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

Manuscript received February 14, 2000
Accepted for publication July 21, 2000

**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; RELICHHOVA 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 MCIALE (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; TSANTIS 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. Micó, 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.) Heynh. 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. Röbbe- len 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°C ± 1°C 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*₂ progeny of crosses between mutant and En-2 wild-type plants and further confirmed in the inbred *F*₃ generation.

Complementation groups were defined after the observation of the *F*₂ 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 (*Ler*) 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 (Maps; 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 0.925 units/μl of Bio Taq polymerase (BioLine), and no hot start was performed. Unlike other genes, the mapping of *ICU* 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*₂ siblings obtained by selfing *F*₁ 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* and *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*₂ families were derived from individual *F*₁ 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% OsO₄ 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 CO₂, 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°C 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°C 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 Kirk-Hime 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 represented among the icu mutants studied. The map positions of the ICU loci were determined after polymorphic SSLP markers in a population consisting of phenotypically recessive F₁ 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 were not found either between reciprocal crosses or between crosses involving a given mutant strain and Ler or En-2 ecotypes. The analysis of the corresponding F₁ and inbred F₂ 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 F₁ individuals (see MATERIALS AND METHODS). The complementation groups were defined as follows.

**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 2.—Rosette phenotypes of single and double *incurvata* mutants. (A) Wild-type En-2 rosette. (B) *df-18*/*df-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 *df-18*/*df-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) *df-18*/*df-18;icu2/icu2* double mutant displaying strongly involute leaves and a very small rosette as compared to each single mutant. (H) *df-18*/*df-18;hst-5/hst-5*, (I) *df-18*/*df-18;icu4-1/icu4-1*, and (J) *df-18*/*df-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 *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, *df-18*, *df-21*, *df-22*, *df-24*, and *df-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

<table>
<thead>
<tr>
<th></th>
<th>N313</th>
<th>N328</th>
<th>N345</th>
<th>N346</th>
<th>N347</th>
<th>N350</th>
<th>N351</th>
<th>N419</th>
<th>N314</th>
<th>N329</th>
<th>N400</th>
<th>N401</th>
<th>pif-1</th>
<th>pif-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>♂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N313 (df-1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N328 (df-17)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N345 (df-18)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N346 (df-21)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N347 (df-22)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N350 (df-19)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N351 (df-24)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N314 (hst-5)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N329 (icu2)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N400 (icu4-1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N401 (icu4-2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N379 (icu5)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pif-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pif-2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

All mutant strains were homozygous. Symbols indicate wild-type (+) or mutant (−) phenotype of F1 progeny from the crosses indicated. The F1 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.

Appearance of wild-type individuals in the F2 of the corresponding crosses.

a Absence of wild-type individuals in the F2 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 elf 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, 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-S6 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 elf, 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-
leaves and rosettes were similar in size (Figure 1, B and C), and leaf curling in double mutants such as *clf-18/C*, and Figure 2, B and C), those of *leaf phenotype*. Whereas *clf-18* selected from the alleles of *CLF* tissue were smaller and more rounded in *clf-18* revealed that the pavement cells in the adaxial epidermis clearly showed obvious differences from the wild type, allowing us to observe under a scanning electron microscope responses. Fully expanded *Incurvata* leaves were expected to resemble single mutants (Figure 5, B and C).

**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). *df-18* was selected from the alleles of *CLF* because of its extreme leaf phenotype. Whereas *df-18* and *icu2/icu2* leaves and rosettes were similar in size (Figure 1, B and C, and Figure 2, B and C), those of *df-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 *df-18/icu2/icu2* leaves (Figure 4D) than in both single mutants (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).

