Journal of Experimental Botany, Vol. 63, No. 3, pp. 1423–1433, 2012
doi:10.1093/jxb/err376 Advance Access publication 3 December, 2011
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

ATH1 and KNAT2 proteins act together in regulation of plant inflorescence architecture

Yang Li, Limin Pi*, Hai Huang and Lin Xu†

National Laboratory of Plant Molecular Genetics, Shanghai Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, China

* Present address: Institute of Biology III, Faculty of Biology, University of Freiburg, D-79104 Freiburg, Germany.
† To whom correspondence should be addressed. E-mail: xulin01@sibs.ac.cn

Received 20 June 2011; Revised 5 September 2011; Accepted 27 October 2011

Abstract

The inflorescence of flowering plants is a highly organized structure, not only contributing to plant reproductive processes, but also constituting an important part of the entire plant morphology. Previous studies have revealed that the class-I *KNOTTED1*-like homeobox (*KNOX*) genes *BREVIPEDICELLUS* (*BP* or *KNAT1*), *KNAT2*, and *KNAT6* play essential roles in inflorescence architecture. Pedicel morphology is known to contribute greatly to inflorescence architecture, and *BP* negatively regulates *KNAT2* and *KNAT6* to ensure that pedicels have a normal upward-pointing orientation. These findings indicate that a genetic network exists in controlling pedicel orientation, but how this network functions in the developmental process remains elusive. Here it is reported that the *ARABIDOPSIS THALIANA HOMEOBOX GENE1* (*ATH1*) gene, which belongs to the *BELL1*-like homeodomain gene family, is a new member participating in regulating pedicel orientation in the class-I *KNOX* network. In a genetic screening for suppressors of *isoginchaku-2D*, a gain-of-function *ASYMMETRIC LEAVES2* mutant that displays downward-pointing pedicels, a suppressor mutant was obtained. Characterization of this mutant revealed that the mutation corresponds to *ATH1*. Genetic analysis indicated that *ATH1* acts mainly in the *KNAT2* pathway. Yeast two-hybrid and bimolecular fluorescence complementation assays demonstrated that ATH1 physically interacts with KNAT2. The data indicate that the ATH1–KNAT2 complex acts redundantly with KNAT6, both of which are negatively regulated by *BP* during pedicel development.

Key words: *Arabidopsis*, *ATH1*, *BP*, inflorescence architecture, *KNAT2*.

Introduction

In flowering plants, the inflorescence is a highly organized structure bearing flowers connected by pedicels. The pedicel characteristics are one of the key contributors to the display of the whole inflorescence architecture, which is highly diverse among flowering plant species (Douglas *et al.*, 2002; Venglat *et al.*, 2002). In recent years, inflorescence architecture, especially pedicel development, has been studied extensively. In *Arabidopsis*, several members in the class-I *KNOTTED1*-like homeobox (*KNOX*) gene family were reported to play central roles in regulating pedicel development (Douglas *et al.*, 2002; Venglat *et al.*, 2002; Ragni *et al.*, 2008).

The class-I *KNOX* genes in *Arabidopsis* comprise four members, namely *SHOOT MERISTEMLESS* (*STM*), *BREVIPEDICELLUS* (*BP*, also called *KNAT1*), *KNAT2*, and *KNAT6* (Hake *et al.*, 2004). A loss-of-function *bp* mutant exhibits defective inflorescence architecture, with downward-pointing pedicels (Douglas *et al.*, 2002; Venglat *et al.*, 2002). Consistent with these abnormal *bp* phenotypes, *BP* is strongly expressed in pedicels, as well as in other inflorescence parts such as young flowers (Lincoln *et al.*, 1994; Douglas *et al.*, 2002; Alonso-Cantabrana *et al.*, 2007). A genetic study revealed that the pedicel phenotype of *bp* is caused by increased expression of two additional class-I *KNOX* genes, *KNAT2* and *KNAT6*, in pedicels, and double mutations in these two genes in the *bp* background fully rescue the pedicel phenotype caused by the *bp* single mutation (Ragni *et al.*, 2008). *BP* and *KNAT2* are negatively regulated by two leaf development-controlling

genes, *ASYMMETRIC LEAVES1* (*AS1*) and *AS2*. These two genes encode transcription factors that form a protein complex (Xu *et al.*, 2003) to regulate *BP* and *KNAT2* directly in leaves (Guo *et al.*, 2008). Overexpression of *AS2* strongly represses *BP* in the inflorescence, resulting in downward-pointing pedicels, mimicking those in the *bp* mutant (Lin *et al.*, 2003; Xu *et al.*, 2003). Although recent progress has greatly improved our understanding of pedicel development, a detailed network for such regulation remains unresolved. In the current work it is reported that the *ARABIDOPSIS THALIANA HOMEOBOX GENE1* (*ATH1*) gene, which plays an important role in the *KNAT2* pathway, regulates pedicel development.

ATH1 is a member of the *BELL1*-like (*BELL*) transcription factor subfamily, which together with the *KNOX* subfamily belongs to the three-amino-acid-loop-extension (*TALE*) superfamily (Hake *et al.*, 2004). In *Arabidopsis*, the *BELL* subfamily comprises 13 members (Hamant and Pautot, 2010). It was reported that two other members, *PENNYWISE* (*PNY*, also called *BELLRINGER* and *REPLUMLESS*) and *POUND-FOOLISH* (*PNF*), act redundantly in flowering initiation and inflorescence architecture. Both PNY and PNF proteins are able to form heterodimers with class-I KNOX proteins (Byrne *et al.*, 2003; Smith and Hake, 2003; Smith *et al.*, 2004). The *ATH1* gene was first identified as a target of the photomorphogenic genes *CONSTITUTIVE PHOTOMORPHOGENIC1* (*COP1*) and *DEETIOLATED1* (*DET1*), as *ATH1* was derepressed in *cop1* and *det1* mutants (Quaedvlieg *et al.*, 1995). In addition, the *ath1* mutant displayed elongated rosette internodes, similar to those of the photoreceptor mutants *phytochromeA* (*phyA*), *phyB*, and *cryptochrome1* (Devlin *et al.*, 1996; Mazzella *et al.*, 2000). Further studies revealed that *ATH1* functions in multiple developmental processes. For example, *ATH1* is required for development of the basal boundaries of shoot organs (Gomez-Mena and Sablowski, 2008), shoot apical meristem activity (Rutjens *et al.*, 2009), and repression of flowering via activation of *FLOWERING LOCUS C* (*FLC*) (Proveniers *et al.*, 2007). Moreover, ectopic expression of *ATH1* resulted in plants with irregular internodes (Cole *et al.*, 2006; Gomez-Mena and Sablowski, 2008; Rutjens *et al.*, 2009).

In this study, it is reported that *ath1* is a suppressor of the *AS2* overexpression lines, which produce shortened, downward-pointing pedicels. It is demonstrated that *ATH1* acts in the *KNAT2* pathway to regulate pedicel development. Furthermore, it is shown that ATH1 physically interacts with KNAT2, and the ATH1–KNAT2 protein complex is required for normal pedicel morphology.

Materials and methods

Plant materials and growth conditions

Seeds of *isoginchaku-2D* (*iso-2D*), *as2-5D*, *ath1-1*, *bp-9*, *knat6-1*, *knat2-5*, *knat6-1 bp-9*, and *knat2-5 bp-9* were kindly provided by M. Matsui, R. S. Poethig, R. Sablowski, S. Hake, and V. Pautot, respectively (Mele *et al.*, 2003; Nakazawa *et al.*, 2003; Gomez-Mena and Sablowski, 2008; Ragni *et al.*, 2008; Wu *et al.*, 2008). Seeds of *pBP:GUS* and *p35S:BP* were obtained from the Arabidopsis Biological Resource Center (ABRC). Plants were grown on soil as previously described (Chen *et al.*, 2000).

