

# Overexpression of *AtAHL20* causes delayed flowering in *Arabidopsis* via repression of *FT* expression

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## Research article

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# Abstract

**Background:** The 29-member Arabidopsis *AHL* gene family is classified into three main classes based on nucleotide and protein sequence evolutionary differences. These differences include the presence or absence of introns, type and/or number of conserved AT-hook and PPC domains. *AHL* gene family members are divided into two phylogenetic clades, Clade-A and Clade-B. A majority of the 29 members remain functionally uncharacterized. Furthermore, the biological significance of the DNA and peptide sequence diversity, observed in the conserved motifs and domains found in the different *AHL* types, is a subject area that remains largely unexplored.

**Results:** Transgenic plants overexpressing *AtAHL20* flowered later than the wild type under both short and long days. Transcript accumulation analyses showed that *35S:AtAHL20* plants contained reduced *FT*, *TSF*, *AGL8* and *SPL3* mRNA levels. Similarly, overexpression of *AtAHL20*'s orthologue in *Camelina sativa*, Arabidopsis' closely related *Brassicaceae* family member species, conferred a late-flowering phenotype via suppression of *CsFT* expression. However, overexpression of an aberrant *AtAHL20* gene harboring a missense mutation in the AT-hook domain's highly conserved R-G-R core motif abolished the late-flowering phenotype. Data from targeted yeast-two-hybrid assays showed that *AtAHL20* interacted with itself and several other Clade-A Type-I *AHL*s which have been previously implicated in flowering-time regulation: *AtAHL22*, *AtAHL27* and *AtAHL29*.

**Conclusion:** We showed via gain-function analysis that *AtAHL20* is a negative regulator of *FT* expression, as well as other downstream flowering time regulating genes. A similar outcome in *Camelina sativa* transgenic plants overexpressing *CsAHL20* suggest that this is a conserved function. Our results demonstrate that *AtAHL20* acts as a photoperiod-independent negative regulator of transition to flowering.

## Background

The 29-member Arabidopsis AT-HOOK MOTIF CONTAINING NUCLEAR LOCALIZED (*AHL*) gene family is found in all sequenced plant species, ranging from the moss *Physcomitrella patens* to flowering plants such as *Arabidopsis thaliana*, *Sorghum bicolor*, *Zea mays* and *Populus trichocarpa* (Zhao et al., 2013; Zhao et al., 2014). *AHL* proteins are characterized by two conserved structural units: the AT-hook motif and the PLANT AND PROKARYOTE CONSERVED (PPC) domain (Aravind and Landsman, 1998; Fujimoto et al., 2004).

The AT-hook is a small DNA-binding protein domain which was first characterized in the HIGH MOBILITY GROUP (HMG) non-histone chromosomal protein HMG-I(Y), *AHL* homologues in mammals (Aravind and Landsman, 1998). Arabidopsis *AHL*s contain a conserved arginine-glycine-arginine-proline (R-G-R-P) core motif in the AT-hook domain (Street et al., 2008; Zhao et al., 2013; Zhao et al., 2014). When AT-hook domain sequences from all sequenced land plant species are aligned, only arginine-glycine-arginine (R-G-R) amino acid residues remain 100% conserved, suggesting that they are important for function (Zhao et

al., 2014). Studies in HMG proteins and AT-hook motif-containing peptides showed that the AT-hook domain binds to the minor groove of AT-rich DNA via the R-G-R motif (Reeves and Nissen, 1990; Huth et al., 1997; Aravind and Landsman, 1998; Rodriguez et al., 2015). Mutations in this core motif have been shown to abolish DNA binding as well as AHL protein function (Himes et al., 2000; Street et al., 2008; Gordon et al., 2011; Yun et al., 2012; Zhao et al., 2013).

*Arabidopsis* AHLs evolved into two major phylogenetic clades: Clade-A and Clade-B (Zhao et al., 2013). Clade-A and Clade-B AHLs underwent multiple gene duplication events which resulted in the expansion of the gene family (Zhao et al., 2014). Functional characterization of single and multiple gene loss-of-function mutants suggest that genetic redundancy exists among multiple AHL genes in *Arabidopsis* (Street et al., 2008; Xiao et al., 2009; Zhao et al., 2013). In previous AHL gene knockout studies, single *T-DNA* insertion mutants including *ahl22-1* (Xiao et al., 2009), *sob3-4* and *esc-8* (Street et al., 2008) did not show obvious phenotypes, unless other closely related family members were also knocked out. Zhao et al. (2013) reported that when specific AHL genes were knocked out in higher order combinations, such as in the quadruple *sob3-4 esc-8 ahl6 ahl22*, the resultant plants showed more dramatic phenotypes compared to lower order gene knockout mutant combinations. Furthermore, Zhao et al. (2013) also showed that *sob3-6*, a dominant negative mutant carrying a missense allele in the R-G-R core of the AT-hook motif, displayed more dramatic hypocotyl phenotypes compared to the *sob3-4 esc-8 ahl6 ahl22* quadruple mutant. Based on these data, a molecular model was proposed where AHLs interact with each other and themselves, as well as other nuclear proteins, such as transcription factors (TFs), to form a “DNA-AHL-TF complex” (Zhao et al., 2013). Overall, these data suggest that genetic redundancy exists among AHLs. It is hypothesized that most AHLs function as complexes, and that mutations in the DNA-binding AT-hook motif may render that entire complex non-functional (Zhao et al., 2013).

Both loss-of-function and gain-of-function studies in *Arabidopsis* have demonstrated a role for AHLs in plant growth and developmental processes, including auxin, brassinosteroid and gibberellic acid signaling, hypocotyl elongation, petiole growth, root system architecture, environmental stress responses, vascular tissue development, floral organ initiation, organ size, flowering time, and pollen wall development, (Zhao and Grafi, 2000; Matsushita et al., 2007; Street et al., 2008; Xiao et al., 2009; Zhao et al., 2009; Gallavotti et al., 2011; Jin et al., 2011; Kim et al., 2011; Yun et al., 2012; Xu et al., 2013; Jia et al., 2014, 2014; Lou et al., 2014; Favero et al., 2016; Favero et al., 2017; Wong et al., 2019; Favero et al., 2020; Sirl et al., 2020). Out of 29 *Arabidopsis* AHL gene family members, 13 have been characterized; *AtAHL 1*, *AtAHL3*, *AtAHL4*, *AtAHL 10*, *AtAHL 15*, *AHL 16*, *AtAHL 18*, *AtAHL 19*, *AtAHL20*, *AtAHL22*, *AtAHL25*, *AtAHL27* and *AtAHL29* (Fujimoto et al., 2004; Matsushita et al., 2007; Street et al., 2008; Xiao et al., 2009; Lu et al., 2010; Yadeta et al., 2011; Yun et al., 2012; Zhou et al., 2013; Jia et al., 2014; Wong et al., 2019; Sirl et al., 2020). Interestingly, several of the functionally characterized AHLs (*AtAHL 16*, *AtAHL 18*, *AtAHL22*, *AtAHL27* and *AtAHL29*) have been directly or indirectly implicated in the regulation of flowering time in a redundant manner (Xiao et al., 2009; Yun et al., 2012; Xu et al., 2013). *AtAHL 16*, *AtAHL 18*, *AtAHL22*, *AtAHL27* and *AtAHL29* are all Clade-A AHLs (Zhao et al., 2013).

In this study, we used a gain-of-function analysis strategy to avoid potential issues associated with genetic redundancy to characterize *AtAHL20*, a Clade-A *AHL*. *AtAHL20* was initially selected for functional analysis together with *AtAHL6* (Clade-B *AHL* gene family member) as part of a comparative functional characterization study between *AHLs* that belong to two distinct phylogenetic clades and contain different AT-hook domain types. That work is ongoing; therefore, this study only focuses on *AtAHL20*. Transgenic plants overexpressing *AtAHL20* flowered later than the wild type in long-day (LD) and short-day (SD) conditions. Transcript abundance analysis of the key flowering time regulator, *FLOWERING LOCUS T (FT)*, showed that its expression was repressed in *35S:AtAHL20* plants. In addition, *FT*'s redundant homologue, *TSF*, and other downstream flowering pathway genes, *AGL8* and *SPL3*, were also repressed in *35S:AtAHL20* plants. We demonstrated that the second arginine residue in the conserved R-G-R core motif in the AT-hook domain was important for the manifestation of *AtAHL20*'s overexpression phenotypes. Targeted yeast-two hybrid assay results showed that *AtAHL20* interacted with itself, its closest family member *AtAHL19* as well as other Clade-A *AHLs*, *AtAHL22* and *AtAHL29*, which have previously been shown to regulate flowering time. Overall, we demonstrated that *AtAHL20* is a photoperiod-independent repressor of *FT* expression and other downstream flowering pathway genes.