**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.

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
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 (Mizu-kami 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 (Yano-fsky 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 F1 and F2 families (Tables 2 and 3) as well as F1 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>
<thead>
<tr>
<th>TABLE 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analysis of F2 families studied in a search for clf ag-1 and icu2 ag-1 double mutants</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of families studied</th>
<th>F1 parental</th>
<th>F1 progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phenotype</td>
<td>Inferred genotype</td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>AG/ag-1;CLF/clf-18</td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>AG/AG;CLF/clf-18</td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>AG/ag-1;CLF/clf-22</td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>AG/AG;CLF/clf-22</td>
</tr>
<tr>
<td>3</td>
<td>WT</td>
<td>AG-ag-1;ICU2/icu2</td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>AG/AG;ICU2/icu2</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>TABLE 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analysis of F3 families studied in a search for clf ag-1 and icu2 ag-1 double mutants</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of families studied</th>
<th>F2 parental</th>
<th>F3 progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phenotype</td>
<td>Inferred genotype</td>
</tr>
<tr>
<td>10</td>
<td>WT</td>
<td>AG/AG;CLF/CLF</td>
</tr>
<tr>
<td>24</td>
<td>WT</td>
<td>AG/AG;CLF/clf-18</td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>AG/ag-1;CLF/CLF</td>
</tr>
<tr>
<td>5</td>
<td>WT</td>
<td>AG-ag-1;CLF/clf-18</td>
</tr>
<tr>
<td>1</td>
<td>Icu</td>
<td>AG/AG;clf-18/clf-18</td>
</tr>
<tr>
<td>1</td>
<td>Icu</td>
<td>AG-ag-1;clf-18/clf-18</td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>AG/AG;CLF/CLF</td>
</tr>
<tr>
<td>4</td>
<td>WT</td>
<td>AG/AG;clf-22/clf-22</td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>AG-ag-1;clf-22/clf-22</td>
</tr>
<tr>
<td>3</td>
<td>Icu</td>
<td>AG/AG;clf-22/clf-22</td>
</tr>
<tr>
<td>2</td>
<td>Icu</td>
<td>AG-ag-1;clf-22/clf-22</td>
</tr>
<tr>
<td>1</td>
<td>Icu</td>
<td>AG/AG;ICU2/icu2</td>
</tr>
<tr>
<td>12</td>
<td>Icu</td>
<td>AG-ag-1;icu2/icu2</td>
</tr>
<tr>
<td>11</td>
<td>Icu</td>
<td>AG/AG;ICU2/icu2</td>
</tr>
<tr>
<td>12</td>
<td>Icu and Er</td>
<td>AG-ag-1;icu2/icu2</td>
</tr>
<tr>
<td>4</td>
<td>Icu and Er</td>
<td>AG-ag-1;icu2/icu2</td>
</tr>
<tr>
<td>4</td>
<td>Icu and Er</td>
<td>AG/AG;icu2/icu2</td>
</tr>
</tbody>
</table>

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

* Plants displayed wild-type leaves but earlier flowering time than the wild type.

+ icu2/icu2;er/er individuals showed a flowering time intermediate between those of wild-type and icu2/icu2;Er/Er plants.
a role for AG derepression in the icu2 mutant. After selfing of F1 ICU2/ICU2;AG/AG-1 individuals, their F2 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 F2 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 elf-18/elf-18;ag-1/ag-1 and elf-22/elf-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 RIECHMANN 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, elf-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 elf-61 allele (data not shown; this mutation was initially named icu1-10 in BERNÁ et al. 1999), but not in elf-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 F2 and F3 families studied to obtain the elf-18 ag-1, elf-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 elf/elf, as well as flowering time of icu2/ICU2;ER/ER plants, were intermediate between those of wild-type and elf/elf;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 Ler genetic background (BERNÁ et al. 1999). These results suggest that an allele modifying both the flowering time and leaf curling characteristic of elf and icu2 mutants exists in the Ler 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-25, 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 M1 plant.

---

**Figure 8.** Electrophoregrams illustrating results of multiplex RT-PCR amplifications performed on total RNA from wild-type and mutant *Arabidopsis thaliana* plants. (A) Ler flowers (a mixed sample of flower buds and mature flowers). (B) Ler 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 (Pe-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 Arabidopsis 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 phenotypetype 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 HYOCOTYL2 (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; Tsukimoto et al. 1993), tomato (TAGI; Pnueli et al. 1994), and cucumber (CUMI; 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 (Tsukimoto 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 (Tsukimoto 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 petaloid 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 Ler 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 df and icu2 mutants is caused by the ectopic expression of AG. As previously reported for df 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 df 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 df 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 Ler 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 df-2 mutant and Ler, its wild-type ancestor (Goodrich et al. 1997), whereas we found a difference of 2 wk for df 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 Ler background delays the flowering time of df 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 df-2 ag-1 double mutant under long-day conditions with a Ler 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 Ler 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, ify 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.

We thank the NASC for providing seeds of mutants, S. Gerber and J. M. Serrano for their technical help, J. M. Martinez-Zapater for kindly providing the pfi mutants, and J. Goodrich, A. Martinez-Laborda, V. Quesada, A. Vera, and two anonymous referees for comments on the manuscript. This research was supported by PB91-0749, ACP95-019, and PB95-0685 grants from the Ministerio de Educación y Cultura de Spain. H. Candela, J. M. Pérez-Pérez, P. Piqueras, and J. Serrano-Cartagena were fellows of the Conselleria de Cultura, Educación i Ciencia del Generalitat Valenciana. P. Robles was fellow of the Dirección General de Enseñanza Superior e Investigación Científica de Spain.

LITERATURE CITED

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


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.
Tsuchi, T., H. Tsukaya and H. Uchimiya, 1996 Two independent


Communicating editor: V. Sundaresan