Plant genetics and map-based cloning

To construct double and triple mutants, candidate plants in the F_2 progeny of each cross were genotyped by polymerase chain reaction (PCR). To generate *iso-2D* suppressors, >4000 *iso-2D* seeds (Columbia-0) were mutagenized with ethyl methanesulphonate (0.2%). The mutagenized seeds (M_1) were planted in soil, and plants with impaired *iso-2D* phenotypes were identified in the M_2 generation. Mapping of the suppressor locus was performed by analysis of an F_2 population from a cross between one of the suppressor mutants and the polymorphic Landsberg *erecta* (L*er*) plants. The suppressor locus was mapped to the proximal arm of chromosome 4, between two simple sequence length polymorphim (SSLP) markers nga1139 and F1N20 in a 4700 kb region. Because the *ATH1* locus is located in this region and the *ath1* mutants show similar phenotypes to certain plants in the F_2 mapping population, the *ATH1* locus was thus sequenced and it was confirmed that *ath1* is the suppressor of the *iso-2D* phenotypes (see Results).

Quantitative reverse transcription-PCR (qRT-PCR)

RNA extraction was performed as described previously (Xu *et al.*, 2003) using inflorescences from plants ~5 weeks old, and reverse transcription was performed using a kit (Fermentas, Lithuania). Quantitative PCR was performed in the presence of the double-stranded DNA-specific dye SYBR green following the manufacturer's instructions (TOYOBO, Japan), with the following gene-specific primers: 5′-CCTCCAAACCGTTTCCTTCT-3′ and 5′-TTTATGCATTGCTTGGCTCATCA-3′ for *ATH1*; 5′-CTTTGAGGCTCGACAACA-3′ and 5′-TAATGCAACTCCCACCAC-3′ for *BP*; 5′-GAACTCGCTACCGCTTTGTCCTC-3′ and 5′-ATCGCGGTCATTGCTTCTTTGT-3′ for *KNAT2*; 5′-CGAGTCAGACAAGAACTC-3′ and 5′-GGATCTCTACATGCAAGC-3′ for *KNAT4*; 5′-CTCCGCCGGTGAAAATCGTGT-3′ and 5′-GGTTCCGTAGCTGCATCTCAATCT-3′ for *KNAT6*; 5′-ATGAAAGAGAGACAACGTTGG-3′ and 5′-GGGGCGGTCTAATCTGCAA-3′ for *AS1*; 5′-ATGGCATCTTCTTCAACAAAC-3′ and 5′-AGACGGATCAACAGTACGGC-3′ for *AS2*; and 5′-TGGCATCA(T/C)ACTTTCTACAA-3′ and 5′-CCACCACT(G/A/T)AGCACAATGTT-3′ for *ACTIN*.

Yeast two-hybrid analysis

Full-length cDNA fragments of *KNAT2* and *KNAT6* and the N-terminal portion of *ATH1* were PCR-amplified using the following primers: 5′-agatctATGGATAGAATGTGTGGTTTCC-3′ and 5′-gtcgacCTCGGTAAAGAATGTTTCATT-3′ for *KNAT2*; 5′-agatctATGGATGGAATGTACAATTTCC-3′ and 5′-gtcgacTTCCTCGGTAAAGAATGATCCA-3′ for *KNAT6*; and 5′-gagctcggatccATGGACAACAACAACAACAAC-3′ and 5′-gtcgacTTAAGGTCTCCAAATCTGATGGTTC 3′ for the *ATH1* N-terminal portion. In each of the above primer sequences, the lower case letters represent additional nucleotides to introduce restriction sites. All constructs were verified by sequencing. Construct combinations pGADT7-KNAT2/pGBKT7-ATH1-N and pGADT7-KNAT6/pGBKT7-ATH1-N were co-transformed into the yeast strain PJ69-4A, and the interaction between the proteins was determined according to the manufacturer's recommendation (Clontech, USA).

Transient protein expression and bimolecular fluorescence complementation (BiFC) assay

Full-length cDNA fragments of *ATH1*, *KNAT2*, and *KNAT6* were subcloned into the plant transformation vector pC131 under the

control of a 35S promoter and with a 3′ in-frame fusion to sequences encoding yellow fluorescent protein (YFP), using the following primers: 5′-gagctcggatccATGGACAACAACAACAACAAC-3′ and 5′-tctagagtcgacTTTATGCATTGCTTGGCTCATC-3′ for *ATH1*; 5′-agatctATGGATAGAATGTGTGGTTTCC-3′ and 5′-gtcgacCTCG GTAAAGAATGTTTCATT-3′ for *KNAT2*; and 5′-agatctATGGAT GGAATGTACAATTTCC-3′ and 5′-gtcgacTTCCTCGGTAAA-GAATGATCCA-3′ for *KNAT6*. Fragments of YFP were truncated at residue 155 (designated YN and YC) as previously described (Kerppola, 2006), using the following primers: 5′-gtcgacGGAG-GAGGCTCA*GCGGACTACAAAGATGACGATGACAAA*ATGGT-GAGCAAGGGCGAGGA-3′ and 5′-gagctcTTAGGCCATGATAT AGACGTTGT-3′ for *YN*; and 5′-gtcgacGGAGGAGGCTCA*GCG-GACTACAAAGATGACGATGACAAA*GACAAGCAGAAGAAC GGCAT-3′ and 5′-gagctcTTACTTGTACAGCTCGTCCATGC-3′ for *YC*. In each of the above primer sequences, the lower case letters represent additional nucleotides to introduce restriction sites; and the italic letters represent a FLAG tag. Sequences between restriction sites and the FLAG tag encode the GGGS linker peptide to facilitate the association between target proteins and YN/YC as previously described (Kerppola, 2006). All constructs were verified by sequencing. To construct BiFC plasmids, the YN fragment was inserted into the C-terminus of KNAT2 and KNAT6, and the YC into the C terminus of ATH1 of the above constructs to replace the YFP fragment. Leaves of 4- to 8-week-old *Nicotiana benthamiana* plants were co-infiltrated with strains containing P19, a viral silencing suppressor gene (Voinnet *et al.*, 2003), and localization of the BiFC fluorescence was observed 2–7 d after infiltration using a confocal laser scanning microscope (LSM 510 META, ZEISS, Germany).