## Results

### ***AtAHL20* tissue expression pattern**

We analyzed *AtAHL20*'s expression pattern via a transcriptional fusion with the  $\beta$ -glucuronidase (*GUS*) reporter gene. Strong global *GUS* signal was observed in all tissues, including root hairs suggesting that *AtAHL20* is constitutively expressed in seedlings (Figure 1A). 12-day old *pAtAHL20-GUS* transgenic plants displayed *GUS* activity in leaf minor veins and trichomes (Figure 1B). In floral structures, *GUS* activity was detected in petals, petal vasculature, anthers, stigma and the upper part of the style, but the signal was weaker in the pedicel and peduncle vasculature (Figure 1C, 1D). Semi-quantitative PCR analysis also showed differential *AtAHL20* tissue expression pattern in whole seedlings, seedling roots, hypocotyls, flowers and siliques, a trend similar to *GUS* histochemical staining pattern (Figure 1E). Largely, *AtAHL20* showed a ubiquitous tissue expression pattern and overlaps with *pFT-GUS* expression pattern in young rosette leaves minor veins (Takada and Goto, 2003).

### ***35S:AtAHL20* plants flowered later than the wild type**

Gene overexpression studies and activation-tagging screens have been key tools used to characterize *AHL* gene function (Street et al., 2008; Xiao et al., 2009; Lu et al., 2010; Yadeta et al., 2011). Multiple independent transgenic plants overexpressing *AtAHL20* displayed a dwarf phenotype (Figure 2A, B) and a late-flowering phenotype compared to the wild type and *ahl20-1/ahl20-2* *T-DNA* insertion lines under both LDs (Figure 2C, 2D) and SDs (Figure 2E, 2F). Previously, a conserved arginine amino acid residue in the AT-hook domain was shown to be necessary for *AtAHL22* and *AtAHL29*'s gain-of-function phenotypes (Street et al., 2008; Yun et al., 2012; Zhao et al., 2013). Therefore, we next examined whether this was the case for *AtAHL20*. We generated constructs carrying *AtAHL20* coding sequence harboring a point

mutation (*AtAHL20m*) in the conserved R-G-R core motif, changing arginine residue number 72 to a histidine (R72>H) (Figure S1). The resultant *35S:AtAHL20m* transgenic plants overexpressing the mutant protein lost both the dwarf and late-flowering phenotypes observed in *35S:AtAHL20* plants (Figure 2A-F). Instead, the *35S:AtAHL20m* transgenic plants displayed an early-flowering phenotype compared to wild-type and *T-DNA* insertion lines under both SDs and LDs (Figure 2C-F) suggesting that *AtAHL20* is a photoperiod-independent floral repressor. These results imply that the second conserved arginine residue is required for the manifestation of *AtAHL20*'s overexpression phenotypes.

### Conserved function of Arabidopsis and Camelina *AHL20* orthologues

Since Arabidopsis and *Camelina sativa* are closely related *Brassicaceae* family member species (Beilstein et al., 2008; Kagale et al., 2014), we hypothesized that some *AHL* orthologues would share similar or overlapping biological functions. To test this hypothesis, one of three Camelina *AHL20-like* copies (*CsAHL20*), which has high similarity to *AtAHL20* at the nucleotide and protein sequence level (Figure S2) was cloned into a binary vector under the *35S CaMV* promoter. The resultant construct was used to transform *Camelina sativa* (L.) Crantz var. Calena wild-type plants. We isolated multiple T<sub>3</sub> homozygous single-locus insertion overexpression lines, which exhibited late-flowering and dwarf phenotypes compared to wild-type controls (Figure 3A). These phenotypes were similar to those observed in *35S:AtAHL20* transgenic plants, inferring that Camelina and Arabidopsis *AHL20* genes have similar biological functions.

### *AtAHL20* represses *FT* expression

To further investigate the cause of the late flowering-time phenotype observed in *35S:AtAHL20* transgenic plants, we measured transcript levels of the key regulatory flowering gene, *FLOWERING LOCUS I* (*FT*) (Corbesier et al., 2007) via reverse transcription quantitative polymerase chain reaction (RT-qPCR). *FT* transcript levels in *35S:AtAHL20* transgenic plants dropped to ~30% of wild-type levels (Figure 4A). This result was similar to that reported in plants overexpressing another Clade-A *AHL* gene family member, *AtAHL22* (Xiao et al., 2009). In contrast, *35S:AtAHL20m* plants contained elevated *FT* levels compared to both wild-type and *35S:AtAHL20* plants (Figure 4A), which is consistent with the early-flowering phenotype observed in these plants.

Since *35S:CsaHL20* transgenic Camelina plants displayed a late-flowering phenotype compared to the wild type (Figure 3A), we hypothesized that this was due to suppression of *CsFT* expression. RT-qPCR data showed that transcript levels of one of three *CsFT* genes in *35S:CsaHL20* plants were repressed four-fold compared to wild-type plants (Figure 3B). Sequence alignment revealed high similarity between *AtFT* and *CsFT* nucleotide and peptide sequences (Figure S3).

Transcriptional profiling using high throughput next-generation ribonucleic acid (RNA) sequencing (RNA-Seq) is a valuable tool to identify differentially expressed genes on a global level (Yang and Wei, 2015; Yang et al., 2015). To gain further insights into the overall flowering-time pathway transcriptome perturbations in *35S:AtAHL20* transgenic plants compared to wild-type plants, we performed RNA-Seq

analysis. Kal's Z-test was performed to identify differentially expressed genes between the wild type and *35S:AtAHL20* transgenic plants (Data S1-2). We identified 1,628 downregulated and 2,179 upregulated genes in *35S:AtAHL20* transgenic plants compared to the wild type. Gene ontology (GO) analysis was performed based on the down-regulated gene list (Data S1) in *35S:AtAHL20* plants compared to the wild type. This led to the identification of three flowering time regulating genes in a small enriched subset of reproductive development GO terms; *AGAMOUS-LIKE 8 (AGL8/AT5G60910)*, *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 (SPL3/AT2G33810)* and *TWIN SISTER OF FT (TSF/AT4G20370)*. This result was confirmed via RT-qPCR analysis, which showed repression of all three genes in *35S:AtAHL20* plants compared to the wild type (Figure 4B). However, *AGL8*, *SPL3* and *TSF* transcript accumulation levels were unchanged in *35S:AtAHL20m* plants (Figure 4B).

### **AtAHL20 interacts with other Clade-A AHLs implicated in flowering time regulation**

A few AHL proteins have been shown to interact with themselves and other non-AHL proteins (Lim et al., 2007; Street et al., 2008; Lu et al., 2010; Yun et al., 2012; Zhao et al., 2013). Interestingly, several Clade-A AHLs have been associated with flowering time phenotypes, suggesting that genetic redundancy exists among these genes (Xiao et al., 2009; Yun et al., 2012; Xu et al., 2013). Therefore, we tested whether any Clade-A AHLs formed homo- and/or heterodimers via targeted yeast-two-hybrid (Y2H) assays. To avoid false positive protein-protein interactions, yeast transformed with bait protein constructs were plated on synthetic defined (SD) media supplemented with a predetermined inhibitory concentration of 1 mM 3-amino-1,2,4-triazole (3-AT) (Figure 5A). Successful co-transformation of yeast with the two bait and prey protein constructs was demonstrated by growth on SDII media (Figure 5B). We showed that AtAHL20 interacted with itself to form a homodimer (Figure 5C). Next, we tested whether other Clade-A AHLs that have been implicated in flowering time regulation interacted with each other to form heterodimers. Indeed, AtAHL20 interacted with AtAHL19, AtAHL22 and AtAHL29. We further asked whether it was possible that all AHLs interacted with each other and tested for interaction between a Clade-B member AtAHL6, and a Clade-A member AtAHL20. There was no interaction between AtAHL6 and AtAHL20, indicating that not all AHLs interact with each other (Figure 5C, Table 1).

