**ATHB4 and HAT3, two class II HD-ZIP transcription factors, control leaf development in Arabidopsis**

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**Abbreviations:** HD-ZIP, homeodomain leucine-zipper; R:FR ratio, red to far-red ratio

In response to plant proximity or canopy shade, plants can react by altering elongation growth and development. Several members of the class II homeodomain-leucine zipper (HD-ZIPII) transcription factor family have been shown to play an instrumental role in the responses to shade. HD-ZIP members of the class III (HD-ZIPIII) expression by the plant phytochrome system, which rapidly influences hormonal responses and a downstream transcriptional network to alter the transition to flowering. 1-3 Changes in R:FR are perceived by the plant photoreceptor system, which rapidly influences hormonal responses and a downstream transcriptional network to alter the transition to flowering. 1-3

**Introduction**

Plants are sessile organisms and to maximize reproductive success, they have to adjust their growth behavior to their environment. Light is one of the most important environmental cues as it provides both energy and information. Plants have evolved refined mechanisms to detect both light quality and quantity and to measure the duration of the light period. Important growth responses and developmental decisions, such as plant architecture and the transition to flowering, are influenced by a combination of cues such as light quality and day length. In nature, plants live in communities with other plant species that might compete for resources. To avoid living under a canopy, they can detect plant proximity and canopy shade as changes in the red (R) to far-red (FR) ratio (R:FR ratio) of light and translate these changes into growth responses, collectively known as the shade avoidance syndrome (SAS) that include enhanced hypocotyl elongation, reduced leaf expansion, decreased branching and accelerated flowering. 1-3 As plant leaves reflect FR-light, neighboring plants can sense subtle decreases in R:FR ratio (‘neighbor proximity detection’) and react by inducing hypocotyl growth. 4 In case of true plant shade, canopy plant leaves selectively absorb light from the photosynthetic active radiation, which includes R light. Therefore, both the R:FR and the overall quantity of the photosynthetic active radiation (400–700 nm) is decreased (canopy shade conditions), which is also translated into growth-induction of the hypocotyl. 1-3 Changes in R:FR are perceived by the plant phytochrome system, which rapidly influences hormonal responses and a downstream transcriptional network to alter the mentioned aspects of plant development and architecture. 5-7

The transcriptome of Arabidopsis changes significantly in response to shade, 5-9 and numerous shade-induced genes are known. 10-11 Several of these rapidly shade induced genes belong to the class II homeodomain leucine-zipper (HD-ZIPII) family of transcription factors, that are mostly known to be involved in the regulation of adaptive responses to the environment. 12-13 The recent finding that HAT1/IA1BA, a HD-ZIPII protein, is also involved in the regulation of meristem activity 14 hints toward additional functions of HD-ZIPII such as regulation of plant development per se. We could recently show that the expression of

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several HD-ZIPII genes is directly controlled by the HD-ZIPIII transcription factor REVOLUTA (REV).\textsuperscript{15} HD-ZIPIII factors have known roles in controlling embryo, shoot and root patterning\textsuperscript{16-18} and our previous finding that they are involved in an adaptive process such as the SAS, suggested that they function at the nexus of adjusting growth to the environment. Previously it was shown that double mutant plants in two HD-ZIPII genes (ATHB4 and HAT3) display strong alterations in their development.\textsuperscript{3,10} Using a genetic approach, we have investigated whether HD-ZIPII transcription factors also have a prominent role in regulating leaf development. It is unknown whether and how HD-ZIPIII are activated by shade and whether TAA1/YUC5 play a role in leaf development.

Results and Discussion

Our analysis of REV target genes revealed several HD-ZIPIII transcription factors that are directly and positively regulated by REV.\textsuperscript{15} Some HD-ZIPIII transcription factors are known to be involved in shade signaling\textsuperscript{10} and our recent analysis showed that HD-ZIPIII are also involved in shade growth (Fig. 1).\textsuperscript{15} Using available double mutant plants in HD-ZIPIII genes (hat1 hat2 and athb4 hat3) and various hd-zipIII mutant plants (rev-5, 35S::miR165a and 35S::ZPR3), we performed comparative leaf growth studies. When grown side-by-side, athb4 hat3 double mutant plants were severely impaired in development and retarded in growth before reaching the reproductive phase (Fig. 2A). The hat1 hat2 double mutant did not display a mutant phenotype in regard to altered leaf polarity (Fig. 2A). Together, these studies revealed that, like HD-ZIPIII, also some HD-ZIPIII play a prominent role in regulating polar leaf development in Arabidopsis. We next examined the vascular strands of petioles of different hd-zipII/35S::ZPR3 mutant plants to detect more subtle polarity-associated defects. Vascular strands of wild type plants, as well as athb4 hat3 double mutant, showed a typical sandwich-structure with xylem on top (colored in blue), cambium cells in the middle (colored in red) and phloem on the bottom (green). In plants with reduced HD-ZIPIII activity (35S::miR165a, rev-5, 35S::ZPR3), the vasculature showed different degrees of abaxialized and radialized characteristics, with phloem surrounding the xylem. Histological analyses of vascular strands of leaves of athb4 hat3 double mutant plants showed strong abaxialization, manifested by radialization of transport elements and also a severe disruption of the overall organization (Fig. 2A). Thus, the athb4 hat3 mutant phenotype somewhat resembles hd-zipIII mutant plants. Interestingly, in our growth conditions, the mutations caused strong leaf patterning defects in the early post-embryonic growth phase and both cotyledons and early leaves showed strong developmental defects (Fig. 2B). Later in development, the mutant athb4 hat3 phenotype was alleviated and leaf development resumed to a more normal state (Fig. 2B, lower panel), an effect that was not observed in hd-zipIII mutant plants. These findings illustrate that these two HD-ZIPIII transcription factors play an important role in leaf patterning, very likely downstream of HD-ZIPIII action. Because they affect more strongly the early post-embryonic growth phase, their action might be less required for the development leaves formed by older plants.

