

# A zinc knuckle protein that negatively controls morning-specific growth in *Arabidopsis thaliana*

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Growth in plants is modulated by a complex interplay between internal signals and external cues. Although traditional mutagenesis has been a successful approach for the identification of growth regulatory genes, it is likely that many genes involved in growth control remain to be discovered. In this study, we used the phenotypic variation between Bay-0 and Shahdara, two natural strains (accessions) of *Arabidopsis thaliana*, to map quantitative trait loci (QTL) affecting light- and temperature-regulated growth of the embryonic stem (hypocotyl). Using heterogeneous inbred families (HIFs), the gene underlying one QTL, *LIGHT5*, was identified as a tandem zinc knuckle/PLU3 domain encoding gene (At5g43630; *TZP*), which carries a premature stop codon in Bay-0. Hypocotyl growth assays in monochromatic light and microarray analysis demonstrate that *TZP* controls blue light associated growth in a time-of-day fashion by regulating genes involved in growth, such as peroxidase and cell wall synthesis genes. *TZP* expression is phased by the circadian clock and light/dark cycles to the beginning of the day, the time of maximal growth in *A. thaliana* in short-day conditions. Based on its domain structure and localization in the nucleus, we propose that *TZP* acts downstream of the circadian clock and photoreceptor signaling pathways to directly control genes responsible for growth. The identification of *TZP* thus provides new insight into how daily synchronization of growth pathways plays a critical role in growth regulation.

blue light | circadian | fine-mapping | quantitative

The embryonic stem or hypocotyl is an excellent model for studying both internal and external factors controlling growth in plants (1). Genetic screens in common laboratory accessions have yielded direct molecular insight into how light- and hormone-dependent signaling pathways interact with the circadian clock to regulate the final length of the hypocotyl (1). The power of the hypocotyl assay is its simplicity, as well as its obvious meaningfulness. When germinating seeds are exposed to low levels of light, such as those caused by a covering layer of debris, the hypocotyl has to grow for a while. Only after the surface has been broken by the tip of the hypocotyls can the embryonic leaves, the cotyledons, unfold. Conversely, if a seed has fallen on open ground, there is no need for the hypocotyls to be particularly long. Because of the ease and reproducibility with which hypocotyl length can be measured in thousands of individuals, it has also been a powerful model in mapping genes with more subtle effects on light and hormone regulated growth, by using methods of quantitative genetics (2). Multiple light signaling genes controlling hypocotyl length have been characterized in quantitative trait locus (QTL) studies (3–6).

In this study, we use the hypocotyl assay to identify QTL controlling growth in 2 light and 2 temperature conditions. We identified a recessive large effect QTL on chromosome five controlling 40% of the growth variation segregating in Recombinant Inbred Lines (RILs) derived from the Bay-0 and Shahdara accessions of *Arabidopsis thaliana*. The QTL was fine mapped and the causal factor shown to be a mutation affecting a tandem zinc knuckle/PLUS3 protein (At5g43630; *TZP*), which is encoded by a

single-copy gene in all completely sequenced plant genomes. We show that *TZP* acts downstream of the circadian clock and light signaling, directly regulating blue light-dependent, morning (dawn)–specific growth, during seedling development and beyond. Based on its nuclear localization and its novel domain structure, we argue that *TZP* functions at the transcriptional level to control growth-promoting pathways. *TZP* represents a new component of the growth pathway that was not previously identified by using traditional genetic screens.

