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ARTICLE ADDENDUM

Arabidopsis thaliana homeodomain-leucine zipper type I transcription factors contribute to control leaf venation patterning

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ABSTRACT

Venation patterning is a taxonomic attribute for classification of plants and it also plays a role in the interaction of plants with the environment. Despite its importance, the molecular physiology controlling this aspect of plant development is still poorly understood. Auxin plays a central role modulating the final vein network and patterning. This addendum discusses recent findings on the role of homeodomain-leucine zipper (HD-Zip) transcription factors on the regulation of leaf venation patterning. Moreno-Piovano *et al.* reported that ectopic expression of a sunflower *HD-Zip I* gene, *HaHB4*, increased the asymmetry of leaf venation. Even more, this work showed that auxin transport in the leaf through LAX carriers controls venation patterning. Here, we provide evidence indicating that some *Arabidopsis thaliana HD-Zip I* genes play a role in the determination of the final leaf venation patterning. We propose that these genes contribute to regulate vein patterning, likely controlling auxin homeostasis.

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The leaf vascular network is determined early in leaf development.[1-3] The vascular patterning is associated to local sites of accumulation of the auxin hormone in developing leaves.[4] It is now clear that auxin plays a key role in determining the leaf venation patterning including the sites and timing of auxin synthesis or application.[5,6] The synthetic auxin-responsive promoter DR5 driving the expression of the β-glucuronidase (GUS) gene revealed, by histochemistry, high GUS activity in hydathodes of developing leaves.[5-7] Even more, the vascular patterning was dramatically affected when the endogenous accumulation of auxin was altered using exogenous treatments of auxin or auxin-inhibitors in Arabidopsis and tomato leaves.[2,3,8] Concurrently, Arabidopsis mutants with altered auxin accumulation showed a defective vein patterning.[7,9] All these results suggest that auxin distribution is tightly associated to the final vascular patterning of the leaf.[5,6] The cell-to-cell auxin transport is tightly regulated by the activity of carriers, including PIN efflux carriers and AUX/LAX influx carriers.[10] Only a mutant deficient in the *LAX2* gene showed vascular breaks in cotyledons.[11] Interestingly, it was recently shown that auxin influx carriers control vasculature development in Arabidopsis.[12] Even more, it was shown that Arabidopsis *AUX/LAX* genes regulate vascular bundle differentiation in a redundant way since the altered phenotype was only observed in the quadruple *aux1/ lax1/ lax2 /lax3* mutant, but not in single *aux/lax* mutants.[12] The vascular bundle organization of this high-order mutant also showed increased number of procambial and xylem cells in shoots and roots.[12] Much work was done to clarify the role of auxin at early developmental stages, however the information on the role of auxin on the final vascular network of adult leaves is still scarce.[5,6]

In a previous report,[13] we showed that auxin influx carriers are required for the normal development of vein network since several *aux/lax* single mutants showed enhanced venation asymmetry compared to wild type plants. The altered vein asymmetry was originally described in Arabidopsis and soybean transgenic plants which ectopically express a sunflower transcription factor: *HaHB4*, a class I homeodomain-leucine zipper (*HD-Zip I*) gene. *HaBH4* expression was driven by a *35S* promoter in Arabidopsis and by its native promoter in soybean. Concisely, *HaHB4*-transgenic plants, both in Arabidopsis and soybean, showed augmented asymmetry on venation patterning and enhanced xylem formation in vascular tissues.[13] This venation patterning phenotype was associated to the repression of *LAX2*, or *AUX/LAX* homologue genes from soybean.[13] Here, we show that *HaHB4*, *lax2-1* and *lax2-2* mutants share additional phenotypes related to the venation hierarchy of the leaf lamina. We recorded the total length of primary, secondary and tertiary order veins and vein density (total vein length/area). The primary and secondary vein length was measured along the entire leaf lamina. Whereas third order vein length was estimated as the average of three random areas including a secondary order vein located at the center region of the leaf lamina. In consonance with our previous study, the vein density was higher

