* plant curled up (Figures 1C and 2C) and not as strongly as those of *clf* mutants (Figures 1B and 2B). Patches of epidermal tissue with a reduced cell size (Figure 4E), resulting in an uneven leaf surface, were consistently present as a regular feature of the Icu2 phenotype. Apparently, such patches were randomly distributed on the adaxial side of the leaves. Similar to *clf* plants, *icu2* mutants showed low fertility and a thin flowering stem when compared to the wild type (data not shown).

TABLE 1

Complementation testing of *incurvata* mutants

♂	♀													
	N313	N328	N345	N346	N347	N350	N351	N419	N314	N329	N400	N401	*pif-1*	*pif-2*
N313 (*clf-1*)		−		−										
N328 (*clf-17*)	−													
N345 (*clf-18*)		−		−	−	−		−		+		−[a]		−
N346 (*clf-21*)	−				−	−				+				
N347 (*clf-22*)			−	−		−	−			+				
N350 (*clf-19*)				−				−		+				
N351 (*clf-24*)	−		−					−		+				
N419 (*clf-25*)			−				−							
N314 (*hst-5*)		+	+	+						+		−[a]		
N329 (*icu2*)		+	+	+		+	+						+	+
N400 (*icu4-1*)			−[a]						−[a]			−[b]		
N401 (*icu4-2*)									−[a]	−[a]	−[b]			
N379 (*icu5*)			−[a]						−[a]	−[a]		−[a]		
pif-1			−											
pif-2			−											

All mutant strains were homozygous. Symbols indicate wild-type (+) or mutant (−) phenotype of F_1 progeny from the crosses indicated. The F_1 progenies of crosses involving both a semidominant and a recessive mutant displayed a phenotype intermediate between those of the wild type and the plant homozygous for the semidominant mutation.

[a] Presence of wild-type individuals in the F_2 of the corresponding crosses.

[b] Absence of wild-type individuals in the F_2 of the corresponding crosses.

INCURVATA3 (ICU3): The only mutant allele of this locus, carried by N314, is recessive. This mutant displayed involute leaves (Figures 1D and 2D) and early flowering, although less than *clf* and *icu2* mutants (∼20 days after sowing). In addition, the fertility of *icu3* mutants was reduced due to morphological abnormalities in the gynoecium, where carpels were often badly fused (data not shown). The *ICU3* gene locates on chromosome 3 at a very short distance from nga172, since no recombination events were found between these loci after studying 50 chromosomes. The mutant allele carried by N314 was named *hasty-5* (*hst-5*) by Telfer and Poethig (1998), a designation that will be followed from here on.

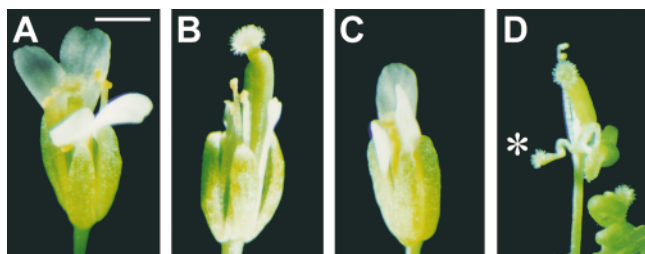*INCURVATA4 (ICU4):* The two alleles of this locus,

FIGURE 3.—Flower phenotypes of single and double *incurvata* mutants. (A) Wild-type Enkheim-2 flower. (B) *clf-18/clf-18* and (C) *icu2/icu2* mutant flowers displaying partial absence of petals. (D) *clf-18/clf-18;icu2/icu2* double-mutant flower showing extreme homeotic transformations similar to those of strong *apetala2* mutant alleles lacking petals and most stamens. *, sepal displaying carpelloid features. Bar, 1 mm.

carried by N400 and N401, were found to be semidominant since leaves from heterozygous plants differed from those of homozygous individuals in their weaker phenotype. The Icu phenotype of heterozygous *ICU4/icu4-1* plants was more easily seen in the early stages of leaf expansion, when leaf curling was most conspicuous. Later in development, these leaves were more similar to those of the wild type. Interestingly, leaf curling in the first two leaves of the *icu4-1/icu4-1* plants was more extreme than in adult vegetative leaves (Figures 1E and 2E). Neither early flowering nor flower aberrations were observed in these mutants. The *ICU4* gene has been mapped to chromosome 1, 31.5 ± 7.0 cM below T27K12-Sp6 and 16.9 ± 4.5 cM above nga128.

INCURVATA5 (ICU5): This complementation group is defined by a completely dominant mutation, carried by the N379 line, which did not display any flower abnormality. This gene also maps to chromosome 1, 8.2 ± 3.0 cM away from the AthACS telomeric marker. Leaves of *icu5* individuals (Figures 1F and 2F) were dark green and slightly curled up when compared to *clf*, *hst-5*, and *icu4* mutants, with no phenotypic differences being observed between homozygous and heterozygous mutant plants. This strain was subjected to morphological analysis by Rüffer-Turner and Napp-Zinn (1980), who found that the numbers of stomata and epidermal cells per surface unit were considerably higher than in its En-2 wild-type ancestor. Due to its compact rosette, we previously assigned this mutant to the Compact rosette (Cro) phenotypic class, which included AIS Form Mutant lines that were found to show an altered photomor-
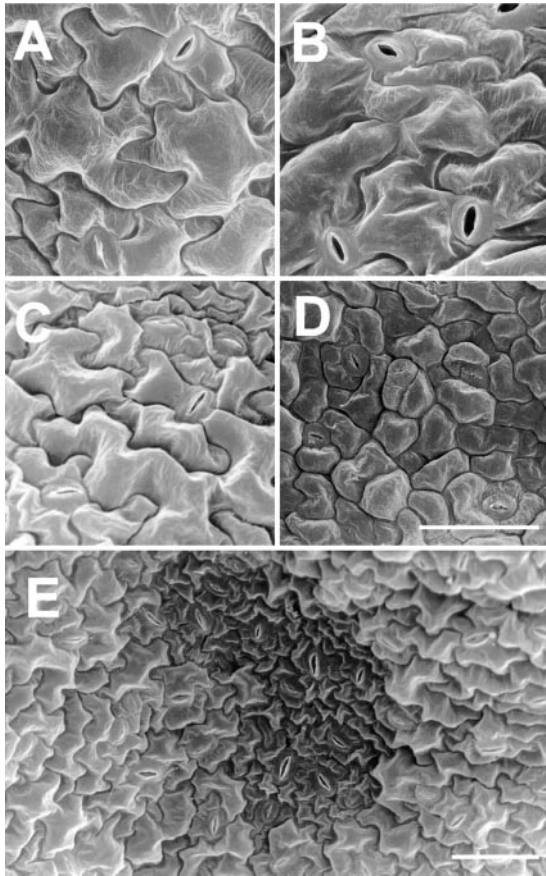
FIGURE 4.—Scanning electron micrographs of the adaxial epidermis of wild-type and *incurvata* mutant leaves. (A) Leaf tissue of the Enkheim-2 ecotype displaying wild-type cell size and shape. (B) *clf-18/clf-18* leaf. (C) *icu2/icu2* leaf. (D) *clf-18/clf-18;icu2/icu2* double-mutant leaf displaying reduced and abnormally shaped pavement cells and stomata. (E) *icu2/icu2* leaf showing a patch of different cell size. Bars, 50 μm. Magnification is the same for A to D. Plant tissue was collected 20 days after sowing.

phogenic response (SERRANO-CARTAGENA *et al.* 1999). All the mutants studied in this work were grown in the dark, which revealed that the hypocotyl length was reduced only in *icu5* in comparison to the wild type (data not shown).

