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Short Report

Interactions between venation pattern formation genes in *Arabidopsis thaliana*

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ABSTRACT Aiming to contribute to the understanding of the genetic mechanisms underlying venation pattern formation in the vegetative leaves of *Arabidopsis thaliana*, we have previously analyzed the natural variability of this trait and searched for venation pattern mutants with normally shaped leaves after chemical and insertional mutagenesis. The rarity of such mutants suggested that vein patterning and leaf morphogenesis might not be independent processes. To test this hypothesis, we have examined 97 abnormally shaped leaf mutants, each one belonging to a different complementation group, and found 2 whose venation patterns differed from that of the wild type. We have focused our attention on one of these mutants, *rotunda1* (*ron1*), and in a recessive monogenic trait, *hemivenata* (*hve*), which was identified in an ecotype. Our study reveals that the Hve phenotype is pleiotropic, including an extremely simple venation pattern, increased stem branching, altered root waving, and low fertility. We have obtained double mutants involving mutations at several genes known to participate in vascular development, and/or auxin transport and perception. Linkage analysis has allowed us to assign the *HVE* gene to a 200 kb interval in the short arm of chromosome 2. Further genetic and molecular analyses are in progress to identify *HVE* among the available candidate genes.

Because multicellular plants and animals evolved independently, they differ in their developmental mechanisms. The cell migrations that occur in animal development are prevented by the rigid wall of plant cells and, therefore, position-dependent cell differentiation is central to plant development. Leaves are plant organs that arise from the apical meristem as lateral appendages. They consist of only a few cell types arranged in a relatively simple anatomy, which make them useful for the study of general developmental processes in plants. We have undertaken the genetic analysis of venation pattern formation in the vegetative leaves of *Arabidopsis thaliana* as a model to study how plant vascular cells determine their position and differentiate accordingly (Candela *et al.*, 1999).

As recognized by Alonso-Blanco *et al.* (2000), the natural variability of a trait can successfully be used to identify genetic variants otherwise difficult to obtain using mutational approaches. Such is the case for mutants with altered venation patterns but otherwise normal leaf shape, which we have found to be infrequent among EMS and T-DNA mutagenized populations. In an earlier report, we took advantage of the availability of a large collection of *Arabidopsis thaliana* ecotypes, deposited at the Nottingham *Arabidopsis* Stock Centre, to search for divergent leaf venation patterns, finding that the leaves of the Eifel-5 (Ei-5) ecotype displayed an extremely simple venation pattern (Figure 1C-D), a phenotype that was shown to be due to a recessive allele of the *HEMIVENATA* (*HVE*) gene (Candela *et al.*, 1999).

We have found that the simple leaf venation pattern displayed by Ei-5 cosegregates with a highly branched inflorescence and an altered root waving in the F_2 and F_3 progenies of an Ei-5 $\times$ Ws-2 cross. Our results are compatible with these traits being due to a single, pleiotropic allele of the *HVE* gene. Interestingly, both the lack of apical dominance in the inflorescence and the reduced vasculature of the leaves can be explained as a consequence of a diminished auxin signal, in accordance with current models based on the involvement of this hormone in such developmental processes (Dengler and Kang, 2001; Sachs, 1991; 2000).

We have also looked for mutants with abnormal venation in the collection of leaf shape mutants obtained in our laboratory, which were selected in a large-scale effort to genetically dissect leaf development in *Arabidopsis thaliana* (Berná *et al.*, 1999). Complementation and linkage analyses of these mutants allowed us to conclude that at least 97 different genes are required for the leaf to attain its final wild-type shape (Berná *et al.*, 1999; Robles and Micol, 2001). We have examined the vasculature of the first vegetative leaf in mutants carrying representative alleles of each of those genes, finding that the *rotunda1* (*ron1*) and *apiculata7* (*api7*) mutants display abnormal venation patterns. While *ron1* leaves display a higher number of free-ending veins near the margin of the lamina (Figure 1B), the midveins of *api7* leaves often split and rejoin abnormally.

To investigate the functional relationship of *HVE* with other genes, such as *HVE*, *LOPPED1* (*LOP1*), *PIN-FORMED1* (*PIN1*), *MONOPTEROS* (*MP*), *COTYLEDON VASCULAR PATTERN2* (*CVP2*), *AUXIN RESISTANT1* (*AXR1*) and *ROTUNDA1* (*RON1*), putatively involved in vein pattern formation or auxin perception or transport, we have obtained the *hve ron1*, *hve mp*, *hve pin1*, *hve lop1*, *hve axr1*, and *hve cvp2* double mutants. Since their phenotypes were found to be additive (Figure 1H-L), we conclude that *HVE* acts in a pathway which does not involve the other genes. In support of this notion, we have found that Hve plants are sensitive to the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) and to the auxin transport inhibitor triiodobenzoic acid (TIBA) when these compounds are added to the culture medium, suggesting that the *HVE* gene is not involved in auxin perception or transport. The fact that Hve plants also display altered root waving suggests a possible role in the metabolism of tryptophan and auxin (Rutherford *et al.*, 1998). We have also obtained transgenic Hve plants that express the *Athb-8-GUS* transgene (Baima *et al.*, 1995), which