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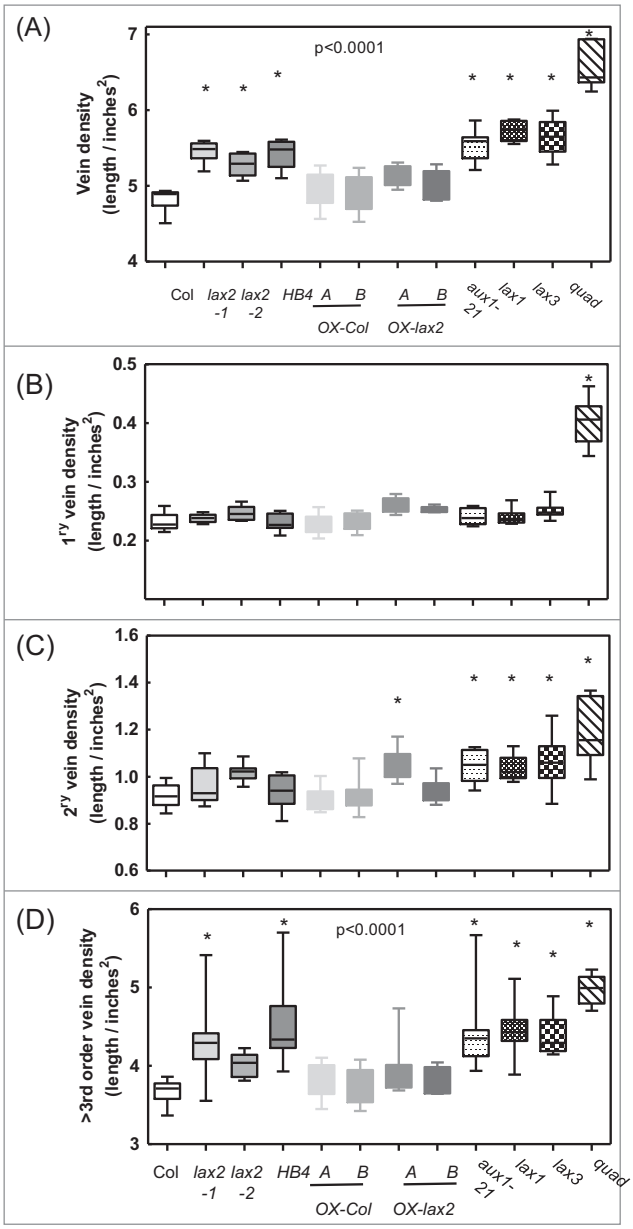

Figure 1. Arabidopsis mutants lacking functional *ATHB* genes show leaves with enhanced lateral-vein asymmetry index. The leaf venation patterning was measured on a fully developed leaf of a 30-day-old plant of the following genotypes: Col-0, *lax2-1*, *lax2-2*, *HB4 (35S:HaHB4)*, *aux1-21*, *lax1*, *lax3*, and independent transgenic lines for *35S:mCitrine:LAX2* generated in Col-0 or *lax2-2* genetic backgrounds. (A) Average distance between the two attachment sites of the lateral veins of the third vascular pair. (B) Lateral-vein asymmetry index. The parameters were scored in the entire leaf of six to eight independent plants per genotype. Thin bars represent SE. Different letters indicate significant differences ($P < 0.05$, Tukey test).

on *HaHB4* Arabidopsis plants relative to the wild type and similar to the vein density of *lax2-1* and *lax2-2* mutants (Fig. 1A). The most of this variation was explained by an increment in the density of 3rd order veins, since the total length of 1st and 2nd order veins remained constant for most genotypes (Fig. 1B-D). A stronger pattern was observed for *lax1*, *lax3* and *aux1-21* mutants, where the increment in vein density was the result of more 2nd and 3rd order veins than in the wild type (Fig. 1B-D). Interestingly, the ectopic expression of LAX2 as an N-terminal fusion protein with mCitrine driven by *35S CaMV* promoter, here named *OX-LAX2*, restored the venation hierarchy and density of *lax2-2* mutant plants. These results were consistent with previous observations related to vein symmetry and support the hypothesis that auxin homeostasis in the leaf is a central regulator of this developmental process. Taken together, these results also shows that auxin influx carriers affects not only vein patterning but vein order and vein density in the leaf.

Since our previous study involved the expression of a sunflower transcription factor, it was difficult to understand if this was part of a conserved role for *HD-Zip I* genes or a consequence of the ectopic expression in a heterologous system. In order to answer this question, we used Arabidopsis mutants and transgenic plants to evaluate the role of native *HD-Zip I* (*ATHB*) genes in vascular patterning. The Arabidopsis genome encodes 17 members of class I *ATHB* that are classified in six clades.[14]