Double-mutant analysis: All the possible double-mutant combinations involving a representative allele from each of the above-described complementation groups were obtained in an attempt to identify genetic interactions between *ICU* loci (Figure 2, G–P). *clf-18* was selected from the alleles of *CLF* because of its extreme leaf phenotype. Whereas *clf-18/clf-18* and *icu2/icu2* leaves and rosettes were similar in size (Figure 1, B and C, and Figure 2, B and C), those of *clf-18/clf-18;icu2/icu2* double mutants were much smaller (Figure 2G). Observation under a scanning electron microscope revealed that the pavement cells in the adaxial epidermal tissue were smaller and more rounded in *clf-18/clf-18;icu2/icu2* leaves (Figure 4D) than in both single mu-

tants (Figure 4, B and C) and the wild type (Figure 4A). Interestingly, the vegetative leaves of a single plant displayed carpelloid features such as stigmatic papillae, suggesting that a shift from vegetative to reproductive fate had taken place (data not shown). Double-mutant flowers displayed a phenotype similar to that of strong *ap2* alleles (BOWMAN *et al.* 1989), that is, lacking petals and most stamens (Figure 3D). In these plants, some sepals showed homeotic transformations to carpels, becoming fused and presenting ovules and stigmatic papillae. All such abnormalities were stronger in the latest flowers. The phenotype of these double-mutant individuals was considered to be synergistic, suggesting that the wild-type functions of both genes may overlap in controlling the expression of *AG* in both leaves and floral organs. Other *clf icu2* double mutants involving different *clf* alleles were also obtained, which showed similar synergistic phenotypes (data not shown).

hst-5/hst-5;icu4-1/icu4-1 rosettes (Figure 2N) appeared to be very small when compared to either the single mutants or the wild type. In contrast to *hst-5* and *icu4-1* mutants, cotyledons of double-mutant seedlings were strongly curled up and often showed a darker color (Figure 2N). These double mutants were late flowering and produced a large number of abnormal vegetative leaves before bolting. One out of three putative double mutants that were transferred to soil developed a fasciated stem and a structure similar to an aerial rosette (data not shown). Thus, the double-mutant phenotype was also interpreted as synergistic, since double-mutant plants showed properties that could not be predicted from the study of either single mutant.

The leaf phenotype of the remaining double-mutant combinations could be explained by mere additivity, suggesting that several independent pathways are necessary for a proper leaf expansion. For instance, *clf-18/clf-18;icu5/icu5* and *icu2/icu2;icu5/icu5* double mutants were early flowering and showed homeotic transformations of floral organs and their leaves were altered as in both recessive mutants, but their basal rosettes were reminiscent of those of *icu5* mutants (Figure 2, J and M). The presence of patches of small cells in double mutants involving *icu2*, such as the *icu2/icu2;icu4-1/icu4-1* and *icu2/icu2;icu5/icu5* double mutants, may also be interpreted as a result of additivity (data not shown).

Histological analysis: The Incurvata phenotype as seen in transverse section is illustrated in Figures 5 and 6. Transverse sections cut across the entire leaf width provide an alternative view of the additivity of the Icu phenotypes, as shown by the more pronounced degree of leaf curling in double mutants such as *icu4-1 icu5/icu4-1 icu5* (Figure 5D) when compared to their corresponding single mutants (Figure 5, B and C).

Fully expanded Incurvata leaves were expected to reveal obvious differences from the wild type, allowing us to ascertain the nature of the cell defects responsible for the mutant phenotype. With this aim, photographs
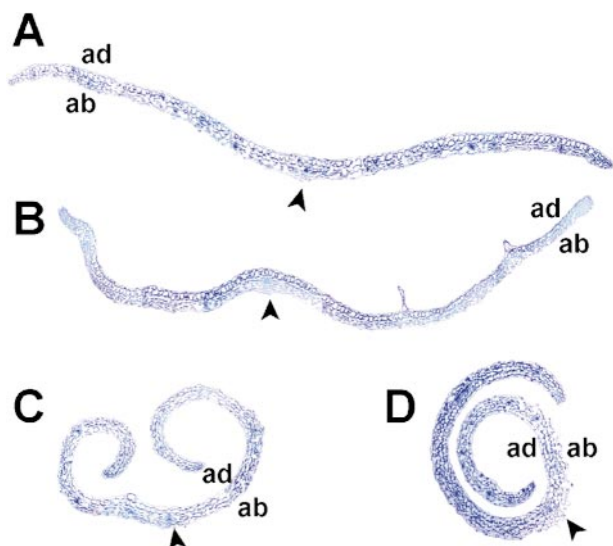
FIGURE 5.—Additivity of the Icu4 and Icu5 phenotypes as shown in transverse sections. (A) Wild-type Enkheim-2 leaf showing its characteristic flattened appearance. (B) *icu5/icu5* and (C) *icu4-1/icu4-1* mutant leaves displaying weak and intermediate Incurvata phenotypes, respectively. Since the midvein (indicated by an arrowhead) is at the center of the images it is clearly shown that the leaves curl toward their adaxial surface. (D) *icu4-1 icu5/icu4-1 icu5* leaf showing the severe phenotype found in the double mutant. ad, adaxial surface. ab, abaxial surface. The genotypes of leaf sections in A, B, C, and D are the same as those of plants shown in Figure 2A, 2F, 2E, and 2P, respectively.
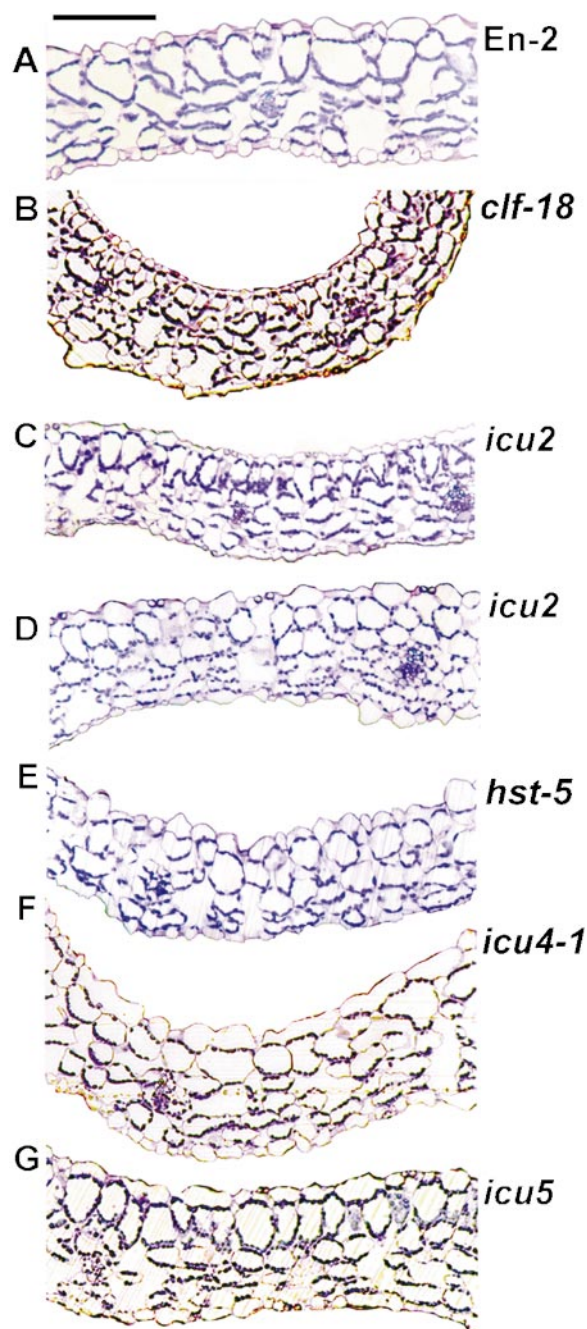