## Results and Discussion

**Mapping QTL for Hypocotyl Elongation in the Bay-0 × Shahdara RIL Population.** Intraspecific variation provides a fertile source of genetic combinations that can be used to map new genes (or new alleles) involved in complex traits such as growth. To investigate natural variation for hypocotyl elongation response to light and temperature, we phenotyped a core set of 164 RILs from the Bay-0 × Shahdara cross in 4 different environments combining 2 white-light [17  $\mu\text{mol}/\text{m}^{-2}\text{s}^{-1}$  (L1) and 10  $\mu\text{mol}/\text{m}^{-2}\text{s}^{-1}$  (L2)] and 2 temperature (22°C and 26°C) conditions [supporting information (SI) Fig. S1A]. The parental phenotypes reveal that in our conditions Shahdara responds poorly to temperatures above 22°C or light below 17  $\mu\text{mol}/\text{m}^{-2}\text{s}^{-1}$ , or a combination of both. In contrast, Bay-0 responds strongly to both temperature and light, with a synergistic interaction between both factors. Variation among the RILs seems to follow parental variation with signs of bimodality [especially at 26°C/L1 and 26°C/L2] suggesting the segregation of some large-effect QTL. Transgression was also prevalent in all conditions and in both directions. Overall, genotypic variation was significant in each environment and broad-sense heritability of the trait was accordingly high, greater than 70% (www.inra.fr/vast/Files/Loudet.PNAS\_SITables.xls). Although RIL response to contrasted temperature and light treatments was significant ( $P < 0.001$ ), RIL × light interactions were not significant at either 22°C or 26°C, and the RIL × temperature interaction was significant only under L1 (data not shown). This indicates that most of the phenotypic variation between RILs is stable and conserved across environments.

The genetic architecture of variation in hypocotyl elongation under these environmental conditions is presented in Fig. S1B. Two

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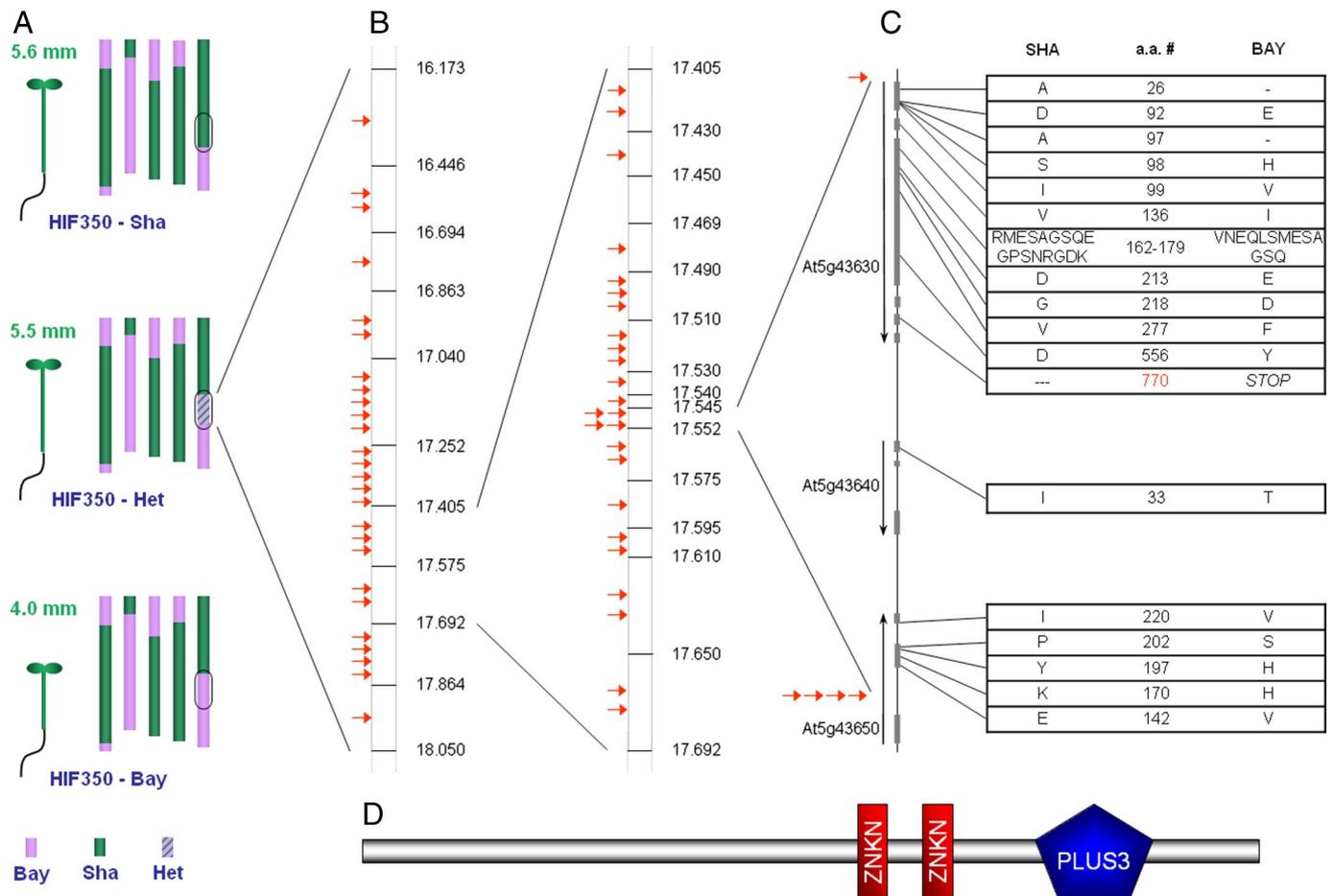
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**Fig. 1.** Confirmation and fine-mapping of the *LIGHT5* QTL to 3 genes, among which *At5g43630* is highly polymorphic between Bay-0 and Shahdara. (**A**) Confirmation of *LIGHT5* using HIFs. The Sha allele is fully dominant over the Bay allele. Two rounds of recombinant screening from HIF350 followed (**B**). Horizontal marks on the chromosomes are markers with physical position in Mb indicated to the right. Red arrows indicate the approximate position of different recombination events that were individually tested in rHIF to establish the QTL position. Fine-mapping identified a 7kb-region containing three genes (**C**). Gray boxes along the vertical axes represent exons from three genes highlighted by vertical arrows. Horizontal red arrows indicate the exact physical position of the last five recombinants defining the QTL candidate region. Amino acid changes between Bay-0 and Shahdara within the interval are presented in the table and linked to their respective physical position along the gene model. (**D**) Model of the protein structure of TZIP1 (*At5g43630*).