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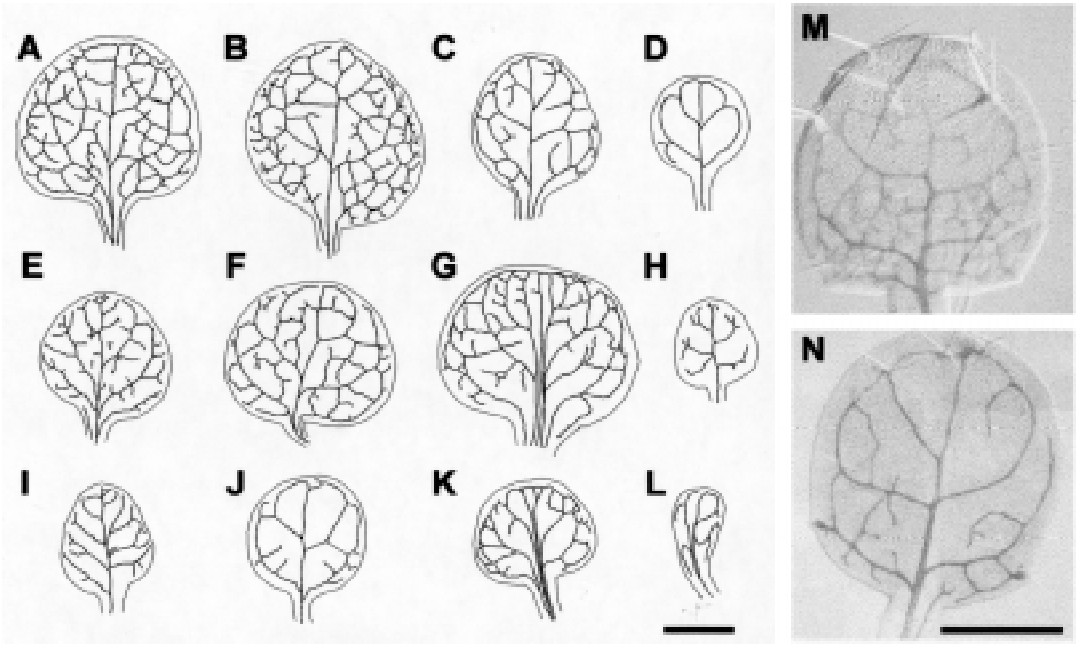

Fig. 1. Venation patterns of first node rosette leaves excised from different *Arabidopsis thaliana* strains, as shown by camera lucida drawings (A-L) and micrographs of GUS-stained tissues (M-N). (A) *Enkheim-2 wild type (En-2),* **(B)** ron1, **(C,D)** hve, **(E)** cvp2-1, **(F)** axr1-12, **(G)** pin1-1, **(H)** hve ron1, **(I)** hve cvp2-1, **(J)** hve axr1-12, **(K)** hve pin1-1, **(L)** hve lop1-65, **(M)** Athb-8-GUS, *and* **(N)** hve Athb-8-GUS. *The diagrams represent leaves from plants grown on Petri dishes, harvested 22 (A-L) or 12 (M-N) days after sowing. The drawings C and D represent two examples of the variability found in Hve leaves. Continuous lines other than those representing the leaf margin denote completely lignified xylem strands (A-L). The scale bars indicate 1 mm (A-L) or 0.5 mm (M-N).*

allows visualization of the venation pattern before the differentiation of xylem cells (Figure 1M-N).

After testing several polymorphic microsatellites for linkage, we found that the *HVE* gene locates on the short arm of chromosome 2, near the nga1145 SSLP marker. We have taken a positional approach to the isolation of this gene, which will help in understanding the role played by its product during vascular development. Additional PCR-based markers were used to genotype 1,132 Hve plants from the F_2 progeny of a Ei-5 x Ws-2 cross. This allowed us to identify recombinants, thereby shortening the candidate region to a 200 kb interval encompassed by three BAC clones.

Materials and Methods

Plants were grown as previously described (Ponce *et al.*, 1998), at 20±1°C and 60-70% relative humidity under continuous fluorescent light (7,000 lx). Leaves were treated with chloral hydrate before being mounted on slides for microscopy (Candela *et al.*, 1999).

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