## **Discussion**

### **Overexpression of *AtAHL20* confers a late-flowering time phenotype in Arabidopsis**

Our gain-of-function study showed that overexpression of *AtAHL20* confers a late-flowering time phenotype under LDs (Figure 2). This result is consistent with previous work implicating several Clade-A Arabidopsis AHLs (*AtAHL18*, *AtAHL22*, *AtAHL27* and *AtAHL29*) in flowering time regulation (Street et al., 2008; Xiao et al., 2009; Yun et al., 2012). Specifically, transgenic plants overexpressing *AtAHL22*, *AtAHL27* and *AtAHL29* displayed a late-flowering phenotype (Street et al., 2008; Xiao et al., 2009; Yun et al., 2012). *AtAHL22*, *AtAHL27* and *AtAHL29* single gene knockout mutants did not show any clear flowering-time phenotypes. Only when *AtAHL27*, *AtAHL29*, *AtAHL22* and *AtAHL18* were simultaneously knocked out and/or knocked down, did the quadruple mutant display an early-flowering phenotype (Street et al., 2008;

Xiao et al., 2009; Yun et al., 2012). These data suggested that several *AHLs* may function as part of a complex(es) to regulate gene expression (Tetko et al., 2006; Reeves, 2010; Yun et al., 2012) and that functional redundancy exists between these genes and other Clade-A *AHL* family members. Indeed, Zhao et al. (2013) proposed a similar model suggesting that various *AHLs* formed multi-*AHL* complexes to regulate hypocotyl growth in *Arabidopsis*. Data from our targeted yeast-two-hybrid assays supports this model by demonstrating that *AtAHL20* physically interacted with itself and other Clade-A *AHL* members; *AtAHL19*, *AtAHL22* and *AtAHL29* (Figure 4). It is, therefore, conceivable that these *AHLs* regulate flowering time as part of a complex. *AtAHL20* did not interact with *AtAHL6* (a Clade-B *AHL*) indicating that not all *AHLs* interacted with each other. Overall, we have shown that *AtAHL20* is the fifth Clade-A *AHL* to be implicated in flowering time regulation in *Arabidopsis*.

### ***AtAHL20* is a repressor of *FT* expression**

Gene expression analyses showed that overexpression of *AtAHL20* resulted in a depletion of *FT* transcript levels (Figure 3). This is not surprising considering that several *AHLs*, including *OsAHL1*, *AtAHL5*, *AtAHL10*, *AtAHL12*, *AtAHL16*, *AtAHL20*, *AtAHL22*, *AtAHL27* and *AtAHL29*, have been reported to exhibit promoter binding capabilities or been shown to confer transcriptional repression or activation of downstream target genes (Xu et al., 2013; Xu et al., 2013; Franco-Zorrilla et al., 2014; Franco-Zorrilla and Solano, 2014; Jia et al., 2014; Favero et al., 2016; Zhou et al., 2016; Lee and Seo, 2017; Wong et al., 2019; Favero et al., 2020). In particular, a previous study showed that *AtAHL20* is a negative regulator of defenses in *Arabidopsis* (Lu et al., 2010). We also showed that an *AHL20* orthologue in *Camelina* repressed *CsFT* expression, which suggests a conserved function across the two species (Figure S2B). It can be hypothesized that several *AHLs* modulate gene transcription, individually or as part of protein complexes in *Arabidopsis* and other species (Yun et al., 2012; Zhao et al., 2013; Favero et al., 2016; Lee and Seo, 2017). Our studies did not show a direct biological mechanistic link between *AtAHL20* overexpression and repression of *FT* transcription. However, a close Clade-A *AHL* family member, *AtAHL22*, was shown to repress *FT* expression via a chromatin remodeling process (Yun et al., 2012). This occurs via *FT* chromatin architecture modification through both H3 acetylation and methylation. In addition, (Favero et al., 2016) also showed that *AtAHL29*, a Clade-A *AHL*, directly binds to *YUC8* and *SAUR19* promoters resulting in gene expression repression. Lee et al. (2017) went further and showed that *AtAHL27* and *AtAHL29* bind *YUC9* promoter and suppress gene expression via chromatin modification activities of SWI2/SNF2-RELATED 1 (SWR1) complex. Recently, Favero et al. (2020) showed that *AtAHL29* binds to PIF-targeted loci to reduce binding of PIF to these regions, thereby inhibiting transcriptional activation of growth promoting genes in *Arabidopsis* petioles. We hypothesize that *AtAHL20* may also bind *FT* promoter elements and suppress its expression, perhaps individually or as part of complex. After all, *AtAHL20* has already been shown to have binding affinities for several A/T-containing elements (Franco-Zorrilla et al., 2014; Franco-Zorrilla and Solano, 2014). A definitive answer to the question of the mechanism of gene repression may be provided via future studies that include yeast-one-hybrid and (chromatin immunoprecipitation) ChIP RT-qPCR experiments. Interestingly, *35S:AtAHL20* plants displayed similar seedling hypocotyl growth inhibition and adult plant phenotypes (dwarfism and late flowering) to *35S:AtAHL22* plants (Figures 2, 5), *esc-D/AtAHL27* as well as *sob3-D/AtAHL29* (Street et

al., 2008; Xiao et al., 2009). Targeted Y2H studies showed that AtAHL20 interacted with AtAHL22, ESC/AtAHL27 and SOB3/AtAHL29 (Figure 4). It is therefore plausible that these AHLs function redundantly to regulate hypocotyl elongation and flowering time, possibly as part of a complex that includes AtAHL20, AtAHL22, AtAHL27 and AtAHL29, which have all been shown to regulate both biological processes.

### **Missense mutation in the AT-hook domain abolishes *AtAHL20*'s overexpression phenotype**

We hypothesized that a missense mutation in *AtAHL20*'s AT-hook domain would abolish function based on similar outcomes in other Clade-A AHL gene family members, *AtAHL29* (Street et al., 2008) and *AtAHL22* (Yun et al., 2012). Thus, it was not surprising that *35S:AtAHL20m* transgenic plants (Figure 2A-D) lost the late-flowering phenotype typically observed when the wild-type *AtAHL20* gene is overexpressed. Our working hypothesis based on the works of (Street et al., 2008; Yun et al., 2012; Zhao et al., 2013)

is that the second arginine residue in the AT-hook domain's conserved R-G-R core is important for DNA binding, and without it, AtAHL20 is unable to bind AT-rich DNA and recruit chromatin modifying components required to repress *FT* transcription. This is in line with a deletion mutant study from Lu et al., (2010) who showed that removal of the entire AT-hook domain abolished AtAHL20's suppression function. This raises an interesting question regarding the specific biological importance of the conserved R-G-R amino acid trio found in both type-1 and type-2 AT-hook motifs, versus peptide sequences flanking the AT-hook domain, for example. Would the mutation of the second arginine in the R-G-R core motif in all three AHL types (Type-I, -II, -III) also abolish overexpression phenotypes observed in transgenic plants overexpressing these genes? What role does the divergent nature of amino acid sequences flanking the R-G-R core play in Clade-A versus Clade-B AHLs? Studies in mammalian AHL orthologues, HMGA proteins, showed that the different types of AT-hook domains bind DNA with different affinities (Huth et al., 1997; Dragan et al., 2003). HMGA proteins containing a type-1 AT-hook, similar to the one found in Clade-A AHLs (e.g. AtAHL20, AtAHL22, AtAHL27 and AtAHL29) (Zhao et al., 2013), were found to confer the highest affinity to AT-rich DNA due to the nature of the peptide sequence adjacent to the R-G-R core motif. Interestingly, HMGAs containing a type-2 AT-hook, similar to one found in AtAHL6, have decreased DNA-binding affinity to AT-rich DNA (Huth et al., 1997; Dragan et al., 2003). Notably, preliminary data from *AtAHL6* gain of function studies showed that transgenic plants overexpressing an aberrant gene carrying an R81->H mutation (*35S:AtAHL6m*) did not abolish the overexpression phenotype (early-flowering) observed in *35S:AtAHL6* plants. We can thus speculate whether this due to the fact that AtAHL6's AT-hook domain has low DNA-binding affinity to begin with. In the future, it will be important to further investigate the effect of missense mutations in Clade-A versus Clade-B AHLs which contain different At-hook types, and whose conserved R-G-R core is flanked by divergent amino acid sequences.