To answer this question, we scored leaf vein symmetry as reported before,[13] using the distance between attachment points of lateral veins and the lateral-vein asymmetry index of Arabidopsis plants. Consistent with a previous work, we found that *lax2-1* and *lax2-2* mutants and the *HaHB4* overexpressing line, showed enhanced asymmetry of both parameters related to venation patterning. Here, we included a set of single *athb* mutants of the same class I of *HaHB4* but belonging to different clades of *ATHB* genes.[14] These mutants were previously shown to display unique and non-redundant phenotypes: *athb1-1* showed shorter hypocotyls grown under a short day condition,[15] *athb7* and *athb12* mutants exhibited less open stomata, lost less water during a drought stress treatment but yielded less seeds at the end of the life cycle[16]; whereas *athb13* alleles displayed shorter siliques and defective pollen germination.[17] Since *ATHB7* and *ATHB12* are phylogenetically close to *HaHB4*,[14] we also included the *amiR7/12* transgenic line where the expression of both *ATHB7* and *ATHB12* is simultaneously silenced using an artificial microRNA.[16] We found that all *athb* mutants tested here developed a more asymmetric venation patterning than the wild type genotype (Fig. 2). However, vein symmetry parameters were differentially affected by the lack of *ATHB* genes. Whereas the distance between attachment points of lateral veins remained stable in all single mutants compared to the wild type (Fig. 2A), the lateral-vein asymmetry index was significantly different in *athb1-1*, *athb13-1* and *athb13-2*, *athb7* and *athb12*, as well as in *amiR7/12* line (Fig. 2B). Notably, a similar effect was observed in plants ectopically expressing *ATHB7*, *ATHB12* or *ATHB13* (Fig. 2). These transgenic plants showed enhanced lateral-vein asymmetry index relative to the wild type genotype (Fig. 2B). These results show that *ATHB* genes play a physiological role controlling vein patterning in plants, likely modulating auxin homeostasis in the leaf lamina. The apparent controversy between mutant and overexpressor plants in these parameters could be explained by cross-regulation between paralog *HD-Zip I* encoding genes, previously described.[16,17] When *ATHB13* was knocked-down or knocked-out, the expression of the closest gene paralog, *ATHB23*, was induced.[17] A negative relationship in gene expression was also observed for the pair of paralogs *ATHB7* and *ATHB12*.[16] Since several reports are showing non-linear relationships between gene expression and its targets, it will be relevant to consider this indirect way of regulation {Sparks, 2016 #1782}. For

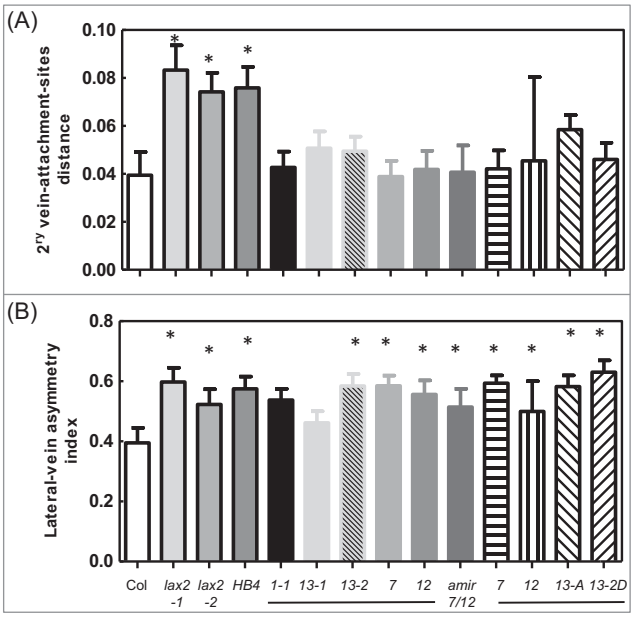

Figure 2. *ATHB* genes modulate venation patterning in adult leaves of Arabidopsis. (A) Vein density on leaf lamina of different genotypes. (B) Length of primary, secondary and tertiary veins of the lamina. The total length of primary and secondary order vein were measured in the entire leaf. The third order vein length was scored in a selected region including a secondary vein. The selected area was 0.5 cm^2 size. Each value is the average of the three regions within the same leaf. Six to eight independent leaves were measured per genotype. Thin bars represent SE. Different small letters indicate significant differences ($P < 0.05$, Tukey test).

example, it was shown that *ATHB13* and *EDF3* were induced in both a mutant and an overexpressor allele of *SHORTROOT* {Sparks, 2016 #1782}. At the mechanistic level, this might be the consequence of complex interactions among TFs involving cooperativity or stoichiometry that might in turn, regulate the *in vivo* function of TFs. Further studies are required to shed light on the underlying regulation that influence vein patterning in leaves.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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