loci with major effects are detected across all environments and called *LIGHT1* and *LIGHT5*, whereas the remaining loci called *HYP* are specific to a single environment with more subtle phenotypic contributions (only 4% each). The identification of these two major effect QTL is in accordance with the meager RIL  $\times$  environment interactions found. The *LIGHT5* locus explains over 40% of the variance, with no *LIGHT5*  $\times$  environment interaction found in any condition (data not shown). Its negative allelic effect is predicted to represent a combined 2.1 to 2.5 mm increase in hypocotyl elongation contributed by Shahdara alleles (Sha) relative to the Bay-0 alleles (Bay). In contrast, Sha alleles at *LIGHT1* are responsible for a decrease in hypocotyl elongation compared with Bay alleles, but with a relatively smaller phenotypic contribution (explaining 25–30% of the variance). The opposite allelic effects of *LIGHT1* and *LIGHT5* are responsible for most of the transgression observed in each environment. *LIGHT1* interacts with temperature under either L1 or L2 conditions and with light at either 22 °C or 26 °C (data not shown). There is no significant epistatic relationship between *LIGHT1* and *LIGHT5*, or among any other pair of loci.

#### Confirmation and Fine-Mapping of *LIGHT5* to 3 Candidate Genes.

Identifying the Quantitative Trait Gene (QTG) underlying a QTL is a challenging task that requires several independent lines of proof that a gene is linked to a trait of interest and that variation in this

gene explains the trait (7). The most general approach is fine-mapping to a very small physical candidate interval, which in the best case allows immediate identification of candidate polymorphisms [quantitative trait nucleotide (QTN)] within the causal gene or regulatory regions (8). For most QTL and situations in *A. thaliana*, phenotyping for a QTL effect remains much more limiting than genotyping many individuals. Therefore, an efficient strategy for fine-mapping is to first isolate recombinants within a segregating nearly isogenic line based on genotype alone and then to phenotypically interrogate only the informative ones (in successive rounds) to reduce the candidate interval to the gene level (9).