### ***FT* repression by *AtAHL20* negatively affects expression of downstream flowering pathway genes**

Quantitative PCR data showed that overexpression of *AtAHL20* also resulted in repression of *TSF*, *AGL8* and *SPL3* expression (Figure 3B). *AGL8* and *SPL3* function downstream of *FT* in the flowering pathway

(Higgins et al., 2010) whereas *TSF* acts redundantly with *FT* as a floral pathway integrator (Yamaguchi et al., 2005). The fact that two redundant floral pathway integrators *FT* and *TSF* transcript levels are regulated in a similar manner in *35S:AtAHL20* transgenic plants, raises an interesting question. Does *AtAHL20* act directly on these two floral pathway integrators, or act upstream of them. Further experiments, including ChIP-Seq, yeast-one-hybrid assays may help identify *AtAHL20*'s direct targets. Previous work showed that overexpression of *AHL29*, a Clade-A Type-I gene (just like *AtAHL20*), also caused delayed flowering in *Arabidopsis* (Street et al., 2008). Interestingly, preliminary Chip-Seq data from our lab showed that *AtAHL29* binds *FT*. Taken together, these data suggest that *AtAHL20* may function in a similar manner, by directly binding to promoters of its downstream targets.

At the same time, it was interesting that overexpression of an aberrant *AtAHL20* protein in *35S:AtAHL20m* transgenic plants only resulted in the elevation of *FT* transcript levels but not in downstream flowering pathway genes *TSF*, *AGL8* and *SPL3*. We speculate that *AtAHL20* indirectly affects expression of downstream targets via the direct repression of the main regulatory component of the flowering pathway, *FT*. Therefore, perhaps the elevation of *FT* transcript levels in *35S:AtAHL20m* transgenic plants is not of enough magnitude to dramatically alter the expression of downstream components.

## Conclusion

In conclusion, overexpression of *AtAHL20* repressed the expression of flowering pathway genes *FT*, *TSF*, *AGL8* and *SPL3*. In contrast, overexpression of an aberrant *AtAHL20* protein harboring a missense mutation in the AT-hook domain abolished these phenotypes. These data suggest that *AtAHL20* is a transcription factor whose function is partly dependent on a conserved R-G-R core motif in the AT-hook domain.

## Methods

### Plant material

All *Arabidopsis thaliana* plants are in the Columbia (*Col-0*) background. *Col-0* and *AtAHL20* T-DNA insertion mutants, *ahl20-1* (Salk\_144620) and *ahl20-2* (Salk\_148971) seeds used in this study were obtained from the Arabidopsis Biological Resource Center (ABRC). Camelina plants *Camelina sativa* (L.) Crantz var *Calena*) were grown in a greenhouse (16 h under the light and 8 h in the dark) at 25°C. Camelina seeds were provided by Dr Scot Hulbert of Washington State University, who obtained them from Dr. Stephen Guy at Washington State University (Guy et al., 2014)

### Cloning and generation of transgenic Arabidopsis and Camelina plants

#### *Arabidopsis thaliana*

#### *AtAHL20* overexpression

Gateway compatible entry plasmids containing Arabidopsis *AHL* gene coding sequences as well as other genes used in this study were obtained from ABRC. To overexpress *AtAHL20*, Gateway entry vector, pENTR223, was used in Gateway LR reactions (Invitrogen, Carlsbad, CA) together with a destination vector pEarlyGate100 binary vector (*35S* constitutive promoter) (Earley et al., 2006). The binary vectors carrying *AtAHL20* cDNA were used to transform *Col-0* wild-type plants via the floral dip method (Clough and Bent, 1998). To generate point mutations in *AtAHL20*'s AT-hook domain we used a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA) using Gateway compatible primers (Table 2). pENTR223 vector carrying *AtAHL20* cDNA was used as a template during the site-directed mutagenesis reaction. The resulting construct was sequenced to confirm the successful mutation of the arginine residues in the respective coding sequences.

## **GUS constructs**

*AtAHL20*'s 1335 bp long promoter region was PCR amplified using Gateway-compatible primers (Table 2) and cloned into the Gateway compatible entry vector pDONR221 via a BP reaction (Invitrogen, Carlsbad, CA). Following the BP reaction, the resultant entry vector was sequenced to confirm the absence of mutations. pDONR221 entry vectors carrying *AtAHL20* promoter were cloned into the Gateway-compatible destination vector pMD163 (ABRC) via the Gateway LR reaction to generate a promoter:GUS expression binary vector.

Transgenic Arabidopsis plants expressing the above mentioned constructs were generated in the wild type *Col-0* background via the floral-dip method (Clough and Bent, 1998). Transgenic seeds were screened on 0.5× Linsmaier and Skoog modified basal medium supplemented with appropriate antibiotics containing 1.0% (w/v) phytigel (Sigma-Aldrich), 1.5% (w/v) sucrose and under continuous white light at 25°C in a Percival E-30B growth chamber.

## ***Camelina sativa***

### **Overexpression of *CsAHL20***

*CsAHL20* coding sequence was extracted from the NCBI database after a BLAST search using *AtAHL20* sequence as a query. Primers (Table 2) were designed from the extracted sequence and were used to amplify *CsAHL20*'s coding sequence. The amplified PCR product was cloned into pDONR221 entry vector via Gateway BP clonase II (Invitrogen, Carlsbad, CA) reaction to generate the pDONR221-*CsAHL20* entry vector. A Gateway LR clonase II (Invitrogen, Carlsbad, CA) reaction between pDONR221-*CsAHL20* and the destination vector pUSH21 was performed to generate pUSH42-2. In this construct expression of *CsAHL20*, coding sequence and the selection marker DsRed were separately driven by CaMV 35S promoters. The binary vector was transformed into *Agrobacterium tumefaciens* strain GV3101 and used for plant transformation via the floral-dip protocol (Lu and Kang, 2008). T<sub>1</sub> seeds harvested from transformed plants were illuminated with a green LED light and fluorescent seeds were visually detected under a red filter (Lu and Kang, 2008). Single insertion T-DNA T<sub>2</sub> mutants were identified by screening for

plants that produced 3:1 fluorescent: nonfluorescent seeds. Homozygous T<sub>3</sub> pUSH42-2-CsAHL20 plants from single locus insertion lines were used for qRT-PCR analysis.

### **Yeast-Two-hybrid plasmids**

A GAL4-based Y2H system was used in protein-protein interaction assays (Weber et al., 2005). Yeast strain *L40ccU3*, bait vector (*pBTM116-GW-D9*) with *TRP1* reporter marker and prey vector (*pACT2-GW*) with *LEU* reporter marker were obtained from Dr. Hanjo Hellmann's lab (Washington State University, Pullman, WA). Gateway entry vectors carrying *AtAHL6*, *AtAHL19*, *AtAHL20*, *AtAHL22* and *AtAHL29's* coding sequences genes were used in LR reactions to clone the respective open reading frames into the bait and prey vectors (*pBTM116-GW-D9*) and (*pACT2-GW*), respectively. Competent yeast cells were transformed with bait and prey plasmid constructs using a standard lithium acetate protocol. Transformed yeast competent cells were incubated for three days at 28°C on SD minimal medium supplemented with Leu and His (SDII). Four randomly selected colonies were diluted 1:2,000 in autoclaved distilled water before 20 µL were simultaneously dropped on both SDII and SDIV lacking tryptophan, leucine, histidine and uracil and containing predetermined levels of 3-amino-1, 2, 4-triazol (3-AT). Yeast was incubated at 28°C for 3-6 days.