FIGURE 6.—Transverse sections of *icu* mutant leaves. Transverse sections were cut through the leaf at the middle of the lamina. Photographs of transects were taken between the midvein and leaf margin. Sectioned rosette leaves are as follows: (A) First En-2 leaf. (B) Fifth *clf-18* leaf. (C) First *icu2* leaf. (D) Seventh *icu2* leaf. (E) Seventh *hst-5* leaf. (F) First *icu4-1* leaf. (G) Fifth *icu5* leaf. Bar, 50 μm.

were taken of transverse sections through the leaf lamina, between the midvein and the leaf margin, at approximately the same distance from the leaf base as the leaf tip. However, tissue sectioning revealed no obvious perturbations that could easily explain the curvature of these leaves (Figure 6). Internal structure of *icu4-1* and *icu5* leaves was very similar to the wild-type En-2, in spite of the fact that *icu4-1* leaf sections showed a noticeable Icu phenotype. The similarity between En-2 and *icu5* could be explained by the weak Icu phenotype of the latter. All adaxial and abaxial epidermal cells, palisade and spongy mesophyll cells, and airspaces were found to be smaller in *clf-18*, *icu2*, and *hst-5* mutant leaves than in those of En-2. Given that *icu2* individuals have some leaves that display a strong Icu phenotype and others without curling, we analyzed both leaf types and found that cell size was only slightly reduced in noncurled *icu2* leaves. However, *icu2* leaves with strong involute phenotype had clearly smaller cells in all their leaf tissues, as did all *clf-18* leaves. Transects of *hst-5* leaves displayed a slight reduction in cell size. KIM *et al.* (1998) have determined that the *clf-25* mutant shows a reduction in both the size and number of cells in vegetative leaves, concluding that the *CLF* gene affects both cell elongation and cell division. Although we have not performed any quantitative analysis, the number of cells in *icu2* and *hst-5* mutants may also be lower than in the

wild type. However, a generalized reduction in the number and the expansion of cells would not be sufficient to explain the Icu phenotype.

Genetic interactions between the *AGAMOUS* gene and *INCURVATA* genes: As mentioned above, *clf* and *icu2* homozygous individuals displayed flowers similar to those of *ap2* mutants, so that sepals and petals were often

TABLE 2

Analysis of F$_2$ families studied in a search for *clf ag-1* and *icu2 ag-1* double mutants

No. of families studied	F$_1$ parental		F$_2$ progeny		
	Pheno-type	Inferred genotype	WT	Ag	Icu
2	WT	*AG/ag-1;CLF/clf-18*	44	9	7
2	WT	*AG/AG;CLF/clf-18*	50	0	12
2	WT	*AG/ag-1;CLF/clf-22*	124	56	49
1	WT	*AG/AG;CLF/clf-22*	30	0	12
3	WT	*AG/ag-1;ICU2/icu2*	84	37	23
1	WT	*AG/AG;ICU2/icu2*	41	0	11

WT, wild-type phenotype; Ag, Agamous mutant phenotype; Icu, Incurvata mutant phenotype.

homeotically transformed into carpels and stamens or staminoid petals, respectively, abnormalities that were extreme in *clf-18/clf-18;icu2/icu2* double mutants (Figure 3D). These transformations are known to appear as a result of the ectopic expression of the floral homeotic gene *AG* in the first two floral whorls, as was observed in transgenic plants constitutively expressing *AG*, which presented in addition leaves that were curled up (Mizukami and Ma 1992). The above observation led Goodrich *et al.* (1997) to test whether the Clf leaf phenotype was a consequence of the ectopic expression of *AG* in the leaves, where this gene is not expressed in wild-type plants (Yanofsky *et al.* 1990).

We aimed to test if not only *CLF* but also *ICU2* played a role as repressors of *AG* both in wild-type leaves and

flowers. Expecting that the Icu2 mutant phenotype would be suppressed in an *ag* background, we obtained *icu2/icu2;ag-1/ag-1* double mutants in crosses performed in parallel with others aimed to isolate *clf-18/clf-18;ag-1/ag-1* and *clf-22/clf-22;ag-1/ag-1* double mutants. *clf-18/clf-18* plants were crossed by NW25 individuals, which bear the recessive mutation *ag-1*, and F$_2$ and F$_3$ families (Tables 2 and 3) as well as F$_4$ families (data not shown) were studied. The leaf lamina normalized while the flowers displayed a clear Ag phenotype in double-mutant individuals. As expected, the leaf phenotype of *clf-18* individuals was considered to be mainly a result of the ectopic expression of *AG*, as was found in the case of *clf* mutants by Goodrich *et al.* (1997). In spite of the similarity between *clf-18/clf-18;ag-1/ag-1* and *CLF/CLF;ag-1/ag-1* individuals, the double mutants could be distinguished due to their flowering time, which was intermediate between those of the wild-type and *clf-18* plants (data not shown). In addition, double-mutant leaves showed a marginal configuration slightly different from that of the wild type, with more prominent teeth. Both the marginal teeth and the remnant early flowering characteristic of the *clf-18 ag-1* double mutant are likely to result from the *clf-18* mutation, independently of the ectopic expression of *AG*. However, we cannot exclude the existence of a residual *AG* expression in the *ag-1* mutant, as indicated by Goodrich *et al.* (1997). No differences were observed in the interactions of *clf-18* and *clf-22* with *ag-1* (Table 3), which excludes allele specificity as an explanation for the observed double-mutant phenotypes.

A similar approach was followed to test if there was

TABLE 3

Analysis of F$_3$ families studied in a search for *clf ag-1* and *icu2 ag-1* double mutants

No. of families studied	F$_2$ parental		F$_3$ progeny		
	Phenotype	Inferred genotype	WT	Ag	Icu
10	WT	*AG/AG;CLF/CLF*	578	0	0
24	WT	*AG/AG;CLF/clf-18*	662	0	196
2	WT	*AG/ag-1;CLF/CLF*	93	29	0
5	WT	*AG/ag-1;CLF/clf-18*	238	90	64
2	Icu	*AG/AG;clf-18/clf-18*	0	0	36
3	Icu	*AG/ag-1;clf-18/clf-18*	0	38[a]	120
2	WT	*AG/AG;CLF/CLF*	69	0	0
4	WT	*AG/AG;CLF/clf-22*	150	0	38
6	WT	*AG/ag-1;CLF/clf-22*	207	74	40
1	Icu	*AG/AG;clf-22/clf-22*	0	0	2
3	Icu	*AG/ag-1;clf-22/clf-22*	0	17[a]	65
2	WT	*AG/AG;ICU2/icu2*	90	0	29
11	Icu	*AG/AG;icu2/icu2*	0	0	394
12	Icu	*AG/ag-1;icu2/icu2*	0	99[a]	323
4	Icu and Er	*AG/ag-1;icu2/icu2*	0	42[a]	116[b]
4	Icu and Er	*AG/AG;icu2/icu2*	0	0	136[b]