Results

Genetic screening for suppressors of the AS2 *overexpression line* iso-2D

Previous data showed that overexpression of the *Arabidopsis AS2* gene results in two types of abnormal phenotypes: (i) all leaves become adaxialized and are curled upwards; and (ii) inflorescences produce shortened and downward-pointing pedicels, which are similar to those of the *bp* mutants (Douglas *et al.*, 2002; Venglat *et al.*, 2002; Lin *et al.*, 2003; Xu *et al.*, 2003). To identify the regulatory network relating to the abnormal pedicel phenotypes when *AS2* is overexpressed, a genetic screening for suppressors of the *AS2* overexpression phenotypes was conducted, using a stable *AS2* overexpression line, *iso-2D* (Nakazawa *et al.*, 2003). One mutant showing compromise of the downward-pointing pedicel was identified, and was further crossed with the wild-type Col-0 to obtain the suppressor single mutant. The isolated single mutant showed similar phenotypes to those of a previously reported mutant *arabidopsis thaliana homeobox gene1* (*ath1*) (Quaedvlieg *et al.*, 1995; Gomez-Mena and Sablowski, 2008). In addition, the mutation locus was mapped to a region between genetic markers F1N20 and nga1139 on chromosome 4, where the *ATH1* locus is positioned (Fig. 1A). The *ATH1* locus in the suppressor mutant was thus sequenced and a single nucleotide substitution from G to A was found in the second exon, resulting in an earlier stop codon in the *ATH1* gene (Fig. 1B). In addition, an allelism test was performed by crossing the suppressor mutant to the previously characterized *ath1-1* mutant (Proveniers *et al.*, 2007; Gomez-Mena and Sablowski, 2008), and all F_1 plants showed the *ath1* phenotypes (Supplementary Fig. S1 available at *JXB* online). Hence, it is concluded that the suppressor is a new *ath1* allele, which was renamed *ath1-4*.

ATH1 *plays an important role in pedicel development*

Wild-type *Arabidopsis* plants form inflorescences that bear flowers and fruits with upward-pointing pedicels (Fig. 1C), whereas pedicels from the *iso-2D* inflorescence are drastically shortened with a downward-pointing orientation (Fig. 1D). Compared with the wild-type and *iso-2D* inflorescences, the pedicel orientation of the *ath1-4 iso-2D* inflorescence was partially rescued, showing a horizontal or only slightly downward-pointing orientation (Fig. 1E). In addition, the shortened pedicels in *iso-2D* were also largely rescued in the *ath1-4 iso-2D* double mutant (Fig. 1E). To confirm further that removal of the *ATH1* gene can rescue the pedicel phenotypes of *AS2* overexpression, *ath1-4* was crossed to another *AS2* overexpression allele, *as2-5D* (Fig. 1F) (Wu *et al.*, 2008), and a previously generated *p35S:AS2* transgenic line, which has the typical *bp*-like pedicel phenotypes (Xu *et al.*, 2003). Both *ath1-4 as2-5D* (Fig. 1G) and *p35S:AS2/ath1-4* (data not shown) plants displayed the rescued pedicels, similar to *ath1-4 iso-2D*. These results indicate that *ATH1* plays a role in formation of normal pedicel morphology. Although the abnormal pedicel phenotype was rescued in *ath1-4 iso-2D* and *ath1-4 as2-5D*, the up-curled rosette leaves caused by the *iso-2D* and *as2-5D* mutations remained hyponastic (Fig. 1H–L). These results indicate that the *ATH1* function is required only for the pathway controlling pedicel morphology, but not for the pathway regulating leaf polarity establishment.

Rescue of pedicel phenotypes in ath1 iso-2D *is not because of recovery of* BP *expression*

Because *AS2* overexpression is known to repress *BP* in the inflorescence (Lin *et al.*, 2003; Xu *et al.*, 2003) and the *bp* mutant itself bears downward-pointing pedicels, it was hypothesized that the *BP* expression level might be recovered in the *ath1-4 iso-2D* inflorescence. Expression of *BP* and several other related genes was thus analysed in *iso-2D* single and *ath1-4 iso-2D* double mutants by qRT-PCR. Surprisingly, although the downward-pointing pedicel phenotypes of *iso-2D* were suppressed in the *ath1-4 iso-2D* plants, the *BP* expression remained at a very low level in *ath1-4 iso-2D*, which was markedly below the wild-type level (Fig. 2). On the other hand, levels of *KNAT2* and *KNAT6* transcripts were also reduced in both *iso-2D* and *ath1-4 iso-2D* compared with those in the wild type. As controls, while the *AS2* transcript level was elevated in both *iso-2D* and *ath1-4 iso-2D*, the *AS1*, *ATH1*, and *KNAT4* levels in *iso-2D* and *ath1-4 iso-2D* showed no significant changes compared with those in the wild type (Fig. 2). To confirm further that *ath1-4 iso-2D* does not affect *BP* expression, a *pBP:GUS* transgenic line was crossed to *iso-2D* and *ath1-4 iso-2D*, respectively, to generate the isogenic *pBP:GUS/iso-2D* and