### **RNA extraction, cDNA synthesis, qRT-PCR, semi-quantitative PCR and data analysis**

Total RNA was extracted from 10-day old camelina seedlings grown on ½ × MS medium using Plant RNA mini kit (Qiagen, Valencia, CA) according to manufacturer's recommendations. For Arabidopsis, total RNA was extracted from rosette leaves collected from 21-day old adult plants (analyzed to quantify *FT*, *TSF*, *AGL8* and *SPL3* via qRT-PCR), as well as from 7-day old seedling roots, whole 7-day old seedling, adult plant rosette leaf, 7-day old seedling hypocotyls, flowers and siliques (used for semi-quantitative PCR for *AtAHL20* tissue specific expression). On-column DNase treatment was performed to digest any potential contaminating genomic DNA. Complementary DNA (cDNA) was synthesized from total RNA (500 ng) using the iScript Reverse Transcription Super mix (Bio-Rad, Hercules, CA). qRT-PCR was carried out using Bio-Rad's SSO Advanced Universal SYBR Green Super Mix (Bio-Rad, Hercules, CA) **and 10-fold diluted** cDNA templates (synthesized above) on a Bio-Rad's CFX96 Touch Real-Time PCR Detection System. Melting curves of SYBR green wells were cross checked to eliminate nonspecific amplification. Data are normalized to MDAR4 messenger ribonucleic acid (mRNA) expression (internal control), and fold changes are displayed relative to control plant lines. Error bars represent standard deviations of technical replicates (n = 3). Three biological replicates were used from each plant line.

### **RNA-Seq library preparation**

Total RNA was extracted from rosette leaves harvested from 20-day old growth-chamber-grown plants using MagJET Plant RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA). The Dynabeads mRNA DIRECT Kit (Thermo Fisher Scientific, Waltham, MA) was used for purification of intact polyadenylated (polyA) mRNA. RNA-Seq libraries used were prepared using the Ion Total RNA-Seq Kit v2 (Life Technologies, Carlsbad, CA) following the manufacturer's protocol.

RNA-Seq datasets were analyzed using CLC Genomics Workbench software (Qiagen, Valencia, CA). RNA-Seq libraries were constructed from RNA extracted from rosette leaf tissue pooled from three independent plants. Following Kal's Z-test (Kal et al., 1999), genes were classified as differentially expressed with a False Discovery Rate (FDR) adjusted p-value < 0.05 and a fold-change absolute value > 3.

### **Read mapping and differential expression**

Reads which already had adaptor sequences removed by the Torrent Suite ver 4.2.1 sequencing software (Thermo Fisher Scientific, Waltham, MA), were quality trimmed using the default setting in CLC Genomics Workbench 7.5 (Qiagen, Valencia, CA). After preprocessing the RNA-Seq data, the reads were mapped to the TAIR10 version of the Arabidopsis genome using CLC Genomics Workbench 7.5 (Qiagen, Valencia, CA). Read counts for each gene were quantified using the RNA-Seq Analysis tool using the default settings. Differential expression of original values was determined with the proportions statistical analysis tool, using Kal's Z-test with FDR correction.

### **Gene ontology enrichment**

Gene ontology analysis was performed using the PLAZA 3.0 Dicots Workbench Analysis tool (Proost et al., 2015)

### **Histochemical GUS analysis**

GUS analysis was performed as described by (Zhang et al., 2009) on six-day old seedlings, rosette leaves from 20-day old and floral structures from flowering plants grown in the greenhouse.

### **Flowering time analysis**

It has been observed that transplanting seedlings to soil can cause stresses that can alter flowering time. Consequently, all seeds were directly sown in pots containing a pre-watered soil mix Sunshine 50 Mix4 (Aggregate) LA4, Green Island Distributors Inc.; Riverhead, N.Y). These pots were subsequently incubated in darkness for seven days at 4°C to induce near-uniform germination. After that, pots were transferred to growth chambers under the following conditions: white light ( $200 \mu\text{mol m}^{-2} \text{sec}^{-1}$ ), 21°C and 60-70 % humidity. Once the seedlings were several days old, they were thinned to one per pot by clipping using small scissors. Experience in the lab suggests that removal of whole seedlings causes root damage to neighboring seedlings, which in turn can cause damage/stress that can potentially lead to altered flowering time. This approach gives the most uniform and repeatable flowering time results for each genotype. To measure flowering time, we counted the number of days from germination time until the floral stem was 0.5 cm above the basal rosette.

### **Seedling growth conditions and hypocotyl measurement**

Seedling growth conditions and hypocotyl measurements were performed as described in (Zhao et al., 2013).

## Sequence alignment

AtAHL20, AtFT, CsAHL20 and CsFT nucleotide and protein sequences were downloaded from NCBI database (Data S4). Both nucleotide and protein sequence alignment were aligned using

BOXSHADE public server [https://embnet.vital-it.ch/software/BOX\\_form.html](https://embnet.vital-it.ch/software/BOX_form.html).

## Statistical analysis

Results are presented as mean values for the combined data. Error bars represent the standard error of the mean (SEM) or standard deviation (SD). For group means multiple comparison to the wild type, we used a One-way ANOVA Dunnett's multiple comparison test or T-test using GraphPad Prism statistical software. \*\*\*\* = p-value < 0.0001, \*\*\* = p-value 0.0001 to 0.001, \*\* = p-value 0.001 to 0.01, \* = p-value 0.01 to 0.05, ns = p-value  $\geq$  0.05.

## Abbreviations

3-AT: 3-amino-1,2,4-triazole

ABRC: Arabidopsis Biological Resource Center

AGL8: AGAMOUS-LIKE 8

AHL: AT-HOOK MOTIF CONTAINING NUCLEAR LOCALIZED

AHL20: AT-HOOK MOTIF CONTAINING NUCLEAR LOCALIZED #20

ANOVA: analysis of variance

AT: Adenine Thymine

At: *Arabidopsis thaliana*

CaMV: cauliflower mosaic virus

*Col-0*: Columbia

Cs: *Camelina sativa*

DNA: Deoxyribonucleic acid

FT: FLOWERING LOCUS T

FDR: false discovery rate

GO: gene ontology

GUS: *beta*-glucuronidase

HMG: HIGH MOBILITY GROUP

LD: long days

SD: short days

MDAR4: MONODEHYDROASCORBATE REDUCTASE 4

mRNA: Messenger Ribonucleic acid

NCBI: National Center for Biotechnology Information

PPC: PLANT AND PROKARYOTE CONSERVED

R-G-R-P: arginine-glycine-arginine-proline

RNA-seq: ribonucleic acid sequencing

RT-qPCR: reverse transcription quantitative polymerase chain reaction

SD: synthetic defined

SEM: standard error of mean

SPL3: SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3

T-DNA: transfer deoxyribonucleic acid

TF: Transcription factor

TSF: TWIN SISTER OF FT

## Declarations

### Ethics approval and consent to participate

Not applicable

### Consent for publication

Not applicable

### Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

### Competing interests

The authors declare that they have no competing interests

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### Authors' contributions

R.T. and M.M.N designed the research. R.T., P.S-K, C.F.P. and B.E.W. performed the experiments. R.T. generated all constructs used in Arabidopsis studies. P.S-K. generated constructs used in *Camelina sativa* studies. P.S-K. generated and analyzed all *Camelina sativa* transgenic material. C.F.P. and B.E.W. participated in generating and screening *AtAHL20* transgenic material. R.T. and M.M.N. wrote the paper. All authors read and approved the final manuscript.