WT, wild-type phenotype; Ag, Agamous mutant phenotype; Icu, Incurvata mutant phenotype.
[a] Plants displayed wild-type leaves but earlier flowering time than the wild type.
[b] *icu2/icu2;er/er* individuals showed a flowering time intermediate between those of wild-type and *icu2/icu2;ER/−* plants.
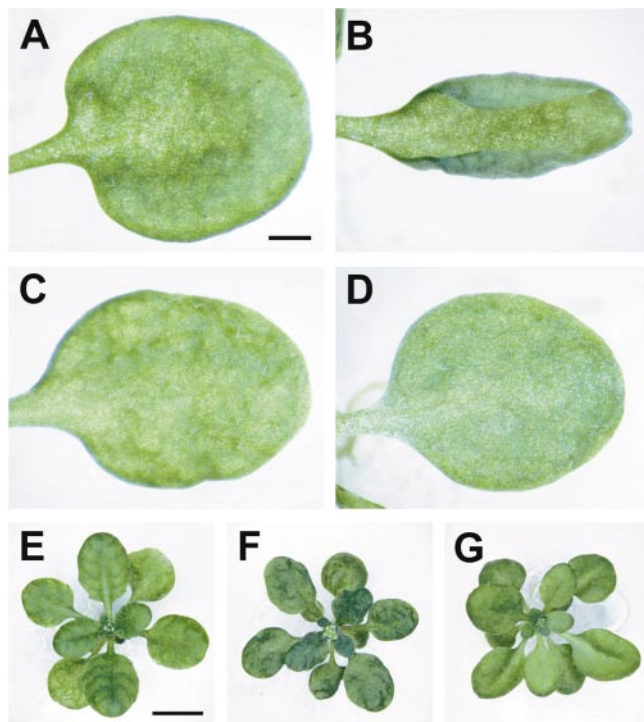
FIGURE 7.—Suppression of the Incurvata2 leaf and rosette phenotypes. (A) Wild-type En-2 rosette leaf showing its characteristically flattened morphology. (B and C) *icu2/icu2* mutant leaves. Although not every *icu2* leaf displays curling, all of them show an uneven leaf surface. (D) *icu2/icu2;ag-1/ag-1* double-mutant leaf displaying its wild-type phenotype. (E) Wild-type L*er*, (F) *icu2/icu2;er/er*, and (G) *icu2/icu2; ag-1/ag-1* rosettes. The latter double mutant displays a wild-type phenotype because of the suppression by *ag-1* of the Icu2 phenotype. Bars, 1 mm (A–D) and 5 mm (E–G). Pictures were taken 20 days after sowing.

a role for *AG* derepression in the *icu2* mutant. After selfing of F$_1$ *ICU2/icu2;AG/ag-1* individuals, their F$_2$ inbred progeny could be classified in accordance with their leaf morphology, finding a 13:3 phenotypic segregation (wild type:Icu, $\chi^2 = 0.55$; $n = 144$) more likely than a 3:1 ($\chi^2 = 5.78$) (Table 3). Sixteen out of 31 F$_3$ families established from *icu2* homozygous plants segregated one-fourth of plants displaying both almost-wild-type leaves (Figure 7) and Ag flowers (Table 3), clearly indicating that *ag-1* was also epistatic to *icu2*, the Icu2 leaf phenotype being suppressed by the *ag-1* mutation. Similar to the *clf-18/clf-18;ag-1/ag-1* and *clf-22/clf-22; ag-1/ag-1* double mutants, *icu2/icu2;ag-1/ag-1* plants also displayed a leaf marginal configuration slightly different from that of the wild type and a flowering time intermediate between those of wild-type and *icu2/icu2* individuals.

Ectopic expression of floral genes in Icu leaves: Transcription of several floral homeotic genes was analyzed, including members of the MADS-box [*AGAMOUS* (*AG*), *APETALA1* (*AP1*), *APETALA3* (*AP3*), and *PISTILLATA* (*PI*); reviewed in RIECHMANN and MEYEROWITZ 1997], and AP2/EREBP [*APETALA2* (*AP2*); reviewed in RIECH-

MANN and MEYEROWITZ 1998] families, together with that of the Polycomb group gene *CLF* (GOODRICH *et al.* 1997). As shown in Figure 8, *A. thaliana* flowers and leaves were tested for the presence of transcripts of the above-mentioned genes (see MATERIALS AND METHODS). By using multiplex reaction mixes including six primer pairs (PONCE *et al.* 2000), we obtained all the six possible amplification products from wild-type flower RNA (Figure 8A), whereas only *AP2* and *CLF* transcripts were shown to be present in wild-type leaf RNA samples (Figure 8B). As regards mutant leaves, no differences were found between the wild-type pattern and those of *hst-5*, *icu4-1*, and *icu5*, all of which displayed noticeable amounts of *AP2* and *CLF* messages (Figure 8, E–G). By contrast, *clf-18* and *icu2* leaves were shown to contain not only *AP2* and *CLF* transcripts but also those from *AG* and *AP3* floral genes (Figure 8, C and D). Ectopic transcription of *AP1* was detected in *icu2* and in the EMS-induced *clf-61* allele (data not shown; this mutation was initially named *icu1-10* in BERNÁ *et al.* 1999), but not in *clf-18*.

The Clf and Icu2 leaf phenotypes are modified by a locus on chromosome 2: As indicated before, both the Clf and Icu2 pleiotropic phenotypes were found to comprise at least three different traits: involute leaves, early flowering, and Apetala flowers. Some observations, made among the F$_2$ and F$_3$ families studied to obtain the *clf-18 ag-1*, *clf-22 ag-1*, and *icu2 ag-1* double mutants, suggested that some other gene may be modifying both leaf curling and flowering time in plants homozygous for either *clf* or *icu2* mutant alleles. On the one hand, leaf phenotype and flowering time of *clf/clf;er/er*, as well as flowering time of *icu2/icu2;er/er* plants, were intermediate between those of wild-type and *clf/clf;ER/ER* or *icu2/icu2;ER/ER* individuals. On the other hand, the *icu2/icu2;er/er* individuals displayed a subtle leaf phenotype, sometimes difficult to distinguish from that of the wild type (Table 3). This may be one of the reasons why no mutant alleles of *ICU2* have been found in a L*er* genetic background (BERNÁ *et al.* 1999). These results suggest that an allele modifying both the flowering time and leaf curling characteristic of *clf* and *icu2* mutants exists in the L*er* background but not in that of En-2. The corresponding gene must be tightly linked to *ER* or be this gene itself.

DISCUSSION

Most plant leaves are flattened structures exhibiting two sides that are similar in area despite their dissimilar dorsoventral identities and differences in the number, size, spatial arrangement, and differentiation of their cells. Hence, mutants deviating from the planarity that characterizes wild-type leaves can be regarded as impaired in putative mechanisms coordinating the growth of the dorsal and ventral tissues of the leaf and as such can be used for testing the existence of such mechanisms. In this article we report the genetic and pheno-

typic studies performed on several mutants from the AIS collection whose leaf laminae curl toward the adaxial side, a phenotype that we have called Incurvata. Our qualitative analyses, based on the study of transverse sections and scanning electron microscopy images of mutant leaves, show a general reduction in cell size, noticeable in the dorsal and ventral tissues of *clf-18*, *icu2*, and *hst-5* mutants. In these mutants, the adaxial cells were always bigger than the abaxial ones, as happens in the wild-type strain. We have not quantitatively analyzed cell size and number in mutant leaves, a kind of study that may not be conclusive to ascertain changes in dorsoventrality. Indeed, KIM *et al.* (1998) were unable

to explain leaf curling in *clf-25* after performing a detailed study of cell size and number. While both parameters were reduced in *clf-25*, the ratio between the adaxial and abaxial epidermal cell surfaces was the same as in the wild type. Hence, no obvious relationship between leaf curling and changes in dorsoventrality could be established from our observations, the only exception being the patches of small cells on the adaxial epidermis of *icu2* mutants, which suggest but do not demonstrate a shift to ventral fate. As a future perspective, the use of specific molecular markers for the adaxial and abaxial cell identities will help to elucidate whether fate changes actually occur in the mutants studied here.