Using scanning-electron microscopy we further characterized the early growth defects of athb4 hat3 mutant plants and could observe that both cotyledons and leaves were radialized to different degrees and lacked adaxial characteristics (Fig. 3B, D, F, H) compared with wild type plants (Fig. 3A, C, E, G). Wild type and athb4 hat3 mutant seedlings were also compared using confocal microscopy and 3D-reconstruction (Figs. 3I and J, Vids. S1 and S2), displaying the alterations in leaf development at higher resolution. Optical sections through developing cotyledons revealed normal polarity of wild-type cotyledons with vascular strands vs. strongly radialized and abaxialized cotyledons with disorganized vascular strands in athb4 hat3 mutant plants (Fig. 3I). The results further corroborated that ATHB4 and HAT3 transcription factors are involved in patterning the adaxial domain in the early leaf primordium.

To find out whether mis-regulation of HD-ZIPIII genes is a consequence of the athb4 hat3 mutant phenotype, we analyzed the expression PHB and PHV, two adaxial marker genes of the HD-ZIPIII family. Plants carrying dominant mutations in either PHB or PHV display dramatic adaxialized phenotypes\textsuperscript{7} and...
thus behave opposite to the developmental defects observed in athb4 hat3 mutant plants. Our expression analysis shows that both PHB and PHV expression is significantly lower in athb4 hat3 mutant plants compared with Col-0 wild type plants (Fig. 3K). These findings suggest that besides acting downstream of REV, ATHB4 and HAT3 might have an additional function upstream of REV. The observation that PHB and PHV expression are reduced in athb4 hat3 mutant plants might suggest that ATHB4 and HAT3 act positively on HD-ZIPIII expression. Based on the auto-activation capacity in a yeast two-hybrid assay, it has been suggested that HAT1/JAIBA may act as a transcriptional activator. However, all HD-ZIPII proteins contain an N-terminal EAR motif, required for transcriptional repression.

It is furthermore known that when overexpressed in plants, several HD-ZIPIIs act as transcriptional repressors over the expression of some genes, for which reason it is unlikely that they act by directly and positively regulating HD-ZIPIII expression. Therefore it seems plausible that the reduced expression of PHB and PHV is an indirect effect, i.e., a mere consequence of reduced adaxial tissue. We therefore conclude that the combined loss of ATHB4 and HAT3 causes strongly abaxialized leaf development, which is reflected by reduced expression of the adaxial identity markers PHB and PHV.

We next examined whether the ectopic expression of HAT3, a REV target gene, can elicit phenotypes associated with either loss- or gain-of-REV function. In order to avoid strong pleiotropic overexpression-phenotypes, we decided to employ the glucocorticoid-receptor (GR) inducible system. Using Gateway recombination (Invitrogen), transgenic 35S::FLAG-GR-HAT3 plants were constructed. Four-week old transgenic T2 plants (n = 20) were grown in short day conditions and treated once a day with DEX by spraying for one week. DEX-induced transgenic 35S::FLAG-GR-HAT3 plants showed strong upward-curving of leaf blades, largely resembling DEX-induced 35S::FLAG-GR-REVd transgenic plants (Fig. 4), a phenotype caused by over-proliferation of adaxial-derived tissue in leaves. These data further support our previous conclusion that HD-ZIPIIIIs and these two HD-ZIPII and HD-ZIPIII act in a common protein complex, indicating leaf blade, are being produced.

Later in development (around 6 weeks after germination; lower panel), mutants produce radial leaves in comparison to Col-0 wild type plants. In wild type Col-0 and hat1 hat2 plants no growth abnormalities were observed. Below the photographs of wild type and the different mutant plants, sections through petioles are shown. The vasculature of wild type Col-0 plants shows the typical sandwich structure tissue containing phloem cells (green), cambium cells (red) and tissue containing xylem elements (blue) on top. Both 35S::miR165a and 35S::ZPR3 transgenic plants show abaxialized vascular strands with phloem nearly surrounding the xylem. In athb4 hat3 mutant plants, the vascular organization is severely disturbed but is also showing abaxialized characteristics.

Fixation, clearing and staining procedure for three-dimensional imaging. For the experiments shown in Figure 3, seeds were germinated and grown in a growth chamber at 22°C under
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RNA expression analysis by reverse transcriptase quantitative PCR. For reverse transcriptase quantitative PCR (qPCR) analyses of gene expression, seedlings were grown on filter paper on top of GM-medium. Seedlings were grown under continuous W for 7 d. qPCR analyses were performed as indicated elsewhere.10 UBQ10 gene was used for normalization. We assayed 3–5 biological replicates for each sample. Primer sequences for qPCR were MSO40 (5'-GCT AAC CCA GCA GGA CTC CT-3') and MSO41 (5'-TAA GCT CGA TCG TCC CAC CGT T-3') for PHB (At2g34710) and MSO42 (5'-GCT AAT CTT CTC TCG ATT GCG GAG GA-3') and MSO43 (5'-GCT CGA TAG TAC CAC CAT TTC CAG TG-3') for PHV (At1g30490). Primers for UBQ10 transcript level analyses were described before.10

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Materials
Supplemental materials may be found here: www.landesbioscience.com/journals/psb/article21824

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