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Short Report

Isolation and characterization of fast-neutron induced mutants with abnormal leaf morphology in *Arabidopsis thaliana*

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ABSTRACT In order to identify novel genes involved in the morphogenesis of plant leaves and to obtain new null alleles of previously identified genes, we screened 23,445 M_2 *Arabidopsis thaliana* individuals, the progeny of 2,931 M_1 Landsberg *erecta* parentals exposed to fast neutron bombardment. We isolated 901 M_2 putative mutants, most of which showed unstable phenotypes that were lost after one or two generations of selfing. We subjected to genetic analysis 25 mutants, whose phenotypes were transmitted with complete penetrance and small variations in expressivity, which fell into 9 complementation groups. Some of them corresponded to genes previously undescribed at the mutational level, their mutant alleles causing a dentate leaf marginal configuration and reduced number of mesophyl cells, some displayed bilaterally asymmetric leaves, and others had either involute or small leaves. Novel alleles of previously described genes, such as *CLF* and *AS1*, were also obtained.

The isolation and molecular analysis of leaf form mutants, together with the wealth of information available on plant leaves at the morphological level, is beginning to unravel the processes underlying the making of a leaf (recently reviewed in Byrne *et al.,* 2001). Our contributions to the genetic dissection of leaf ontogeny in *Arabidopsis thaliana* consisted of the analysis of natural variations in overall leaf morphology (J.M. Pérez-Pérez, J. Serrano-Cartagena and J.L. Micol, submitted) and venation pattern (Candela *et al.,* 1999), the study of mutants with abnormally shaped or sized leaves, belonging to already existing collections (Serrano-Cartagena *et al.*, 1999; 2000), and the isolation and study of novel EMS-induced leaf mutants in a large-scale screening, which were shown to correspond to 94 genes (Berná *et al.*, 1999), the map position of 76 of which has been determined (Robles and Micol, 2001).

With the aim of isolating null or extremely hypomorphic alleles of genes required for leaf development, we performed a mutant search, screening 23,445 M_2 *Arabidopsis thaliana* individuals, the progeny of 2,931 M_1 Landsberg *erecta* (L*er*; Fig. 1A) parentals exposed to fast neutron bombardment, a mutagenesis procedure that causes mainly deletions (Shirley *et al.*, 1992), and selected plants displaying alterations in the morphology of their vegetative leaves. A total of 901 M_2 putative mutants were isolated, most of which showed unstable phenotypes that were lost after one or two generations of selfing. We subjected to genetic analysis a total of 25 mutants, whose leaf phenotypes were transmitted with complete penetrance and small variations in expressivity, which fell into 9 complementation groups. Some of them corresponded to genes previously undescribed at the mutational level, such as those causing a dentate leaf marginal configuration and reduced number of mesophyl cells (*denticulata29* and *30*; *den29* and *den30*); others displayed bilaterally asymmetric leaves (*asymmetric leaves3*; *as3*); some had involute leaves (*incurvata8* and *15*; *icu8* and *icu15*); and others small leaves (*exigua9*; *exi9*). Novel alleles of previously described genes, such as *CURLY LEAF* (*CLF*; Goodrich *et al.*, 1997; Fig. 1G) and *ASYMMETRIC LEAVES1* (*AS1*; Rédei and Hirono 1964) were also obtained.

The *as* mutants display bilaterally asymmetric laminae with respect to the proximo-distal axis defined by the midvein of the leaf. We have isolated a new allele, *as1-14*, of the previously described *AS1* complementation group. The leaves of this mutant are wider than those of the wild type and show more than one midvein (Fig. 1B). We also identified a new *AS* locus, whose *as3* allele causes the leaf lamina to fold downwards obliquely to the midvein (Fig. 1C). Its cotyledons and juvenile leaves are wider than those of the wild type, and marginal indentations appear as early as in the third vegetative leaf and are more prominent as the shoot develops. The mutant is poorly fertile and displays a bushy inflorescence. The surface of some cauline leaves exhibits stigmatic papillae, reminiscent to those found in the wild-type gynoecium.

The leaves of the *den29* mutant are narrow and pointed (Fig. 1D). Adaxial trichomes are less abundant than in the wild type and completely absent from the first two leaves of some individuals. Aberrant trichomes are found on the juvenile leaves, their number decreasing in the adult leaves. The leaf margin is thin and whitish, displaying more pronounced teeth as the rosette develops. The leaf surface of this mutant is uneven, with protuberant veins that are more pigmented than the interveinal tissue, which is pale green. The *den30* mutant (Fig. 1E) displays a phenotype weaker than that of *den29*. Its leaves are spathulate and toothed, with elongated petioles. Trichome shape is affected in much the same way as in the *den29* mutants, but they are more abundant. Transverse sections of Den leaves show a marked reduction in the number of mesophyll cells, which are larger than those of the wild type. It is difficult to distinguish between palisade and spongy mesophyll cells, due to the increased aerial spaces. Although the last kind of perturbation has been found in mutants with an aberrant development of chloroplasts, confocal images do not show clear differences between the chloroplasts of *den* and L*er*.

The *exi9* mutant displays small and pale green vegetative leaves (Fig. 1F). This mutant is dwarf, with slightly epinastic leaves, a compact rosette of less than 1 cm in diameter, and a short inflorescence. It is early flowering, flowers and fruits being normally shaped although small, with a reduced number of seeds per plant. Epidermal cells are also smaller and display fewer protrusions than those of the wild type.