The Icu phenotype may result from the disruption of different developmental phenomena, some of which have already been discussed by previous authors. One such process may be the establishment and/or maintenance of leaf dorsoventral asymmetry, whose perturbation may give rise to uncoordinated growth of the dorsal and ventral tissues of this organ. The *PHANTASTICA* (*PHAN*) gene of *Antirrhinum majus* is the first one reported to be necessary for the early discrimination of dorsal and ventral leaf identities, its mutations causing ventralization of dorsal tissues (WAITES and HUDSON 1995; WAITES *et al.* 1998). A second possibility is that represented by the Arabidopsis *CLF* gene, which seems to be instrumental in the restriction of floral homeotic gene expression domains, as suggested by the phenotype of *clf* mutants, whose leaves curl up as a result of the ectopic expression of *AG* (GOODRICH *et al.* 1997).

When speculating on the molecular nature of mutations carried by the AIS mutants, it must be noted that a major disadvantage is the lack of information on how they were induced (BÜRGER 1971). Moreover, as we do not know how the screening was performed, we cannot exclude that different mutations are actually identical by descent, being derived of a single M_1 plant. Since
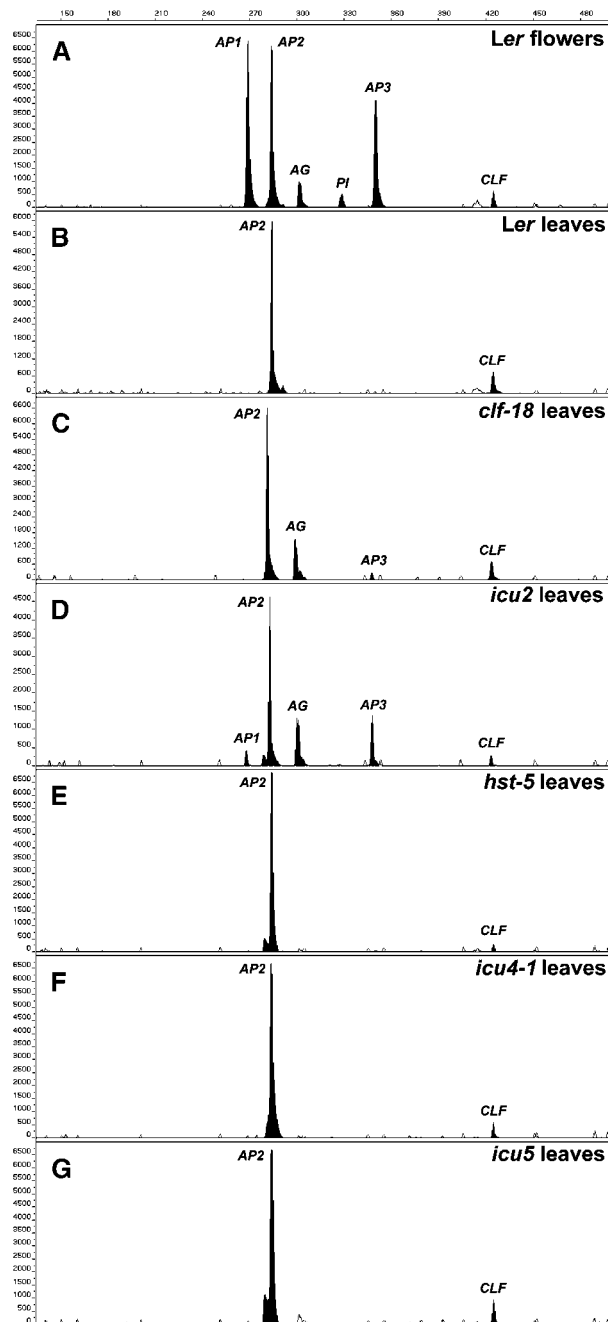

FIGURE 8.—Electrophoregrams illustrating results of multiplex RT-PCR amplifications performed on total RNA from wild-type and mutant *A. thaliana* plants. (A) L*er* flowers (a mixed sample of flower buds and mature flowers). (B) L*er* vegetative leaves. (C–G) *clf-18*, *icu2*, *hst-5*, *icu4-1*, and *icu5* vegetative leaves, respectively. Multiplex PCR products were obtained from reaction mixes including six primer pairs, designed to amplify segments of the genes *AG*, *AP1*, *AP2*, *AP3*, *PI*, and *CLF*. The horizontal and vertical axes indicate, respectively, the size of the electrophoresed molecules (in nucleotides) and the intensity of fluorophore emission (in arbitrary units of fluorescent signal strength). Each electrophoregram corresponds to a single gel lane and contains peaks that represent the molecules obtained from the multiplex PCR amplification of cDNA samples from leaves or flowers excised from individual plants at 21 days and 5 weeks after sowing, respectively. In the electrophoregram, produced by the GENESCAN 2.1 software, every peak is denoted with the name of the corresponding gene. The open peaks in the background correspond to internal molecular weight standards and the black peaks correspond to cDNA from the genes under study.

most *icu* mutations have been found to be recessive, they probably correspond to hypomorphic or null mutations, the only exceptions being those of semidominant *icu4-1* and *icu4-2* and dominant *icu5* alleles. If the latter were neomorphic, antimorphic, or ectopic derepression mutations, the corresponding genes might not necessarily be required for wild-type leaf development. A role for these genes in leaf development could be accepted, however, if they were hypermorphic alleles or mutations in haplo-insufficient loci. Genetic analysis involving triploid plants with a different dosage of dominant or semidominant alleles (TIMPTE *et al.* 1994) will help to discriminate among some of the alternatives above.

Several floral genes are ectopically expressed in *icu2* mutant leaves: Our finding that the Icu2 phenotype is almost completely suppressed in an *ag-1* mutant background suggests that *ICU2* is also required to repress *AG* in the leaves. In addition, the synergistic interaction between *icu2* and *clf-18* suggests that ICU2 may be a member of the multimeric complexes involving Polycomb group proteins.

We have demonstrated the ectopic expression of *AG* and *AP3* floral genes in the leaves of *clf-18*, *clf-61*, and *icu2*, but not in *hst-5*, *icu4-1*, and *icu5* mutants. *icu2* leaves in addition contained *AP1* transcripts. *AP1* and *PI* gene products were not detected in *clf-18* mutant leaves, as described for *clf-2* mutants by means of Northern blots (GOODRICH *et al.* 1997). Hence, the expression patterns revealed by our multiplex RT-PCR method coincide with those obtained previously.

Our multiplex RT-PCR analyses of the ectopic expression of MADS-box genes in *icu2* mutant leaves have allowed visualization and thus confirmation of the proposed interactions between *AG* and *ICU2*. Gene expression patterns were therefore correlated with genetic interactions revealed by the epistatic effect of *ag-1* on *icu2* and by the synergism between *icu2* and *clf-18*. In addition, we have shown that not only *AG* but also *AP1* and *AP3* are misexpressed in *icu2* leaves. Taken together, these observations demonstrate that the *icu2* mutation causes ectopic expression of several floral organ identity genes outside their normal realm of action and indicate that the activity of *ICU2* is required to repress *AP1*, *AP3*, and *AG* expression in wild-type leaves. The genetic and molecular evidences provided in this work clearly indicate that *CLF* and *ICU2* play related roles, making the latter a candidate to belong to the Polycomb group of regulatory genes.

