Short Report

Genetic and molecular analysis of *INCURVATA2,* a negative regulator of floral homeotic genes in the leaves of *Arabidopsis thaliana*

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ABSTRACT One of the largest available collections of plant morphological mutants is the *Arabidopsis* Information Service (AIS) Form Mutants collection. We studied 152 AIS lines already known to display abnormally shaped leaves, finding 22 that exhibited involute, upwardly curled, vegetative leaves, a phenotype that we named Incurvata (Icu). Here we present advances in a positional attempt to clone the *INCURVATA2* gene, whose recessive allele *icu2* is carried by the AIS line N329, and which was mapped near the lower telomere of chromosome 5. The *icu2* mutation causes early flowering and Apetala flowers, together with involute leaves, a phenotypic trait associated to ectopic derepression in the leaves of the *AGAMOUS* and *APETALA3* floral organ identity genes.

As a contribution to a better understanding of the developmental processes underlying leaf elaboration, we studied 152 *Arabidopsis thaliana* mutant lines already known to display abnormally shaped leaves, which belong to the *Arabidopsis* Information Service Form Mutants collection, gathered by A. R. Kranz and currently stored at the Nottingham *Arabidopsis* Stock Centre (Serrano-Cartagena *et al.*, 1999). A group of 13 such mutants, originally isolated by G. Röbbelen, displayed curled, involute leaves, a phenotype that we named Incurvata (Icu). Their complementation analysis indicated that the mutants correspond to five genes, one of which, *ICU2*, was represented by a single allele, *icu2*, carried by the AIS line N329.

We found that the AIS mutants that we initially named *icu1* carry alleles of the Polycomb-group gene *CURLY LEAF* (*CLF*; Goodrich *et al.*, 1997). Involute leaves (Fig. 1B), early flowering (about 15 days after sowing) and Apetala flowers were pleiotropic traits that *icu2* individuals shared with *clf* mutants. However, not all the leaves of a given *icu2* plant curled upwards (Fig. 1C) and not as strongly as those of *clf* mutants. Patches of epidermal tissue with a reduced cell size, resulting in an uneven leaf surface, were consistently present as a regular feature of the Icu2 phenotype. Such patches are randomly distributed on the adaxial side of the leaves. Similar to *clf* plants, *icu2* mutants showed low fertility and a thin flowering stem compared to the wild type.

The leaf phenotype of the *icu2* mutant is suppressed in an *agamous* background, as described for *clf* mutants (Fig. 1D). Furthermore, the phenotype of *clf icu2* double mutants was found to be synergistic. In addition, we developed a non-radioactive, rapid and sensitive method for the simultaneous detection of several mRNA molecules (Ponce *et al.*, 2000), which was used to test for the activity of several genes in flowers and leaves. We analyzed transcription of the Polycomb-group gene *CLF*, together with that of some floral homeotic genes, 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. It was found that some floral organ identity genes are ectopically derepressed in the leaves of *icu2* mutants, as is known to occur in *clf* plants (Fig. 1E). 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 (Serrano-Cartagena *et al.*, 2000).

Linkage analysis to SSLP (simple sequence length polymorphisms) markers was used as a first step towards the positional cloning of *ICU2*. The gene was found to be linked to the nga129 and MBK5 microsatellites, at the lower arm of chromosome 5, using a mapping population of 41 F$_2$ individuals from a cross between homozygous *icu2* individuals (in an En-2 background) and the ecotype L*er*. Since *ICU2* was found to be 8.62±3.18 cM away from MBK5, we took advantage of the genomic sequence available for this region to design nine new SSLP markers located distally to MBK5 (Fig. 2), in an attempt to achieve the high resolution mapping of this gene. After the analysis of 670 F$_2$ plants, the candidate region was limited to a contig of three overlapping transformation-competent artificial chromosome clones (TACs; Liu *et al.*, 1999): K21H1, K3G17 and K8K14.

We are currently sequencing several candidate genes included in this region in order to identify the *ICU2* gene or, alternatively, to find new additional markers to narrow the candidate interval. Transformation by infection of *icu2* mutant plants with the above mentioned TACs is also in progress, in an attempt to restore the wild-type phenotype.

We have proposed that, as flowers evolved, a new major class of genes, including *CLF* and *ICU2*, may have been recruited to safeguard leaf identity, since they contribute to the restriction of the expression of floral organ identity genes in vegetative leaves (Serrano-Cartagena *et al.*, 2000). The cloning of *ICU2* will shed light on the genetic operations that have evolved to repress in the leaves genes whose activity is required in other developmental stages or domains.