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## Tables

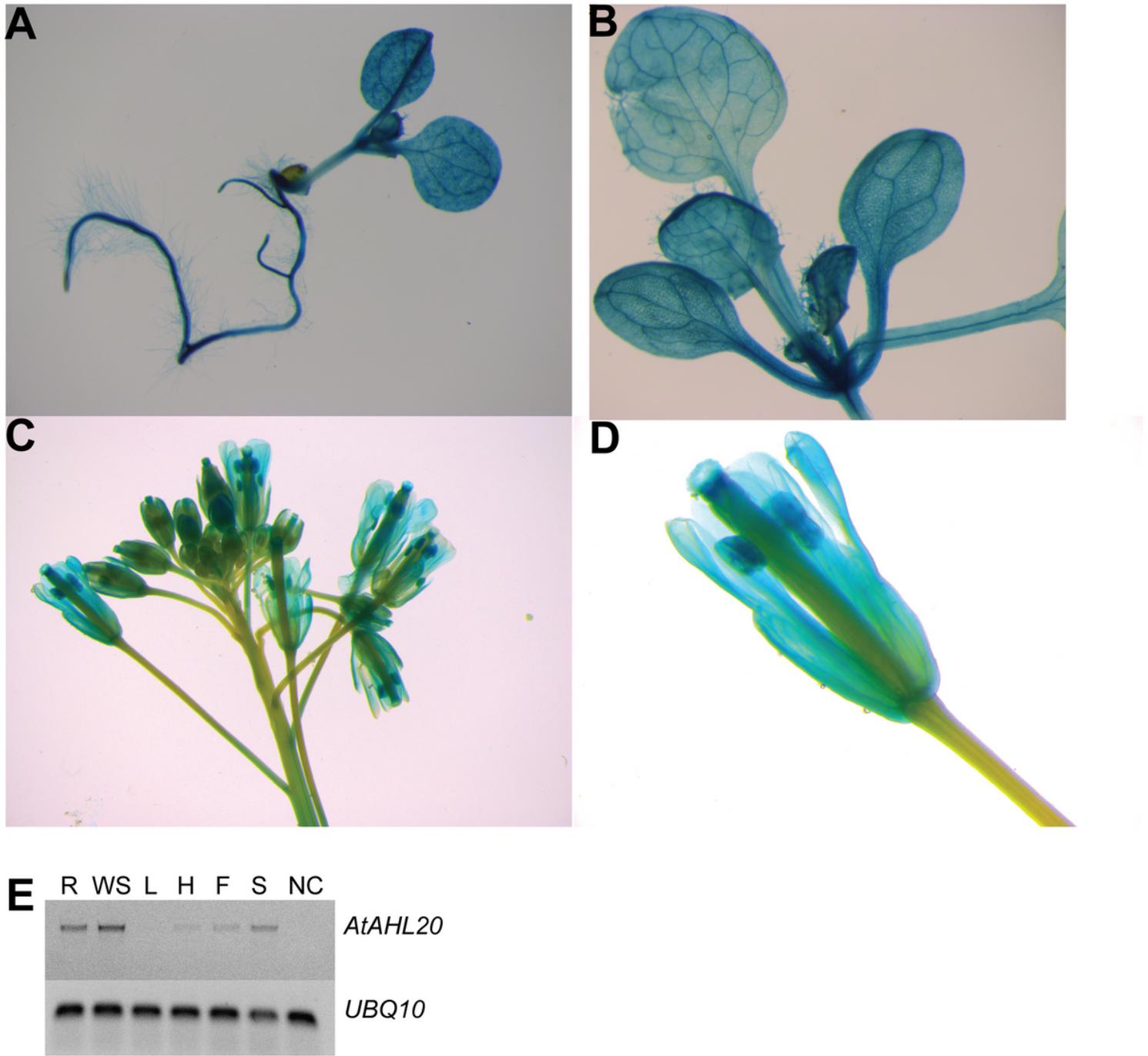
**TABLE 1.** Yeast two hybrid interactions

Clade	Bait	Interacting partner	Interaction
A/A	AtAHL19	AtAHL19	Positive
A/A	AtAHL20	AtAHL20	Positive
A/A	AtAHL20	AtAHL19	Positive
A/A	AtAHL22	AtAHL20	Positive
A/A	AtAHL29	AtAHL20	Positive
A/A	AtAHL29	AtAHL19	Positive
B/B	AtAHL6	AtAHL6	Positive
B/A	AtAHL6	AtAHL20	Negative

**TABLE 2.** Primers used for cloning and gene expression studies in the study.

Primer	Sequence
<i>Arabidopsis thaliana</i>	
AtAGL8qPCR-F	TGCGCTCCAGAAGAAGGATAAAGC
AtAGL8 qPCR-R	TCCGTCAACGACGATGCACCA
AtAHL20CDS-F	ATGGCAAACCCTTGGTGGAC
AtAHL20CDS-R	TCAGTAAGGTGGTCTTGCCT
AtAHL20-ATTB-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCAAACCCTTGGTGGAC
AtAHL20-ATTB-Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGTAAGGTGGTCTTGCCTGGA
AtAHL20qPCR-Fw	CGTTGAGGTGGTCAACCGTA
AtAHL20qPCR-Rv	TTGCCTGCGTCTTGAGAAGT
AtFTqPCR-F	CCAAGTCCTAGCAACCCTCA
AtFTqPCR-R	TACTGTTTGCCTGCCAAG
AtMDAR4q PCR-Fw	GCGGTGGCTATATCGGTATGG
AtMDAR4q PCR-Rv	AAAGAGACGTGCCATGCAGTG
AtSPL3qPCR-F1	CTTAGCTGGACACAACGAGAGAAGG
AtSPL3qPCR-R1	GAGAAACAGACAGAGACACAGAGGA
AtTSFqPCR-F	GAGTCCAAGCAACCCTCACCAA
AtTSFqPCR-R	CACCACAATACGATGAATTCCCGAG
UBQ10 -F	GGCCTTGTATAATCCCTGATGAATAAG
UBQ10 -R	AAAGAGATAACAGGAACGGAAACATAGT
Promoter-GUS	
AtAHL20Prom-ATTB-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTTGTAGCGGTAAATTGTGGCTTAA
AtAHL20Prom-ATTB-Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGATTGACCAAAAACTGGAAATTTCGC
<i>Camelina sativa</i>	
CsAHL20-Fw	AACGGTTTACTTAGCCGGGG
CsAHL20-Rv	GCAGCTATCACCATGACCGA
CsAHL20-ATTB-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGTCAAACCCTTGGTGGACG
CsAHL20-ATTB-Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGTATGGTGGTCTGCGCGCTG
CsFT-Fw	AGGAATTCACCGTGTCTGTA
CsFT-Rv	CGAGTGTGAAGTTCTGGCG
CsMDAR4-Fw	TTGGCGAAATGAGGAGGCTT
CsMDAR4-Rv	AATGCCATGAGAAGGCGAA