Three independent genetic operations at work in Arabidopsis vegetative leaf development: The observation of phenotypic synergism between nonallelic mutations, as opposed to additivity, might be used as a criterion to identify genetic operations at work in a developmental process, as it has been done in a genetic analysis of Drosophila wing venation pattern formation (DÍAZ-BENJUMEA *et al.* 1989; DÍAZ-BENJUMEA and GARCÍA-BELLIDO 1990). Regarding the lines studied in this work,

double-mutant genotypes involving mutations belonging to different groups of synergism gave rise to additive phenotypes, as would be expected if their gene products act in an independent manner. By contrast, synergistic phenotypes may arise not only as a result of defective overlapping or redundant functions but also as the cumulative result of leaky mutations in genes acting on common targets or along the same pathway. Hence, the functions of the genes damaged in such double mutants displaying synergistic phenotypes are related. Three independent genetic operations at work in vegetative leaf development can be proposed, as suggested by the groups of synergism found in this work. It must be emphasized, however, that the three genetic operations mentioned above, which refer only to the five genes affected by the mutants studied here, represent only a subset of the spectrum of processes contributing to the making of a leaf. In fact, in a large scale screening for viable EMS-induced mutants that approached but did not reach saturation of the genome, 94 different genes were found to yield mutations causing abnormal leaf morphology (BERNÁ *et al.* 1999).

The first genetic operation identified in this work is defined by mutations at two genes, *CLF* and *ICU2*, which contribute to the regulation of the expression of the floral homeotic gene *AG* both in leaves and flowers. We suggest that one of the functions of this group of genes is to act as "leaf identity safeguard genes" since they contribute to the restriction of the expression of floral organ identity genes in vegetative leaves. The cloning of *CLF* has shed light on how the expression of *AG* is silenced in wild-type leaves (GOODRICH *et al.* 1997). The similarity of *CLF* to *Enhancer of zeste*, a member of the Polycomb group (Pc-G) of genes of *Drosophila melanogaster* (JONES and GELBART 1993), suggests a role for this gene in maintaining the repressed state of the homeotic genes through cell divisions (MULLER 1995) and highlights the similarity between the mechanisms involved in the control of homeotic functions in animals and plants, which appeared as a result of independent evolution. The Polycomb and trithorax groups were initially defined in animals on the basis of mutant phenotypes and later found to include genes encoding proteins that can bind to DNA forming part of multimeric complexes and participate in the maintenance of the transcriptional status of homeotic genes (KENNISON 1995).

As far as we know, a patchy distribution of groups of small epidermal cells has been found only in transgenic lines overexpressing *FILAMENTOUS FLOWER* (*FIL*; SAWA *et al.* 1999) but has not been previously reported for any *A. thaliana* leaf mutant, *icu2* being the first one. A similar variegated pattern was found for a *trithorax* allele of *D. melanogaster*, originally named *Regulator of bithorax* (*Rg-bx*), which showed patches of mesothoracic structures in the metathorax (GARCÍA-BELLIDO and CAPDEVILA 1978). The patchy phenotype of *icu2* plants may be presumed to arise from a failure in some mecha-

nism of transcriptional memory, which could be clonally inherited throughout leaf expansion. Given that abaxial cells are smaller than the adaxial ones, the above-mentioned patches can alternatively be seen as a consequence of a change from dorsal to ventral cell fate.

The second genetic operation revealed by our analyses is defined by the synergistic interaction found between mutations at the *HST* (*ICU3*) and *ICU4* loci. Although *HST* has been proposed to play a role in regulating phase change (TELFER and POETHIG 1998), several facts point to an additional role for these genes in the regulation of meristem cell division and/or expansion. In the double mutants, development was delayed, producing an increased number of abnormally shaped leaves in an unordered manner, the epidermal cell size in the leaves was found to be reduced, and in some cases a fasciated stem developed. Further data sustaining our conclusion that the *HST* gene plays a role in a genetic operation different from that involving *CLF* and *ICU2* come from the similar leaf phenotypes of *hst ag* and *hst* individuals that indicate that the Hst mutant phenotype is not due to ectopic expression of *AG* (TELFER and POETHIG 1998). The genetic conclusions made by these authors on the basis of the phenotype of *hst ag* double mutants are reinforced in this work, given that we provide molecular evidence for the absence of *AG* transcripts in *hst-5* leaves. The morphogenetic functions of *HST* and *ICU4* in wild-type leaf organogenesis, however, are yet to be established.

The third genetic operation identified is represented by *ICU5*, whose only mutant allele does not interact with those affecting the remaining genes studied here. Since we have found that *icu5/icu5* individuals present a disrupted photomorphogenetic response, one of the possible causes yielding an Icu phenotype is the perturbation of photomorphogenetic processes or those related to the synthesis, perception, or transduction of hormonal signals. Mutations at the *SHORT HYPOCOTYL2* (*SHY2*) locus, originally identified during a screening for suppressors of the long hypocotyl phenotype of *phyB*, have also been reported to cause an Incurvata phenotype in rosette leaves (KIM *et al.* 1996; REED *et al.* 1998). In addition, mutations in the *AUXIN RESISTANT3* (*AXR3*) gene also result in an Incurvata phenotype (ROUSE *et al.* 1998). Both genes, *SHY2* and *AXR3*, map on top of chromosome 1 and are obvious candidates for being allelic to *ICU5*.

Leaf identity safeguard genes: Although a common evolutionary origin for leaves and floral organs is generally accepted, based mainly on the phenotype that results from removal of the three A, B, and C homeotic functions from floral organs (BOWMAN *et al.* 1991), current evidence indicates that the expression of floral homeotic genes is not sufficient to determine a reproductive fate for leaves. For instance, the ectopic expression of the Arabidopsis floral homeotic gene *AG* in transgenic plants (MIZUKAMI and MA 1992), *clf* mutants

(GOODRICH *et al.* 1997), or the *icu2* mutant (this work) changes the reproductive fate of the first two floral whorls, but only affects leaves by producing a curled phenotype. Similar results have been obtained when the *AG* homologues from petunia (*pMADS3*; TSUCHIMOTO *et al.* 1993), tomato (*TAG1*; PNUELI *et al.* 1994), and cucumber (*CUM1*; KATER *et al.* 1998) were expressed in leaves and flowers. The *ovulata* mutations of *A. majus*, which are ectopic expression alleles of the *AG* homolog *plena*, do not normally lead to floral organ development in bracts and vegetative organs, although in exceptional cases they may cause the presence of stigma-like cells at the leaf tips (BRADLEY *et al.* 1993). Similarly, the *blind* mutation of *Petunia hybrida* is phenocopied by the constitutive expression of *pMADS3* (TSUCHIMOTO *et al.* 1993), and a transposition event at the *pMADS3* gene has been reported to cause the same overexpression phenotype (KATER *et al.* 1998). The *blind* mutant was originally suggested to be affected in a negative regulator of *pMADS3* since its leaves and sepals curled up and its flowers were similar to those of *ap2* mutants, although, once again, no transformations of vegetative into reproductive organs were reported (TSUCHIMOTO *et al.* 1993). All the above-mentioned difficulties in inducing a reproductive fate in leaves by expressing floral homeotic genes gave support to the suggestion that additional factors are needed to specify such a fate in the inflorescence. An alternative explanation could be that the expression of floral homeotic genes in the leaves is restricted by controls that preserve the leaf organ identity. As such, the regulatory pathway involving *CLF* and *ICU2* genes accounts for the absence of expression of *AG* and other floral genes in wild-type Arabidopsis leaves. Mutations in genes such as *CLF* and *ICU2* would cause the ectopic expression of one or more floral genes in the leaves, which in turn would coerce leaf cells to divide and expand abnormally. Under the above hypothesis, a visible homeotic transformation of a leaf into any of the floral organs would be obtained only when there was failure of the genes preserving both the initiation and the maintenance states of the organ identity of the leaf. In our view, these so-called leaf identity safeguard genes might operate by repressing floral genes not only in the leaves but also in some floral organs as well as in other plant organs and developmental stages.