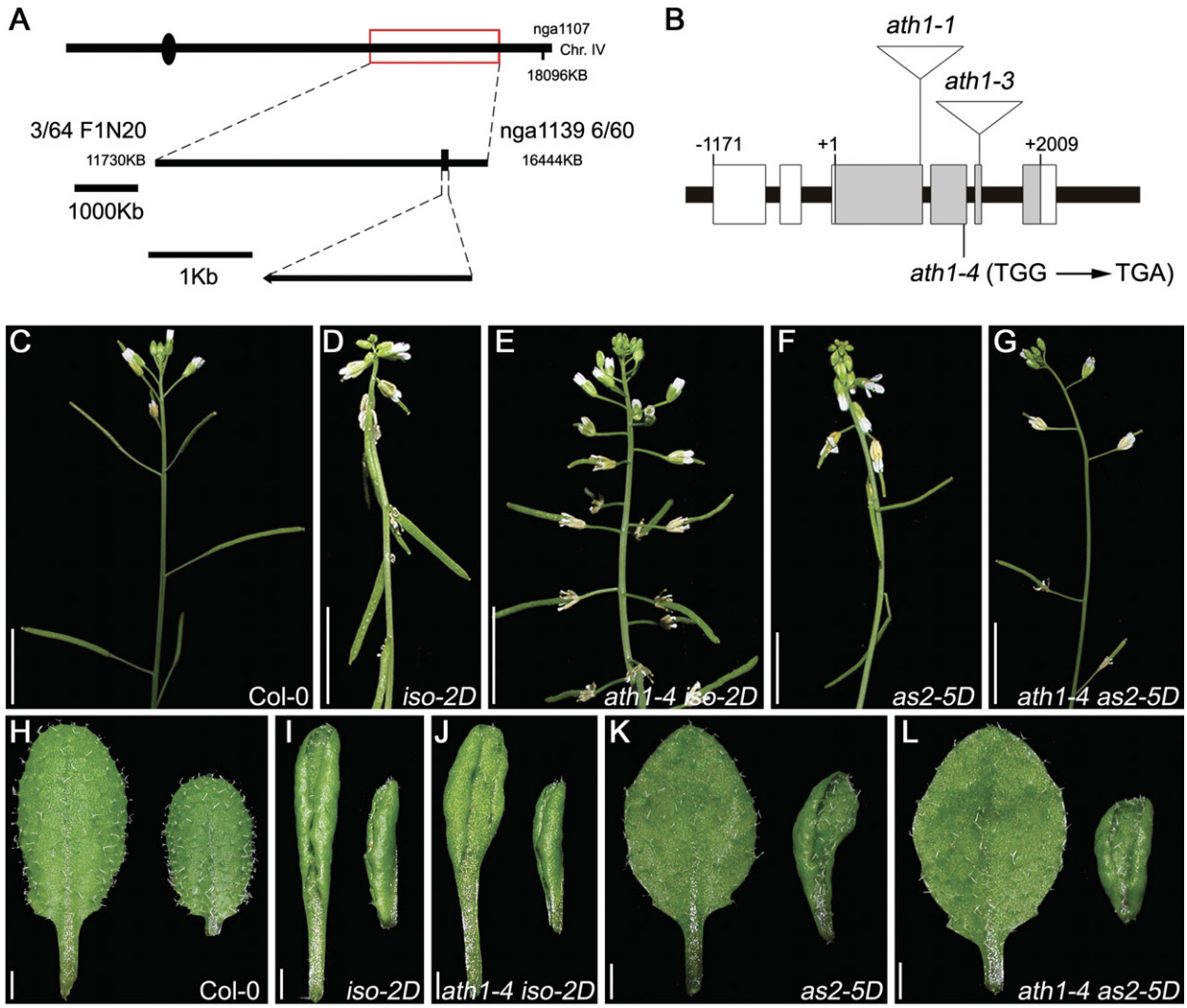

Fig. 1. Abnormal pedicel phenotypes caused by *AS2* overexpression were impaired in the *ath1* mutant background. (A) Map-based cloning to localize an *AS2* suppressor gene on chromosome 4, between markers F1N20 and nga1139. (B) Structure of the *ATH1* gene. Grey and white boxes indicate protein-coding regions and untranslated regions (UTRs), respectively. (C–G) Inflorescence structures of wild type (C), *iso-2D* (D), *ath1-4 iso-2D* (E), *as2-5D* (F), and *ath1-4 as2-5D* (G). (H–L) The fifth (left) and sixth (right) rosette leaves of 19-day-old wild-type (H), *iso-2D* (I), *ath1-4 iso-2D* (J), *as2-5D* (K), and *ath1-4 as2-5D* (L) plants. Note that only phenotypes of the downward-pointing pedicels but not the up-curled rosette leaves in *iso-2D* and *as2-5D* were rescued in *ath1-4 iso-2D* and *ath1-4 as 2-5D*. Bars=1 cm in C–G, and 0.1 cm in H–L.

pBP:GUS/ath1-4 iso-2D sibling plants for further analyses. In wild-type plants, β-glucuronidase (GUS) staining accumulated in the pedicel, especially at the junction between the pedicel and flower (Supplementary Fig. S2A, D at *JXB* online). In contrast, GUS signals were barely detected in inflorescences and flowers of *pBP:GUS/iso-2D* and *pBP:GUS/ath1-4 iso-2D* plants (Supplementary Fig. S2B, C, E, F), consistent with the qRT-PCR results.

It was previously known that *BP* acts to repress *KNAT2* and *KNAT6* to ensure normal inflorescence architecture (Ragni *et al.*, 2008). Therefore, either loss of *BP* function or loss of repression for *KNAT2* and/or *KNAT6* could result in the downward-pointing pedicel phenotype. Whether overexpression of *BP* could rescue the downward-pointing pedicel in *AS2* overexpression lines was investigated by crossing a *p35S:BP* transgenic plant to *iso-2D* and *as2-5D* mutants, respectively, and the F_1 isogenic populations were analysed. Compared with those of *iso-2D*/+ and *as2-5D*/+ (Fig. 3A, C), the downward-pointing pedicels of both *p35S:BP*/+ *iso-2D*/+ (Fig. 3B) and *p35S:BP*/+ *as2-5D*/+ (Fig. 3D) were ameliorated. These results indicate that the pedicel phenotypes of *iso-2D* and *as2-5D* are, indeed, the result of lack of *BP*, and the insufficient down-regulation of *KNAT2* and *KNAT6* might be the major reason for the abnormal inflorescence architecture in the mutants.

Removal of both ATH1 *and* KNAT6 *rescues the* bp *inflorescence phenotype*

To investigate further genetic interaction between *BP* and *ATH1* in morphological control, *ath1-4* and *ath1-1* were introduced into the *bp-9* mutant, which produces

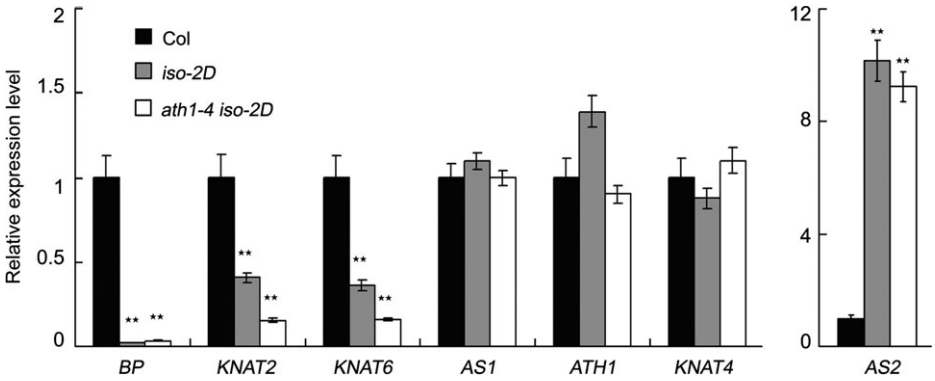

Fig. 2. qRT-PCR analysis of transcript levels of *BP*, *KNAT2*, *KNAT6*, *AS1*, *ATH1*, *KNAT4*, and *AS2* in wild-type Col-0, *iso-2D*, and *ath1-4 iso-2D* inflorescences. Quantification was normalized to that of *ACTIN* and then to the value of wild-type Col-0, whose value was arbitrarily fixed at 1.0. Each cDNA sample was made in triplicate, and the consistent results from two separately prepared RNA samples were used. Bars show the standard deviation, and double asterisks show significant statistical differences by *t*-test ($P < 0.01$).

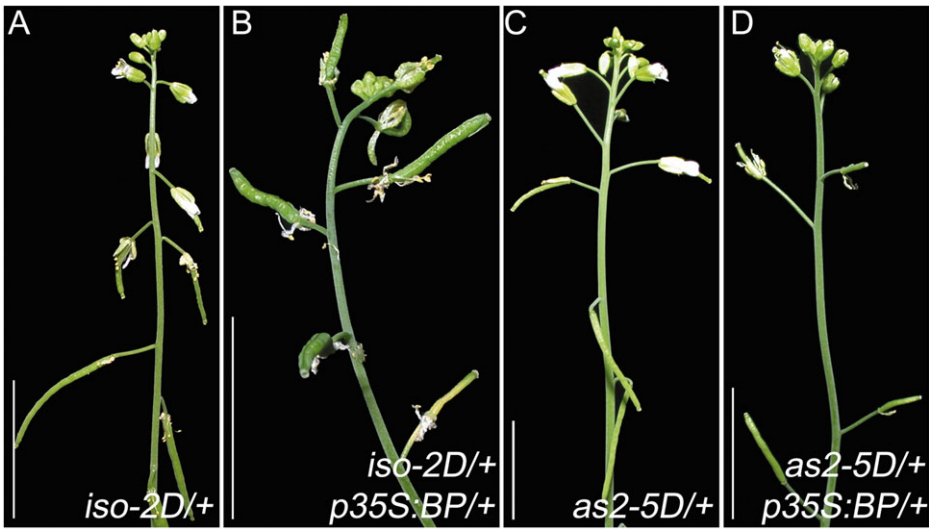

Fig. 3. The overexpression of *BP* rescues the pedicel phenotypes caused by gain-of-function mutations of the *AS2* gene. (A–D) Inflorescence structures of *iso-2D/+* (A), *iso-2D/+ p35S:BP/+* (B), *as2-5D/+* (C), and *as2-5D/+ p35S:BP/+* (D). Note that the abnormal pedicel phenotypes caused by overexpression of *AS2* were rescued in *iso-2D/+ p35S:BP/+* and *as2-5D/+ p35S:BP/+* plants. Note that *iso-2D/+*, *p35S:BP/+*, and *as2-5D/+* indicates heterozygous for *iso-2D*, *p35S:BP*, and *as2-5D*, respectively. Bars=1 cm.