The *icu8* (Fig. 1H) and *icu15* (Fig. 1I) mutants display slightly curled leaves, making a compact rosette. The first two leaves

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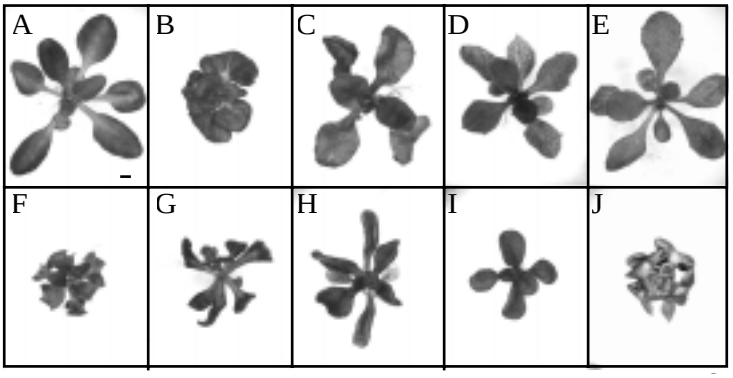

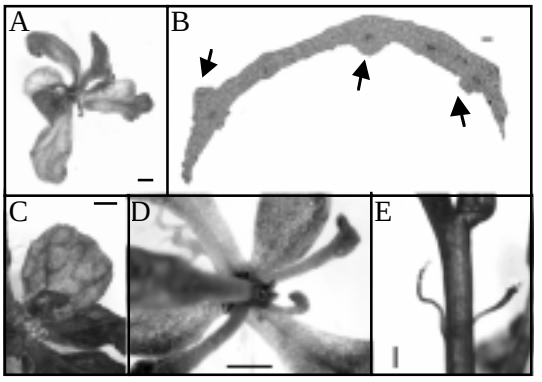

Fig. 1 *(left)***. Representative fast-neutron induced mutants. (A)** *Ler*. **(B)** as1-14. **(C)** as3. **(D)** den29. **(E)** den30. ***(F)*** exi9. **(G)** clf-62. **(H)** icu8-2. **(I)** icu15-3. **(J)** ucu2-1*. Photographs were taken 22 days after sowing. Scale bars,1 mm.*

Fig. 2 *(right)***. Synergistic phenotype of the *as1 den30* double mutant. (A)** *Rosette.* **(B)** *Transverse section of a fifth leaf. Arrows indicate midveins.* **(C)** *Leaf with dark green protuberant veins.* **(D)** *Detail of a rosette displaying some partially filamentous adult leaves.* **(E)** *Filamentous structures on the stem. Photographs (B), (C) and (E) correspond to* as1-1 den30 *plants, and (A), (D) and (F) to* as1-14 den30*. Photograph (E) and the others were taken, respectively, 48 and 25 days after sowing. The scale bar in (B) indicates 100 μm; all others, 1 mm.*

occasionally show trichomes on their abaxial surface, a trait characteristic of adult vegetative leaves. Transverse leaf sections show that both palisade and spongy mesophyll cells are slightly smaller than in the wild type. The inflorescence shows a higher number of lateral branches and siliques are small. Terminal inflorescences contain an increased number of flower buds. The *icu8* mutants display a stronger Icu phenotype than *icu15* mutants. Some *icu8* leaves are pointed, and more involute with a higher number of abaxial trichomes than those of *icu15* individuals. Some terminal inflorescence flowers display a reduced number of floral organs, sometimes transformed into carpel-like organs. Their fertility is very reduced and some of their seeds do not germinate. Last, the vegetative leaves of the dwarf *ucu2* mutant and are spirally rolled downwards along the midvein (Fig. 1J).

We obtained all the possible double mutant combinations involving the above-mentioned mutations. In all cases the double mutants displayed additive phenotypes, the only exceptions being *as1 den30* and *as1 den29* individuals (Fig. 2A). In such double mutants, leaves are rough and narrower than those of *as1* individuals, and their veins are more prominent and greener than in *den* mutants (Fig. 2C). Transverse sections show several midveins per leaf, which were indistinctly found in the dorsal or ventral domains (Fig. 2B), contrary to that found in wild-type leaves, which display a single and completely ventral midvein. Leaves of some of these double mutant individuals are progressively narrower as the rosette develops, the latest ones being filamentous, although not completely cylindrical, since their dorsal surface is flat (Fig. 2D). Filamentous structures are also shown on the stem (Fig. 2E). Some cauline leaves display complete radialization, which in some cases is limited to the basal region.

A role in the specification of leaf axes in *Arabidopsis thaliana* has been recently suggested for *AS1* and *AS2* (Byrne *et al.*, 2000; Ori *et al.*, 2000). We attempted to obtain double mutants involving the *as3* first described in this work and the already known *as1* and *as2* mutations. We found that the *as3 as2-1* double mutants show wrinkled leaves with prominent venation, as in the *as1 den30* or *as1 den29* individuals. No radialized leaves were displayed by *as3 as2* double mutants. The dorsoventral abnormalities shown in double mutants affected in both the *AS* and *DEN* genes suggest their involvement in the specification or maintenance of the adaxial and abaxial domains of the leaf.

Materials and Methods

Plants were grown as previously described (Ponce *et al.*, 1998). The mutant screening was carried out as described in Berná *et al.*, 1999. Tissue sectioning and microscopy was performed as indicated in Serrano-Cartagena *et al.*, 2000.

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