Even though transgenic plants expressing the floral homeotic genes *APETALA3* (*AP3*) or *PISTILLATA* (*PI*) alone did not show any alteration in their vegetative leaves (JACK *et al.* 1994; KRIZEK and MEYEROWITZ 1996; GOODRICH *et al.* 1997), doubly transgenic plants expressing both genes became early flowering and their leaves curled up (KRIZEK and MEYEROWITZ 1996), as happens in *clf* mutants (GOODRICH *et al.* 1997). Some cauline leaves were partially transformed to petalloid organs in these plants, a situation similar to that found in the bracts of transgenic plants expressing *NAG1*, the to-

bacco homolog of *AG*, which developed as carpelloid organs (MANDEL *et al.* 1992). Following from this, KRIZEK and MEYEROWITZ (1996) proposed the existence of a floral character gradient throughout the plant, so that cauline leaves would have more floral character than the vegetative ones, thereby explaining why transformations occur only late in development. Additional support for the idea of Icu leaf phenotypes being caused by the deregulation of MADS-box genes comes from plants expressing an antisense methyltransferase construct. Apart from displaying an Icu phenotype, these transgenic plants showed reduced DNA methylation levels and ectopic expression of *AG* and *AP3* in their leaves (FINNEGAN *et al.* 1996).

Together with those that we have named leaf identity safeguard genes, other repressor genes are required for a proper leaf organogenesis. Such is the case of *rough sheath2* (*rs2*), the maize ortholog of *PHAN*, which operates repressing *KNOX* genes in the leaves (TIMMERMANS *et al.* 1999; TSIANTIS *et al.* 1999). Ectopic expression of *KNOX* genes in *rs2* maize mutants causes leaf cells to adopt a proximal fate, an observation that has been used to reinterpret the Phan phenotype as a proximalization of leaf distal tissues, making them unifacial such as those of the petiole (TSIANTIS *et al.* 1999). Taken together, molecular evidence for the *rs2* maize mutant phenotype, the reinterpreted Phan phenotype, and the results presented in this work indicate that several genetic operations have evolved to repress in the leaves genes whose activity is required in other developmental stages or domains.

Partial suppression of the Incurvata phenotype in a L*er* genetic background: A role for *AG* in determining flowering time has been proposed on the basis of the early flowering phenotype seen in transgenic plants constitutively expressing the gene (MIZUKAMI and MA 1997), raising the possibility that the early flowering phenotype of the *clf* and *icu2* mutants is caused by the ectopic expression of *AG*. As previously reported for *clf* mutants (GOODRICH *et al.* 1997), we have found that the early flowering conferred by the *icu2* mutation was only partially suppressed in an *ag-1* genetic background, raising the possibility of a role for the *ICU2* gene in regulating flowering time independently of *AG*. Nevertheless, as indicated for *clf* mutants by GOODRICH *et al.* (1997), this may be merely a consequence of residual *AG* expression of the *ag-1* allele.

In addition, we have found that the effect of *clf* and *icu2* mutations on flowering time and leaf shape was reduced in lines bearing the *er* mutation. Thus, it is suggested that an allele of *ER* or other closely linked genes present in the L*er* background might modulate the effects in the *CLF* and *ICU2* genes. Actually, flowering time under long-day conditions was reported to differ by only 2 days between the *clf-2* mutant and L*er*, its wild-type ancestor (GOODRICH *et al.* 1997), whereas we found a difference of 2 wk for *clf* alleles compared

to the En-2 ecotype under continuous lighting. A likely explanation for this observation would be that the presence of the modifier in the L*er* background delays the flowering time of *clf* and *icu2* mutants at least under long-day conditions. In agreement with this, GOODRICH *et al.* (1997) observed minimum differences in leaf shape and flowering time between the wild type and the *clf-2 ag-1* double mutant under long-day conditions with a L*er* background. Such a modifier would explain why the differences in flowering time are more conspicuous in the En-2 ecotype and why no mutant allele of *ICU2* was found in a screening for leaf mutants performed in a L*er* background (BERNÁ *et al.* 1999).

Role of *ICU* genes in regulating phase change: Leaf phenotypes such as those of the *icu* mutants studied in this work are displayed by Arabidopsis transgenic lines simultaneously overexpressing two of the genes that regulate the competence to flowering. This is the case of a 35S::*LFY* 35S::*FPF1* strain, whose leaves are similar to those of *hst* mutants (MELZER *et al.* 1999). Constitutive expression of *FPF1* (*FLOWERING PROMOTING FACTOR1*; KANIA *et al.* 1997) causes early flowering and shortening of the juvenile phase, as inferred from the presence of abaxial trichomes in the earlier vegetative leaves (MELZER *et al.* 1999), a trait shared by *hst* mutants (TELFER and POETHIG 1998). In addition, *lfy* mutations do not modify flowering time in either *hst* or 35S::*FPF1* plants, indicating that *HST* and *FPF1* behave similarly (MELZER *et al.* 1999). The synergistic nature of the phenotype of the *hst-5 icu4-1* double mutant suggests that *ICU4* is a novel component of the developmental pathway that involves *HST* and *FPF1*.

LITERATURE CITED

BATESON, W., 1913 *Problems of Genetics*. Mrs. Hepsa Ely Silliman Memorial Lectures. Yale University, New Haven, CT.

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

BERNÁ, G., P. ROBLES and J. L. MICOL, 1999 A mutational analysis of leaf morphogenesis in *Arabidopsis thaliana*. Genetics **152:** 729–742.

BOWMAN, J. L., D. R. SMYTH and E. M. MEYEROWITZ, 1989 Genes directing flower development in Arabidopsis. Plant Cell **1:** 37–52.

BOWMAN, J. L., D. R. SMYTH and E. M. MEYEROWITZ, 1991 Genetic interactions among floral homeotic genes of Arabidopsis. Development **112:** 1–20.

BRADLEY, D., R. CARPENTER, H. SOMMER, N. HARTLEY and E. COEN, 1993 Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the *plena* locus of Antirrhinum. Cell **72:** 85–95.

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.

DÍAZ-BENJUMEA, F. J., and A. GARCÍA-BELLIDO, 1990 Genetic analysis of the wing vein pattern of Drosophila. Roux's Arch. Dev. Biol. **198:** 336–354.

DÍAZ-BENJUMEA, J., M. A. F. GAITÁN and A. GARCÍA-BELLIDO, 1989 Developmental genetics of the wing vein pattern of Drosophila. Genome **31:** 612–619.

EDWARDS, K., C. JOHNSTONE and C. THOMPSON, 1991 A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. **19:** 1349.

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

GARCÍA-BELLIDO, A., and M. P. CAPDEVILA, 1978 The initiation and maintenance of gene activity in a developmental pathway of Drosophila, pp. 3–21 in *The Clonal Basis of Development*, edited by S. SUBTELNY and I. M. SUSSEX. Academic Press, New York.

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.

HAKE, S., and N. SINHA, 1991 Genetic analysis of leaf development, pp. 187–222 in *Oxford Surveys of Plant Molecular and Cell Biology*, edited by B. J. MIFLIN. Oxford University Press, London.

HALL, L. N., and J. A. LANGDALE, 1996 Molecular genetics of cellular differentiation in leaves. New Phytol. **132:** 533–553.

JACK, T., G. L. Fox and E. M. MEYEROWITZ, 1994 Arabidopsis homeotic gene *APETALA3* ectopic expression: transcriptional and posttranscriptional regulation determine floral organ identity. Cell **76:** 703–716.

JONES, R. S., and W. M. GELBART, 1993 The Drosophila Polycombgroup gene *Enhancer of zeste* contains a region with a sequence similarity to *trithorax*. Mol. Cell. Biol. **13:** 6357–6366.

KANIA, T., D. RUSSENBERGER, S. PENG, K. APEL and S. MELZER, 1997 *FPF1* promotes flowering in Arabidopsis. Plant Cell **9:** 1327–1338.