Materials and Methods

Plants were grown as previously described (Ponce *et al.*, 1998), at 20±1°C and 60-70% relative humidity under continuous
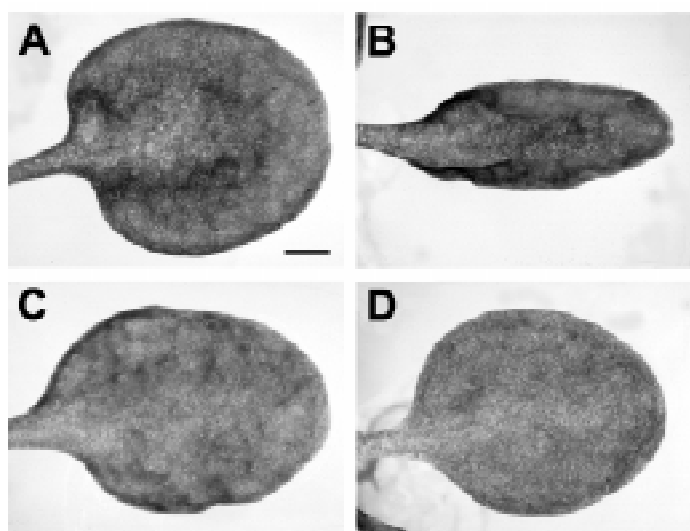
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Fig. 1. The Icu2 leaf phenotype. (A) *Wild-type Enkheim-2 rosette leaf showing its characteristically flattened morphology.* **(B,C)** *icu2/icu2 mutant leaves.* **(D)** *icu2/icu2;ag-1/ag-1 double mutant displaying its wild-type phenotype. Scale bar, 1 mm. Pictures were taken 20 days after sowing.* **(E)** *Electrophoregrams illustrating results of multiplex RT-PCR amplifications performed on total RNA from wild-type (Ler) and mutant plants. The horizontal and vertical axes indicate, respectively, the size of electrophoresed molecules (in nucleotides) and the intensity of fluorophore emission (in arbitrary units of fluorescent signal strength).*
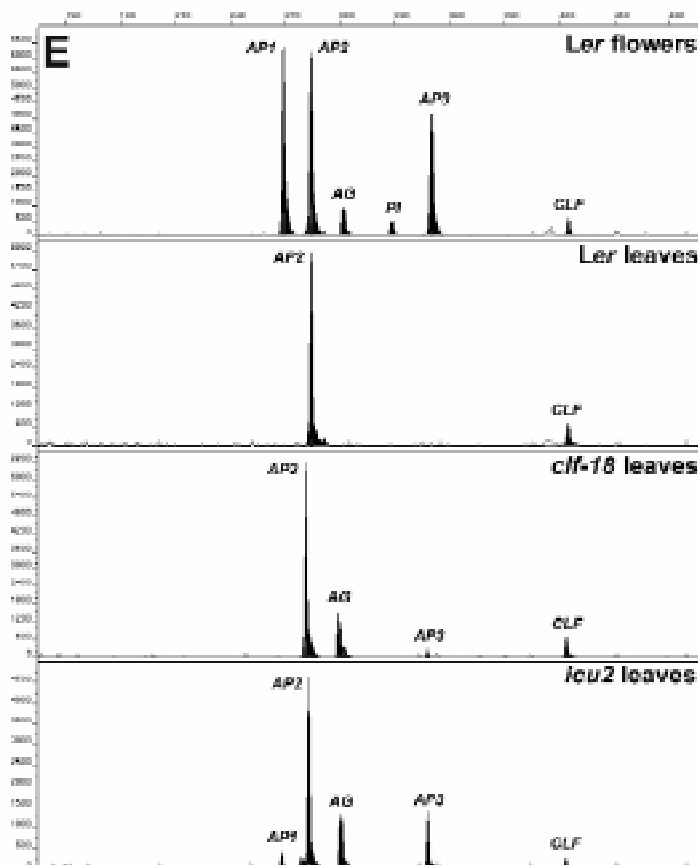
fluorescent light (7,000 lx). Linkage analysis and transcription assays were performed, respectively, as described in Ponce *et al.*, 1999, and Ponce *et al.*, 2000.

Fig. 2. Map-based strategy followed to identify the *ICU2* gene. *1,340 chromosomes were analysed, obtaining 59 recombinants (shown in parentheses) relative to 10 molecular markers designed after testing for polymorphism repetitive sequences in the candidate region. Sequencing of some candidate genes in the informative recombinants rendered new single nucleotide polymorphisms (SNP) that allowed us to locate the ICU2 gene within a 120 kb interval encompassed by 3 overlapping TACs.*

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SERRANO-CARTAGENA, J., CANDELA, H., ROBLES, P., PONCE, M.R., PEREZ-PEREZ, J.M., PIQUERAS, P. and MICOL, J.L. (2000). Genetic analysis of *incurvata* mutants reveals three independent genetic operations at work in *Arabidopsis* 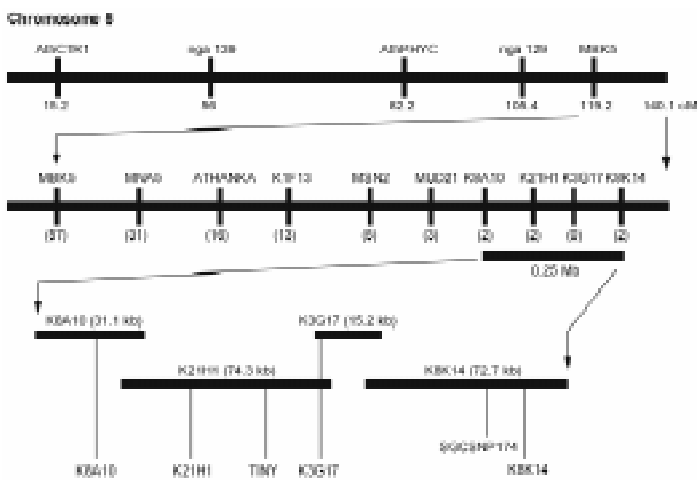leaf morphogenesis. *Genetics* 156: 1363-1377.