downward-pointing pedicels. The abnormal *bp-9* inflorescence (Fig. 4A, J) was almost unaffected in *ath1-4 bp-9* and *ath1-1 bp-9* double mutants (Fig. 4B, C, J). It was previously reported that *knat6*, but not *knat2*, can partially rescue the *bp* downward-pointing pedicel phenotype, and the *knat2 knat6 bp* triple mutant produces completely normal upward-pointing pedicels (Ragni *et al.*, 2008) (Fig. 4D, E, J). The present genetic data showed that the pedicel phenotype of both *ath1-4 knat2-5 bp-9* and *ath1-1 knat2-5 bp-9* triple mutants (Fig. 4F, G, J) was similar to those in the *knat2-5 bp-9* double mutant (Fig. 4D, J), in which most pedicels were downward pointing. Remarkably, both *ath1-4 knat6-1 bp-9* and *ath1-1 knat6-1 bp-9* (Fig. 4H, I, J) substantially rescued the *bp-9* pedicel phenotype, with only a small proportion of pedicels showing the horizontal or downward-pointing phenotype. These results strongly suggest that *ATH1* and *KNAT2* might function in the same pathway, which is separate from the *KNAT6* pathway, to regulate pedicel phenotype.

ATH1 physically interacts with KNAT2

Several recent studies revealed that, through their N-terminal domain, a number of BELL family proteins are able to form complexes with KNOX family proteins (Bellaoui *et al.*, 2001; Muller *et al.*, 2001; Smith *et al.*, 2002). To determine whether ATH1 physically interacts with KNAT2, a yeast two-hybrid assay was performed. The data showed that co-expression of the N-terminal domain of ATH1 and full-length KNAT2 promoted expression of the reporter genes, resulting in cells able to grow on media lacking tryptophan, leucine, adenine, and histidine (Fig. 5A, B). However, in the present experimental conditions, the protein–protein interaction by co-expression of the N-terminal domain of ATH1 and full-length KNAT6

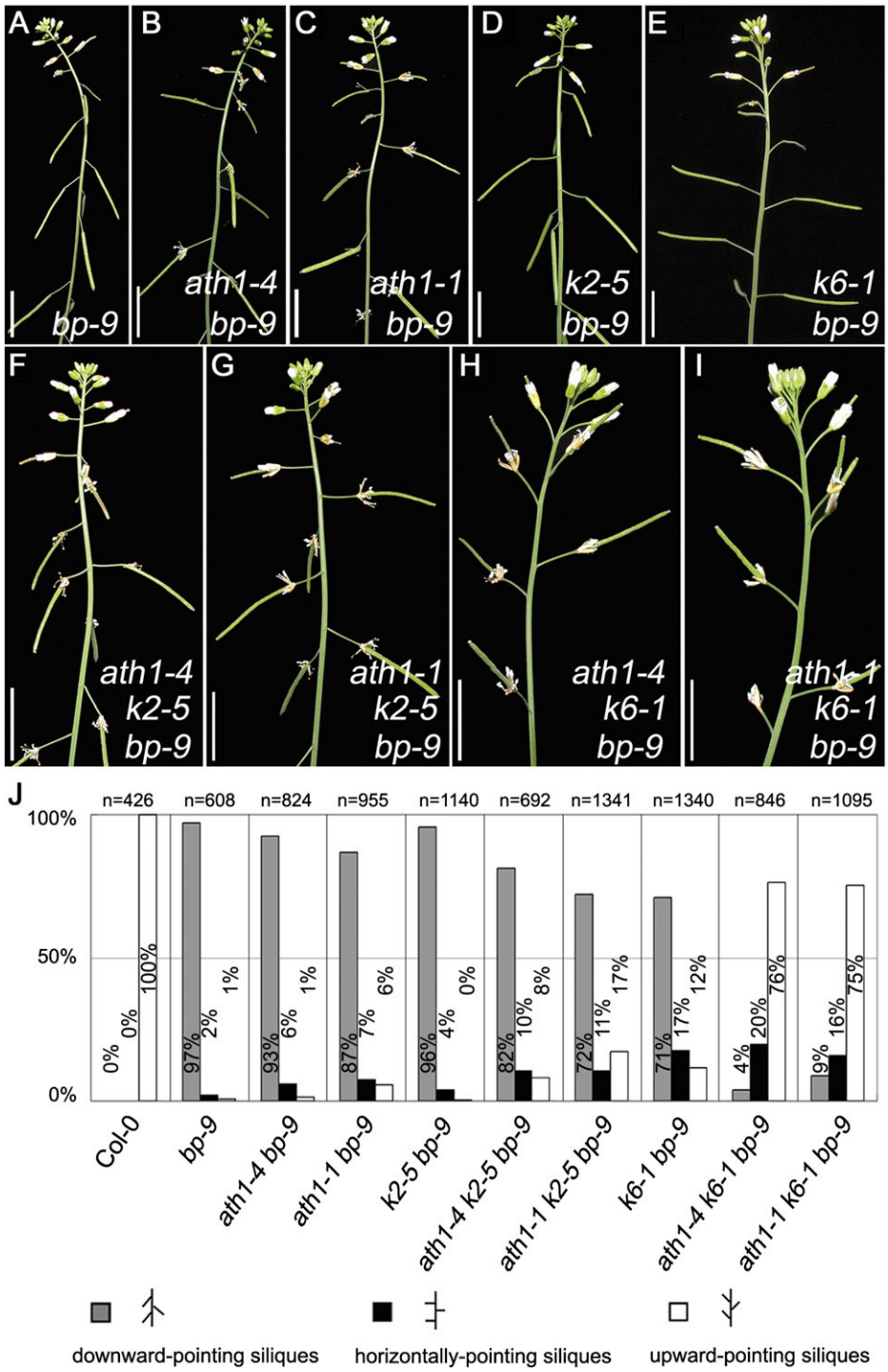

Fig. 4. *bp* pedicel phenotypes were rescued in the *ath1* and *knat6* double mutation backgrounds. (A–I) Inflorescence structures of *bp-9* (A), *ath1-4 bp-9* (B), *ath1-1 bp-9* (C), *knat2-5 bp-9* (D), *knat6-1 bp-9* (E), *ath1-4 knat2-5 bp-9* (F), *ath1-1 knat2-5 bp-9* (G), *ath1-4 knat6-1 bp-9* (H), and *ath1-1 knat6-1 bp-9* (I). (J) Statistical analysis of pedicel orientation. 'n' indicates the number of pedicels scored. *k2-5*, *knat2-5*; *k6-1*, *knat6-1*. Bars=1 cm in A–I.

appeared very weak, as compared with the RecT-Lam and RecT-53 negative and positive controls, respectively (Fig. 5A, B).