KATER, M. M., L. COLOMBO, J. FRANKEN, M. BUSSCHER, S. MASIERO *et al.*, 1998 Multiple *AGAMOUS* homologs from Cucumber and Petunia differ in their ability to induce reproductive organ fate. Plant Cell **10:** 171–182.

KENNISON, J. A., 1995 The Polycomb and trithorax group proteins of Drosophila: trans-regulators of homeotic gene function. Annu. Rev. Genet. **29:** 289–303.

KIM, B. C., M. S. SOH, B. J. KANG, M. FURUYA and H. G. NAM, 1996 Two dominant photomorphogenic mutations of *Arabidopsis thaliana* identified as suppressor mutations of *hy2*. Plant J. **9:** 441–456.

KIM, G., H. TSUKAYA and H. UCHIMIYA, 1998 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.

KOSAMBI, D. D., 1944 The estimation of map distance from recombination values. Ann. Eugen. **12:** 172–175.

KRANZ, A. R., 1978 Demonstration of new and additional population samples and mutant lines of the AIS-seed bank. Arabidopsis Inf. Serv. **15:** 118–139.

KRANZ, A. R., and B. KIRCHHEIM, 1987 Genetic resources in Arabidopsis. Arabidopsis Inf. Serv. **24**.

KRIZEK, B. A., and E. M. MEYEROWITZ, 1996 The Arabidopsis homeotic genes *APETALA3* and *PISTILLATA* are sufficient to provide the B class organ identity function. Development **122:** 11–22.

MANDEL, M. A., J. L. BOWMAN, S. A. KEMPIN, H. MA, E. M. MEYEROWITZ *et al.*, 1992 Manipulation of flower structure in transgenic tobacco. Cell **71:** 133–143.

MCHALE, N. A., 1993 *LAM-1* and *FAT* genes control development of the leaf blade in *Nicotiana sylvestris*. Plant Cell **5:** 1029–1038.

MEINKE, D., and M. KOORNNEEF, 1997 Community standards for Arabidopsis genetics. Plant J. **12:** 247–253.

MELZER, S., G. KAMPMANN, J. CHANDLER and K. APEL, 1999 *FPF1* modulates the competence to flowering in Arabidopsis. Plant J. **18:** 395–405.

MIZUKAMI, Y., and H. MA, 1992 Ectopic expression of the floral homeotic gene *AGAMOUS* in transgenic Arabidopsis plants alters floral organ identity. Cell **71:** 119–131.

MIZUKAMI, Y., and H. MA, 1997 Determination of Arabidopsis floral meristem identity by *AGAMOUS*. Plant Cell **9:** 393–408.

MULLER, J., 1995 Transcriptional silencing by the Polycomb protein in Drosophila embryos. EMBO J. **14:** 1209–1220.

PNUELI, L., D. HAREVEN, S. D. ROUNSLEY, M. F. YANOFSKI and E. LISFSCHITZ, 1994 Isolation of the tomato *AGAMOUS* gene *TAG1* and analysis of its homeotic role in transgenic plants. Plant Cell **6:** 163–173.

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:** 497–501.

PONCE, M. R., P. ROBLES and J. L. MICOL, 1999 High-throughput genetic mapping in *Arabidopsis thaliana*. Mol. Gen. Genet. **261:** 408–415.

PONCE, M. R., J. M. PÉREZ-PÉREZ, P. PIQUERAS and J. L. MICOL, 2000 A multiplex RT-PCR method for fluorescence-based semiautomated detection of gene expression in *Arabidopsis thaliana*. Planta **211:** 606–608.

POZZI, C., K. J. MÜLLER, W. ROHDE and F. SALAMINI, 1999 Leaf development, pp. 145–165 in *Development: Genetics, Epigenetics and Environmental Regulation*, edited by V. E. A. RUSSO, D. J. COVE, L. G. EDGAR, R. JAENISCH and F. SALAMINI. Springer-Verlag, Berlin-Heidelberg.

REED, J. W., R. P. ELUMALAI and J. CHORY, 1998 Suppressors of an *Arabidopsis thaliana phyB* mutation identify genes that control light signalling and hypocotyl elongation. Genetics **148:** 1295–1310.

RELICHOVA, J., 1976 Some new mutants. Arabidopsis Inf. Serv. **13:** 25–28.

RIECHMANN, J. L., and E. M. MEYEROWITZ, 1997 MADS domain proteins in plant development. Biol. Chem. **378:** 1079–1101.

RIECHMANN, J. L., and E. M. MEYEROWITZ, 1998 The AP2/EREBP family of plant transcription factors. Biol. Chem. **379:** 633–646.

ROUSE, D., P. MACKAY, P. STIRNBERG, M. ESTELLE and O. LEYSER, 1998 Changes in auxin response from mutations in an *AUX/IAA* gene. Science **279:** 1371–1373.

RÜFFER-TURNER, M., and K. NAPP-ZINN, 1980 Investigations on leaf structure in several genotypes of *Arabidopsis thaliana* (L.) Heynh. Arabidopsis Inf. Serv. **16:** 94–98.

SAWA, S., K. WATANABE, K. GOTO, E. KANAYA, E. H. MORITA *et al.*, 1999 *FILAMENTOUS FLOWER*, a meristem and organ identity gene of Arabidopsis, encodes a protein with a zinc finger and HMG-related domains. Genes Dev. **13:** 1079–1088.

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. **261:** 725–739.

SINHA, N., S. HAKE and M. FREELING, 1993 Genetic and molecular analysis of leaf development. Curr. Top. Dev. Biol. **28:** 47–80.

SMITH, L. G., and S. HAKE, 1992 The initiation and determination of leaves. Plant Cell **4:** 1017–1027.

TELFER, A., and R. S. POETHIG, 1994 Leaf development in Arabidopsis, pp. 379–401 in *Arabidopsis*, edited by E. M. MEYEROWITZ and C. R. SOMMERVILLE. 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 *Arabidopsis thaliana*. Development **125:** 1889–1898.

TIMMERMANS, M. C., A. HUDSON, P. W. BECRAFT and T. NELSON, 1999 *ROUGH SHEATH2*: a Myb protein that represses knox homeobox genes in maize lateral organ primordia. Science **284:** 151–153.

TIMPTE, C., A. K. WILSON and M. ESTELLE, 1994 The *axr2-1* mutation of *Arabidopsis thaliana* is a gain-of-function mutation that disrupts an early step in auxin response. Genetics **138:** 1239–1249.

TSIANTIS, M., and J. A. LANGDALE, 1998 The formation of leaves. Curr. Opin. Plant Biol. **1:** 43–48.

TSIANTIS, M., R. SCHNEEBERGER, J. F. GOLZ, M. FREELING and J. A. LANGDALE, 1999 The maize *rough sheath2* gene and leaf development programs in monocot and dicot plants. Science **284:** 154–156.

TSUCHIMOTO, S., A. R. VAN DER KROL and N-H. CHUA, 1993 Ectopic expression of *pMADS3* in transgenic petunia phenocopies the petunia *blind* mutant. Plant Cell **5:** 843–853.

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 dicotyledoneous plants. J. Plant Res. **108:** 407–416.

Van Lijsebettens, M., and J. Clarke, 1998   Leaf development in Arabidopsis. Plant Physiol. Biochem. **36:** 47–60.

Villani, P. J., and D. A. Demason, 1997   Roles of the *AF* and *TL* genes in pea leaf morphogenesis: characterization of the double mutant (*af af tl tl*). Am. J. Bot. **84:** 1323–1336.

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.

Yanofsky, M. F., H. Ma, J. L. Bowman, G. N. Drews, K. A. Feldmann et al., 1990   The protein encoded by the Arabidopsis homeotic gene *AGAMOUS* resembles transcription factors. Nature **346:** 35–39.

Communicating editor: V. Sundaresan