We followed the HIF strategy to build nearly isogenic lines from a RIL (RIL350) that was segregating solely for the *LIGHT5* region. Comparing plants homozygous for the Sha allele with plants homozygous for the Bay allele at the QTL region (in an otherwise identical genetic background) confirmed the phenotypic impact of *LIGHT5* on hypocotyl elongation (Fig. 1A). HIF350-Sha hypocotyls are consistently 1.6 mm longer than those of HIF350-Bay (slightly less than predicted by the QTL analysis). The analysis of heterozygous plants showed that the Sha allele of *LIGHT5* is fully dominant over the Bay allele (Fig. 1A). The phenotypic effect observed was identical when first fixing alternate genotypes at the QTL region and then comparing the phenotypes of the descendants produced by those homozygous plants (“fixed progeny”) or when







uncontrolled elongation (1). However, *PIF4* and *PIF5* are expressed at control or slightly lower levels in *TZP-OX* (Fig. S9 G and H). These results support *TZP* acting in parallel with (or downstream of) *PIF4* and *PIF5* in growth control.

## Conclusions

Despite extensive forward genetics screens in *A. thaliana*, natural variation has recently made important contributions to the identification of genes not previously known to impact several different traits (38–41). Apart from being able to exploit allelic variation (in multiple genetic backgrounds) that cannot be generated by conventional mutagenesis, the success of these studies has often been because of the use of quantitative phenotyping, as opposed to the qualitative gauges used in typical mutant screens. We have demonstrated here the power of QTL analysis to reveal a new component of the hypocotyl growth pathway in *A. thaliana*, *TZP*, a unique, tandem zinc knuckle/PLUS3 domain protein encoded by a single copy gene in the vascular plant lineage. *TZP* provides a direct link between light signaling and the pathways that control growth in an environmentally independent fashion.

## Materials and Methods

A detailed and referenced version of this section is available online (*SI Materials and Methods*).

**Plant Material and Phenotyping.** The core population of 164 RILs from the Bay-0 × Shahdara set was phenotyped in four different light and temperature

environments to map QTL affecting hypocotyl elongation ([www.inra.fr/vast/Files/Loudet.PNAS.SITables.xls](http://www.inra.fr/vast/Files/Loudet.PNAS.SITables.xls)). HIF350 was developed from an F7 line (RIL350) that still segregated for a single and limited genomic region around *LIGHT5* locus. Plants still heterozygous for the QTL region were screened with adequate markers to isolate recombinants (rHIF) used in the fine-mapping process. Advanced rHIF crosses were generated from two different rHIFs recombined immediately to the north or immediately to the south of the *LIGHT5* interval giving rise to lines arHIF47. Distinct Bay-0 lines from the stock center were used to find variants at *LIGHT5*. rHIF138-8[Bay] was complemented by over-expressing each of the three positional candidate genes cloned from rHIF138-13[Sha].

**QTL Mapping.** Analyses used hypocotyl length mean values of an average of 16 seedlings (from 2 distinct experiments) per genotype per environment. QTL analyses were performed by using QTL Cartographer, with classical parameters for interval mapping and composite interval mapping.

**Microarray Analysis.** Microarray experiments were carried out per Affymetrix protocols (ATH1 GeneChip), on 7-day-old tissue harvested under either continuous blue at subjective dawn or every 4 hours (starting at dawn) under 12 hours white light/12 hours dark cycles over 1 day (six time points). Hybridization intensities from all microarrays were normalized together by using gcRMA implemented in the R statistical package. The blue dataset was then separated and differentially expressed genes were identified by using linear modeling with the limma bioconductor package in R.

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