A BiFC assay was performed to investigate further the interaction between ATH1 and KNAT2 or KNAT6 *in planta*. The KNAT2–YN and ATH1–YC pair or the KNAT6–YN and ATH1–YC pair was co-expressed in *Nicotiana benthamiana* leaves, with YN and ATH1–YC, KNAT2–YN and YC, and KNAT6–YN and YC pairs serving as negative controls (Fig. 6). The data showed that the presence of KNAT2–YN and ATH1–YC, or KNAT6–YN and ATH1–YC, in tobacco cells produced YFP signals in both the cytoplasm and nuclei (Fig. 6), whereas all the negative controls displayed no fluorescence signal. These

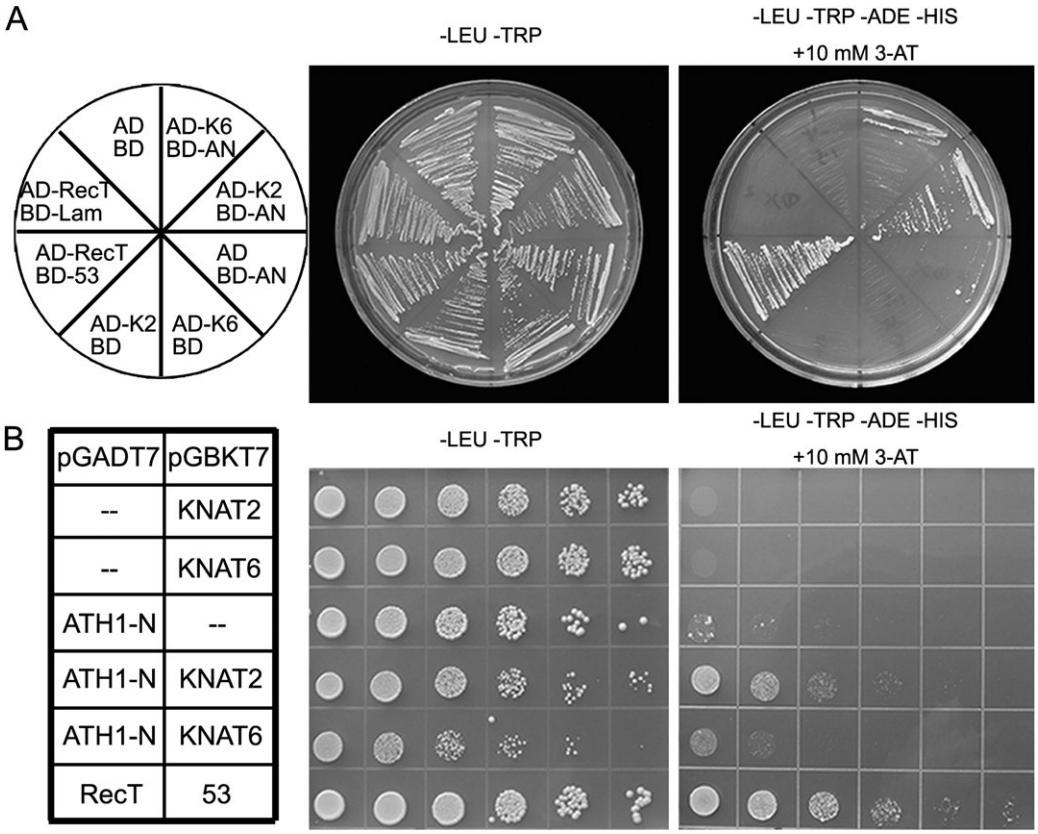

Fig. 5. ATH1 physically interacts with KNAT2 and KNAT6 in yeast cells. (A, B) Yeast two-hybrid assay shows that the N-terminal domain of ATH1 is able to interact with KNAT2, while the interaction between the N-terminal domain of ATH1 and KNAT6 appeared very weak. The RecT-Lam and RecT-53 pair served as the negative and positive controls, respectively. Yeast cultures with a 1:10 dilution series were plated on the media from left to right (B). K2, KNAT2; K6, KNAT6; and AN, N-terminal domain of ATH1.

results indicate that ATH1 potentially has the ability to bind KNAT2 and KNAT6 in plant cells.

Discussion

Formation of inflorescence architecture appears to be a complex developmental process, requiring a number of regulatory components, including those in the class-I *KNOX* genes. In this work, *ATH1* functions in modulating pedicel morphology are reported, adding a new factor to the present regulatory network of inflorescence architecture. In addition, the data reveal the protein–protein interaction between ATH1 and KNAT2, and that the protein complex may act to fulfil the task of regulating pedicel development.

Based on previous data and the results obtained in this study, genetic action models for genes that regulate pedicel phenotypes are proposed (Fig. 7). According to the previous model, for a normal pedicel shape the *BP* gene must down-regulate two redundant genes, *KNAT2* and *KNAT6* (Fig. 7A) (Ragni *et al.*, 2008). In contrast, the *bp* mutation causes derepression of both *KNAT2* and *KNAT6*, and the increased expression of these two genes resulted in the downward-pointing pedicels (Ragni *et al.*, 2008) (Fig. 7B). In the *iso-2D* and *as2-5D* mutants, the increased AS1–AS2 function represses *BP*, *KNAT2*, and *KNAT6*. However, because down-regulation of *BP* in turn derepresses *KNAT2* and *KNAT6*, the transcript levels of these two genes are only reduced moderately (Fig. 7C). The data from gene expression analyses also reveal that the downward-pointing pedicel phenotype relies not only on the increased *KNAT2* and *KNAT6* transcripts, but, more importantly, the functional balance between *BP* and *KNAT2/6*. Both *KNAT2* and *KNAT6* transcript levels were actually reduced in *iso-2D*; however, because the *BP* level in *iso-2D* was even more severely reduced, the downward-pointing pedicel phenotype of the *iso-2D* mutant is evident.

In the *ath1 knat2 bp* triple mutants, removal of *KNAT2* is equivalent to removal of *ATH1*, and the *KNAT6* transcripts must increase due to *bp* mutation. Therefore, the pedicel orientation is either downward pointing or only slightly recovered (Fig. 7D). In *ath1 iso-2D* and *ath1 as2-5D* double mutants, *BP* is repressed and the *KNAT2* pathway is blocked completely because of the *ath1* mutation (Fig. 7E). In this case, the reduced *KNAT6* function only weakly affects pedicel orientation, and the pedicels display a horizontal or normal orientation. Finally, the *ath1 knat6 bp* triple mutation (Fig. 7F) is equivalent to the *knat2 knat6 bp* triple mutation, in which pedicel defects caused by the *bp* mutation could be largely or completely rescued. It would be interesting to validate the models by changing *KNAT2* and *KNAT6* expression levels in the *bp*, *iso-2D*, or *as2-5D* backgrounds in the future.

Heterodimers between several BELL and class-I KNOX proteins have demonstrated important roles in regulating