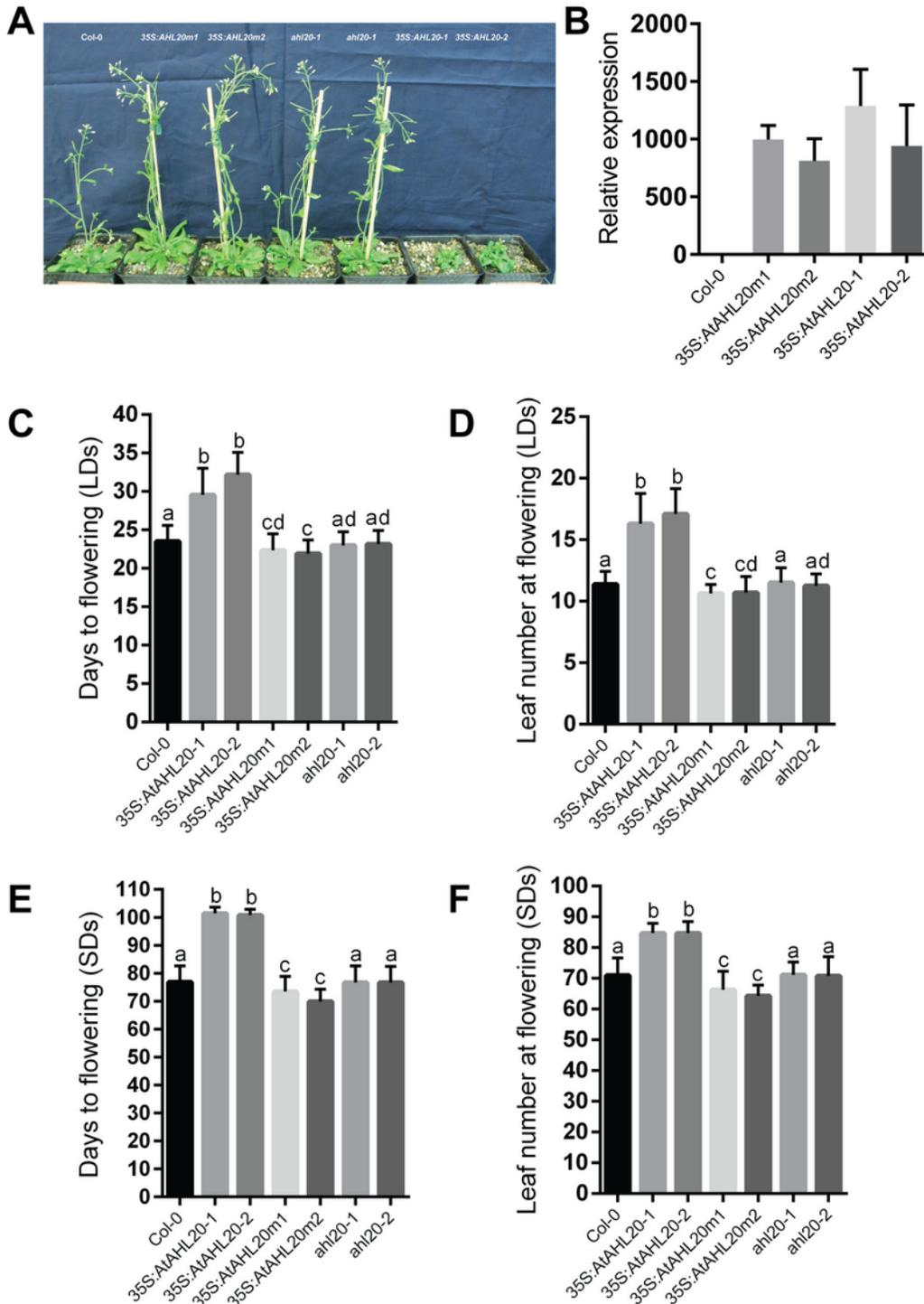
## Figures



**Figure 1**

*AtAHL20* promoter-GUS expression patterns. Wild-type Arabidopsis plants were transformed with a construct encoding a pAtAHL20-GUS transcriptional fusion. Histochemical analysis was conducted on 6-day old seedlings grown on Murashige and Skoog (MS) agar plates under continuous white light, as well as on rosette leaves and floral structures of adult plants grown under LD conditions in the greenhouse. GUS histochemical staining patterns in (A) 6-day old pAtAHL20:GUS seedlings (B), 12-day old plant pAtAHL20:GUS rosette leaf showing expression in minor veins (C), pAtAHL20:GUS floral structure (D), close-up of pAtAHL20:GUS floral structure. GUS activity assays were performed three times with similar results. (E), Semi-quantitative PCR gel showing *AtAHL20* tissue expression pattern in 7-day old seedling

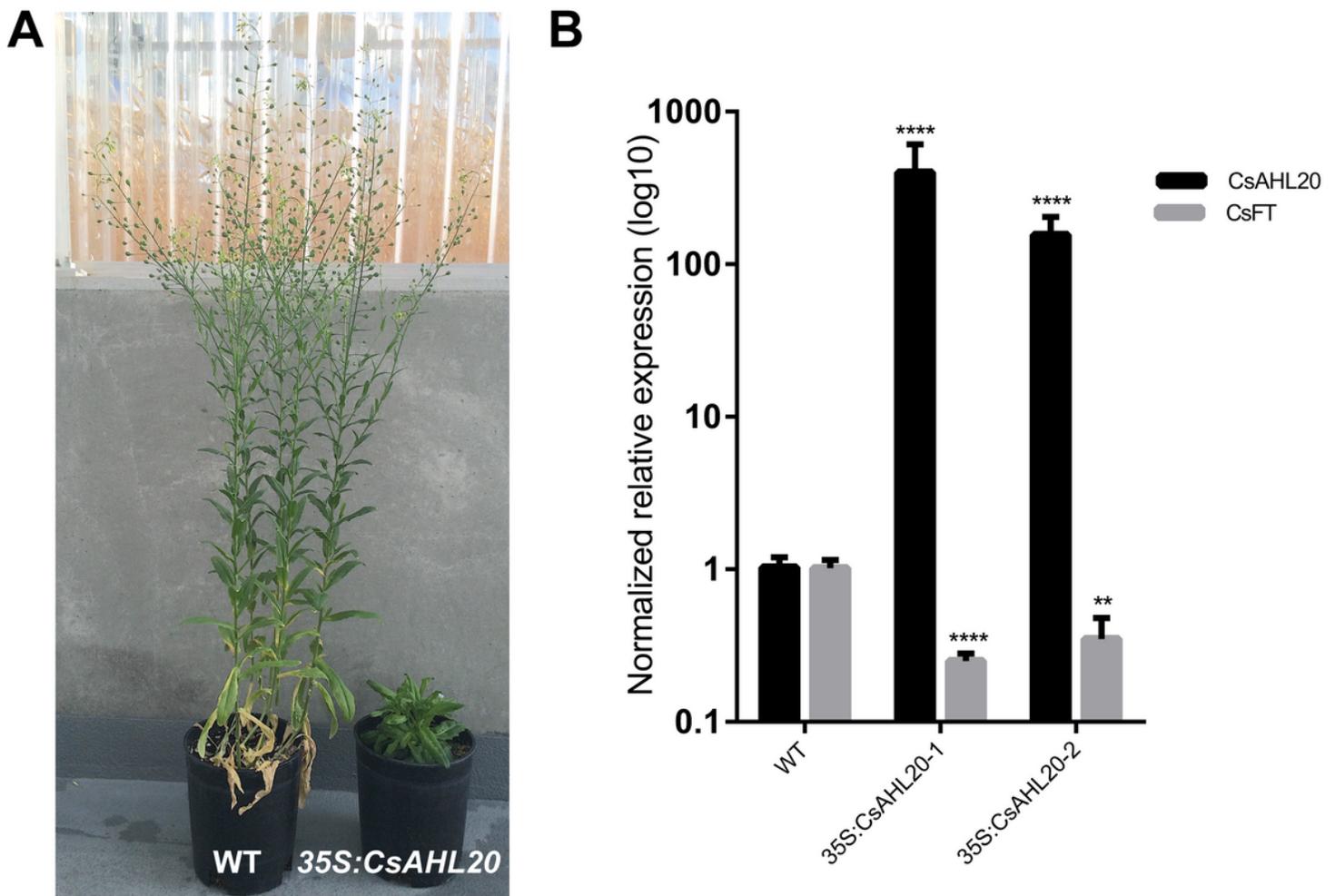
roots (R), 7-day old whole seedling (WS), adult flowering plant rosette leaf (L), 7-day old hypocotyls (H), flower (F), siliques (S) and no reverse transcriptase negative control (NC). UBQ10 was used as a house keeping gene control.



**Figure 2**

Phenotypic and flowering-time analysis of 35S:AtAHL20 transgenic plants. (A) 35S:AtAHL20 transgenic plants displayed dwarf phenotypes compared to the wild type, 35S:AtAHL20m1, 35S:AtAHL20m2, ahl20-

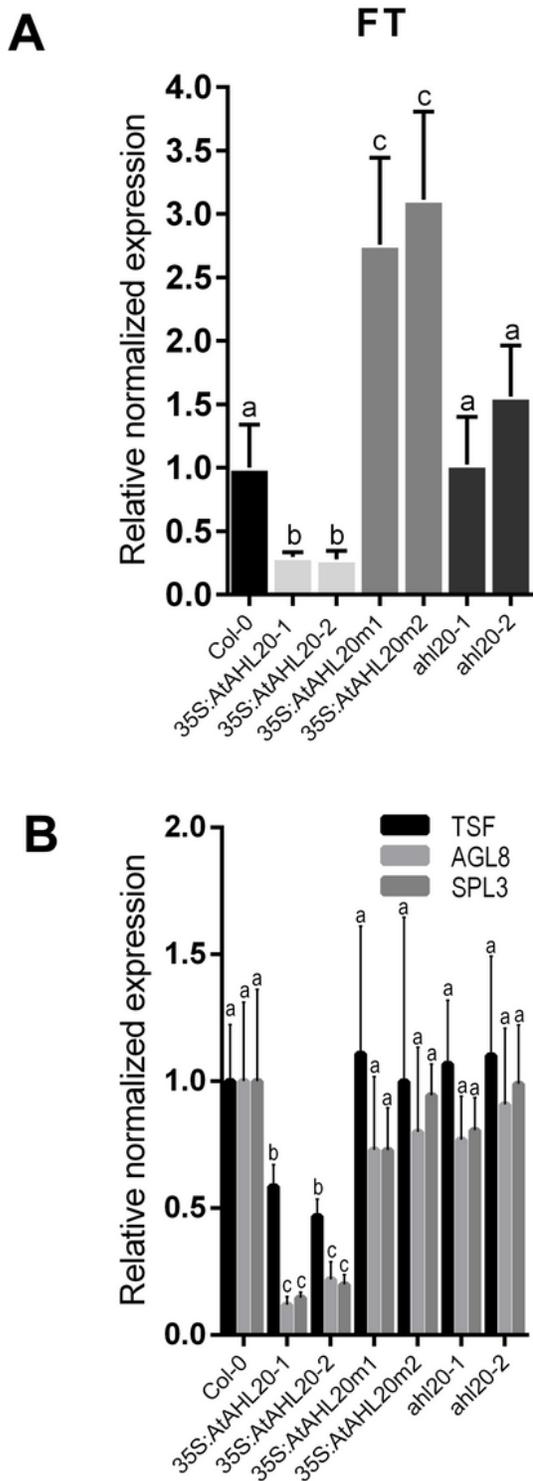
1 and *ahl20-2* plants under LD conditions. (B) *AtAHL20* expression levels in 35S:*AtAHL20* and 35S:*AtAHL20m* plants. (C and D) 35S:*AtAHL20-1* and 35S:*AtAHL20-2* transgenic plants flowered later than the wild type, 35S:*AtAHL20m1*, 35S:*AtAHL20m2*, *ahl20-1* and *ahl20-2* plants under LD conditions. (E and F) 35S:*AtAHL20-1* and 35S:*AtAHL20-2* transgenic plants flowered later than the wild type, 35S:*AtAHL20m1*, 35S:*AtAHL20m2*, *ahl20-1* and *ahl20-2* plants under SD conditions. When we overexpressed *AtAHL20* protein carrying a point mutation in a conserved R-G-R core motif, the resultant transgenic plants flowered at the same time as the wild type. Flowering time was calculated by counting the number days from sowing until the appearance of a 1 cm long primary bolt (B), as well as by counting the total number of primary rosette and cauline leaves present at bolting (C). The error bar denotes standard deviation (SD). Different letters indicate statistical significance (ANOVA;  $P < 0.05$ ).  $n =$  at least 35 plants (between 35 - 48 plants per genotype). The experiment was repeated three times with similar outcomes.