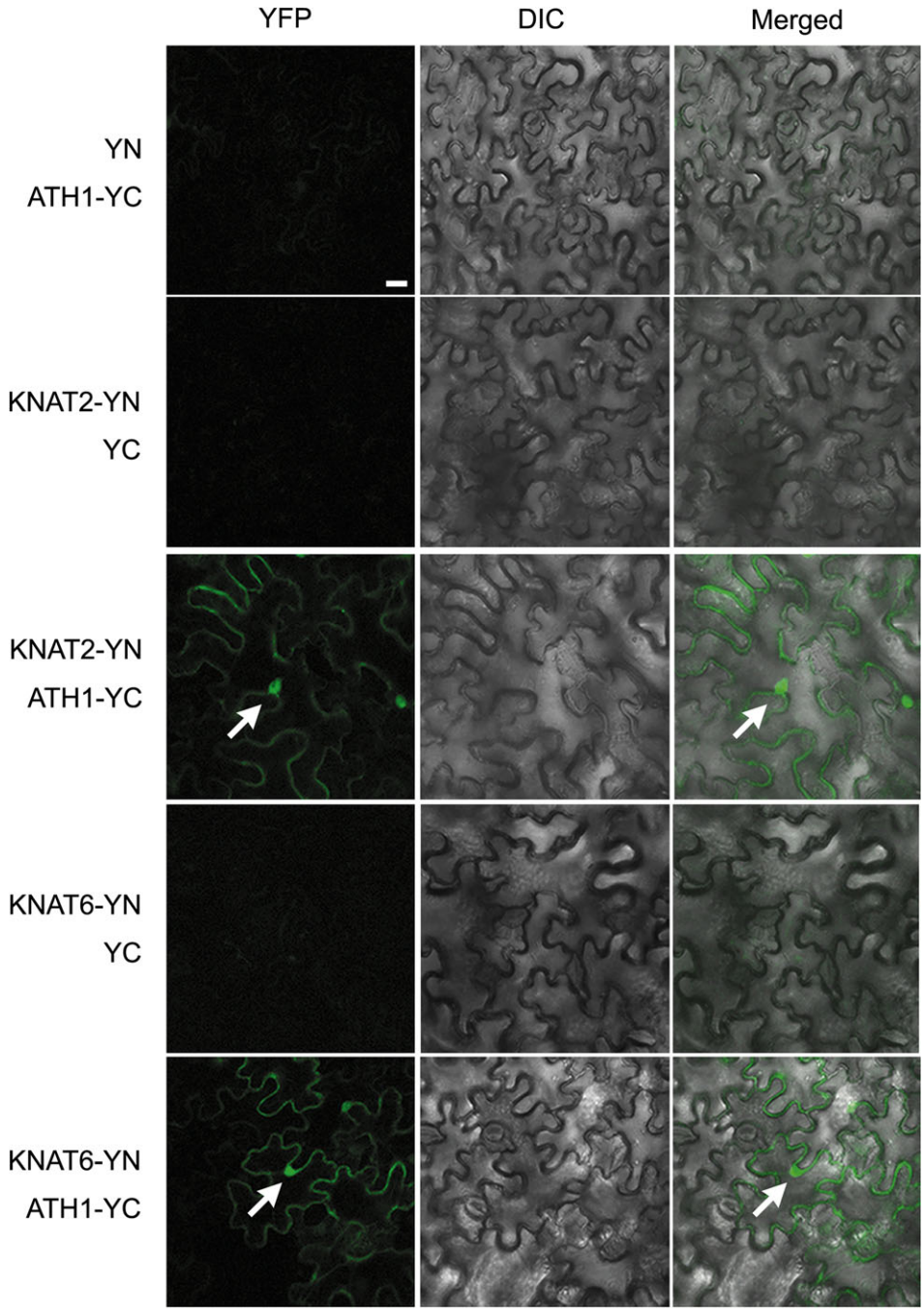

Fig. 6. Bimolecular fluorescence complementation (BiFC) assay shows that ATH1 is able to associate with both KNAT2 and KNAT6. White arrows indicate nuclei. All images are of the same magnification. DIC, differential interference contrast; YN, N-terminal domain of YFP; YC, C-terminal domain of YFP. Bars=20 μm.

plant inflorescence architecture or other developmental processes. The identified heterodimers include PNY–BP, PNF–BP, PNY–STM, and PNF–STM (Byrne *et al.*, 2003; Smith and Hake, 2003; Bhatt *et al.*, 2004; Kanrar *et al.*, 2006). In this study, the results of genetic analyses and yeast two-hybrid and BiFC assays also support the assumption that ATH1 and KNAT2 form a heterodimer to regulate pedicel development. It was found that ATH1 and KNAT6 can also form a protein complex, although the protein–protein interaction in the yeast two-hybrid assay was fairly weak. It is proposed that, because of their similar protein structure, heterodimer formation between the BELL and the KNOX family proteins is common in artificial experimental conditions. However, endogenous protein dimerization may follow different rules. To determine the endogenous protein complex for the two families, genetic evidence is important. Based on the genetic analysis, pedicel phenotypes of *ath1 knat6 bp* are almost normal as compared with that of the *bp* mutant, whereas the abnormal pedicels in *ath1 knat2 bp* were only very weakly rescued. These results indicate that, although the BiFC assay showed ATH1 and KNAT6 interaction, this heterodimer may not

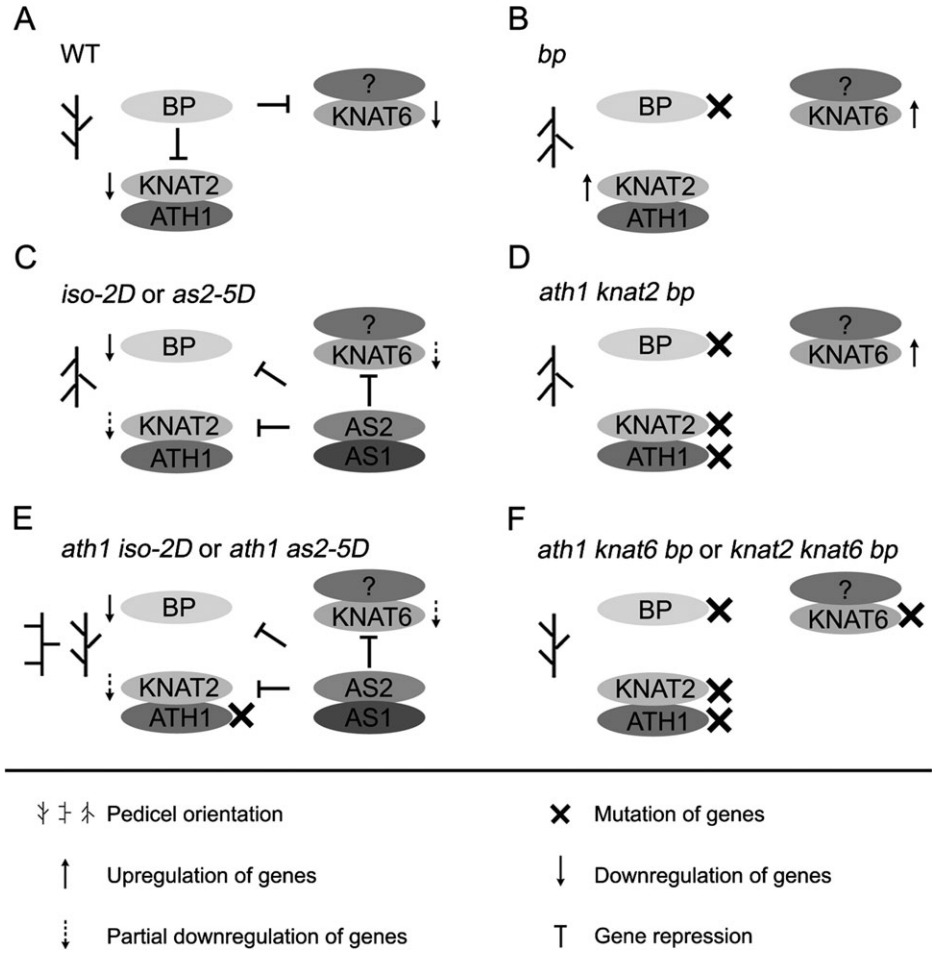

Fig. 7. Action models for *ATH1*, *BP*, *KNAT2*, *KNAT6*, and *AS1-AS2* in regulation of pedicel orientation.

exist *in planta* or is not the major player in regulation of pedicel morphology.