**Figure 3**

*CsAHL20* overexpression represses *CsFT* expression in *Camelina sativa*. (A) 35S:*CsAHL20* plants displayed a dwarf and late-flowering phenotype compared to the wild type. (B) Overexpression of *CsAHL20* in *Camelina sativa* wild-type plants resulted in suppressed *CsFT* transcript levels. T-test, using GraphPad Prism statistical software. \*\*\*\* =  $p$ -value  $< 0.0001$ , \*\*\* =  $p$ -value 0.0001 to 0.001, \*\* =  $p$ -value

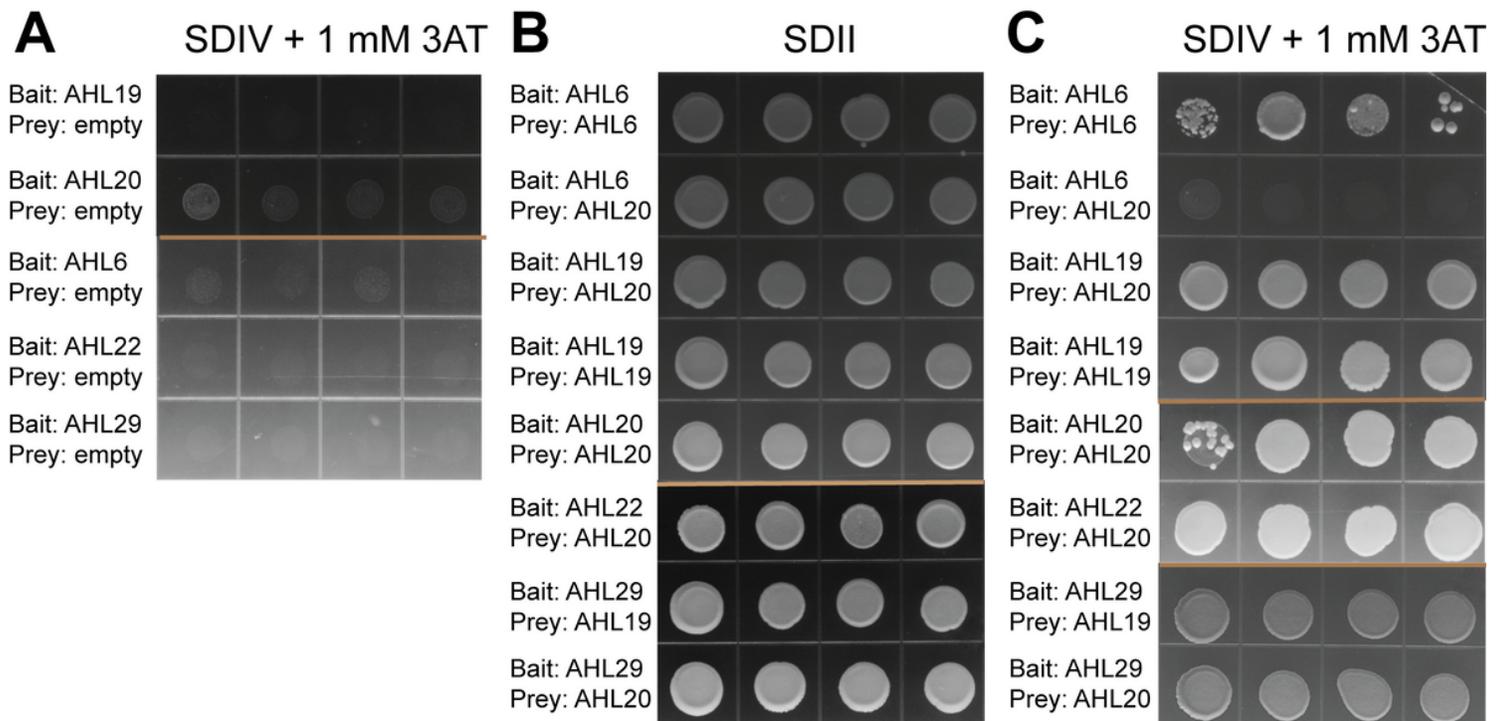
0.001 to 0.01, \* = p-value 0.01 to 0.05, ns = p-value  $\geq$  0.05. ns = not statistically significantly different from the wild type.



**Figure 4**

AtAHL20 overexpression represses FT expression under LD conditions. (A) FT transcript levels were suppressed to ~30% of wild-type levels in 35S:AtAHL20 plants. In contrast, FT transcript abundance was restored to wild-type levels in the dominant-negative mutant 35S:AtAHL20m. (B) RT-qPCR analysis data

showed that AGL8, SPL3 and TSF transcript levels were repressed in 35S:AtAHL20 plants but were unchanged in the wild type and 35S:AtAHL20m plants. RT-qPCR analysis was performed using RNA extracted from rosette leaves of three biological replicates of 21-day old plants grown under LD conditions. The error bar denotes standard error of mean (SEM). Different letters indicate statistical significance (ANOVA;  $P < 0.05$ ).  $n = 3$  biological replicate plants.



**Figure 5**

Targeted yeast-two-hybrid assays. (A) Targeted yeast hybrid assays were performed between AtAHL20, AtAHL6, AtAHL19, AtAHL22 and AtAHL29. 1 mM 3-amino-1,2,4-triazole (3-AT) was used to suppress auto-activation of bait proteins. (B) Yeast cells that were co-transformed with both the bait and prey constructs were plated on synthetic dropout II (SDII) media, which lacked tryptophan and leucine, as positive controls to demonstrate successful transformation. (C) Four individual colonies were picked and plated on synthetic dropout IV (SDIV) media lacking tryptophan, leucine, histidine, and uracil but supplemented with 1 mM 3-AT to suppress autoactivation. Clade-B member AtAHL6 and Clade-A member AtAHL20 physically interacted with themselves but not with each other. AtAHL20 and its closest family member AtAHL19 physically interacted with each other, as well as themselves. AtAHL20 also interacted with other Clade-A AHLs: AtAHL22 and AtAHL29. These results may suggest that AHLs function redundantly to regulate flowering time, possibly as part of homo-, hetero-dimers or multiple AHLs protein complex that includes AtAHL20, AtAHL22, AtAHL27 and AtAHL29. AtAHL6, a Clade-B member, was used as a control to show that not all AHLs interacted with each other. Light brown lines show demarcation between panels of Y2H assays that were performed separately, but have been pasted together to make this figure.

## Supplementary Files

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