Interaction between KNOX and BELL proteins in plants was proposed to guide correct protein subcellular localization. For example, the nuclear localization of STM relies on its interacting with the BELL proteins ATH1, PNY, and BEL1-like homeodomain 3 (BLH3) (Cole *et al.*, 2006; Rutjens *et al.*, 2009). Likewise, previous studies showed that cellular localization of PNY also relies on protein interaction with its KNOX homeodomain partner (Bhatt *et al.*, 2004). It is possible that the established ATH1–KNAT2 dimer may bring the protein complex to its correct subcellular position. The heterodimer may also help to recognize the promoter sequence of specific downstream genes during inflorescence development. A recent study showed that molecular regulation of the haploid–diploid transition in the unicellular green soil alga *Chlamydomonas reinhardti* requires functioning of the Gsp1–Gsm1 protein heterodimer. Gsp1 and Gsm1 correspond to the *Arabidopsis* BELL and KNOX proteins, respectively. These two proteins are contributed by gametes of plus and minus mating types, respectively, physically interact, and translocate from the cytosol to the nucleus upon gametic fusion (Lee *et al.*, 2008). Differing from the monomer Gsp1 and Gsm1 in the gametes, this heterodimer in a diploid background initiates gamete development, probably through recognizing and regulating distinct targets. Although *ATH1* appears to influence multiple aspects of plant development, its roles may be defined by the presence and function of specific interacting partners (e.g. *ATH1*/*KNAT2* in pedicel development).

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. The *iso-2D* suppressor corresponds to the *ATH1* gene.

Figure S2. *pBP*:*GUS* staining in wild-type Col-0 (A, D), *iso-2D* (B, E), and *ath1-4 iso-2D* (C, F).

Acknowledgements

We thank M. Matsui, R. S. Poethig, R. Sablowski, S. Hake, V. Pautot, and the ABRC for providing *Arabidopsis* seeds, J. Huang for marker primers of genetic mapping, Y. Fang for YFP vector, J. Zhang for helpful discussion about the BiFC experiment, and X. Gao for confocal observations. This work was supported by grants from the Chief Scientist

Program of Shanghai Institutes for Biological Sciences, the Chinese National Scientific Foundation (31071064), and Shanghai Institutes for Biological Sciences (2009KIP205).

References

Alonso-Cantabrana H, Ripoll JJ, Ochando I, Vera A, Ferrandiz C, Martinez-Laborda A. 2007. Common regulatory networks in leaf and fruit patterning revealed by mutations in the *Arabidopsis ASYMMETRIC LEAVES1* gene. *Development* **134,** 2663–2671.

Bellaoui M, Pidkowich MS, Samach A, Kushalappa K, Kohalmi SE, Modrusan Z, Crosby WL, Haughn GW. 2001. The Arabidopsis BELL1 and KNOX TALE homeodomain proteins interact through a domain conserved between plants and animals. *The Plant Cell* **13,** 2455–2470.

Bhatt AM, Etchells JP, Canales C, Lagodienko A, Dickinson H. 2004. VAAMANA—a BEL1-like homeodomain protein, interacts with KNOX proteins BP and STM and regulates inflorescence stem growth in *Arabidopsis*. *Gene* **328,** 103–111.

Byrne ME, Groover AT, Fontana JR, Martienssen RA. 2003. Phyllotactic pattern and stem cell fate are determined by the *Arabidopsis* homeobox gene *BELLRINGER*. *Development* **130,** 3941–3950.

Chen C, Wang S, Huang H. 2000. *LEUNIG* has multiple functions in gynoecium development in *Arabidopsis*. *Genesis* **26,** 42–54.

Cole M, Nolte C, Werr W. 2006. Nuclear import of the transcription factor SHOOT MERISTEMLESS depends on heterodimerization with BLH proteins expressed in discrete sub-domains of the shoot apical meristem of *Arabidopsis thaliana*. *Nucleic Acids Research* **34,** 1281–1292.

Devlin PF, Halliday KJ, Harberd NP, Whitelam GC. 1996. The rosette habit of *Arabidopsis thaliana* is dependent upon phytochrome action: novel phytochromes control internode elongation and flowering time. *The Plant Journal* **10,** 1127–1134.

Douglas SJ, Chuck G, Dengler RE, Pelecanda L, Riggs CD. 2002. *KNAT1* and *ERECTA* regulate inflorescence architecture in Arabidopsis. *The Plant Cell* **14,** 547–558.

Gomez-Mena C, Sablowski R. 2008. *ARABIDOPSIS THALIANA HOMEOBOX GENE1* establishes the basal boundaries of shoot organs and controls stem growth. *The Plant Cell* **20,** 2059–2072.

Guo M, Thomas J, Collins G, Timmermans MC. 2008. Direct repression of KNOX loci by the ASYMMETRIC LEAVES1 complex of Arabidopsis. *The Plant Cell* **20,** 48–58.

Hake S, Smith HM, Holtan H, Magnani E, Mele G, Ramirez J. 2004. The role of *KNOX* genes in plant development. *Annual Review of Cell and Developmental Biology* **20,** 125–151.

Hamant O, Pautot V. 2010. Plant development: a TALE story. *Comptes Rendus Biologie* **333,** 371–381.

Kanrar S, Onguka O, Smith HM. 2006. *Arabidopsis* inflorescence architecture requires the activities of KNOX–BELL homeodomain heterodimers. *Planta* **224,** 1163–1173.

Kerppola TK. 2006. Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells. *Nature Protocols* **1,** 1278–1286.

Lee JH, Lin H, Joo S, Goodenough U. 2008. Early sexual origins of homeoprotein heterodimerization and evolution of the plant KNOX/BELL family. *Cell* **133,** 829–840.

Lin WC, Shuai B, Springer PS. 2003. The Arabidopsis *LATERAL ORGAN BOUNDARIES*-domain gene *ASYMMETRIC LEAVES2* functions in the repression of *KNOX* gene expression and in adaxial–abaxial patterning. *The Plant Cell* **15,** 2241–2252.

Lincoln C, Long J, Yamaguchi J, Serikawa K, Hake S. 1994. A *knotted1*-like homeobox gene in Arabidopsis is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. *The Plant Cell* **6,** 1859–1876.

Mazzella MA, Bertero D, Casal JJ. 2000. Temperature-dependent internode elongation in vegetative plants of *Arabidopsis thaliana* lacking phytochrome B and cryptochrome 1. *Planta* **210,** 497–501.

Mele G, Ori N, Sato Y, Hake S. 2003. The *knotted1*-like homeobox gene *BREVIPEDICELLUS* regulates cell differentiation by modulating metabolic pathways. *Genes and Development* **17,** 2088–2093.

Muller J, Wang Y, Franzen R, Santi L, Salamini F, Rohde W. 2001. *In vitro* interactions between barley TALE homeodomain proteins suggest a role for protein–protein associations in the regulation of *Knox* gene function. *The Plant Journal* **27,** 13–23.

Nakazawa M, Ichikawa T, Ishikawa A, Kobayashi H, Tsuhara Y, Kawashima M, Suzuki K, Muto S, Matsui M. 2003. Activation tagging, a novel tool to dissect the functions of a gene family. *The Plant Journal* **34,** 741–750.

Proveniers M, Rutjens B, Brand M, Smeekens S. 2007. The Arabidopsis TALE homeobox gene *ATH1* controls floral competency through positive regulation of *FLC*. *The Plant Journal* **52,** 899–913.

Quaedvlieg N, Dockx J, Rook F, Weisbeek P, Smeekens S. 1995. The homeobox gene *ATH1* of Arabidopsis is derepressed in the photomorphogenic mutants *cop1* and *det1*. *The Plant Cell* **7,** 117–129.

Ragni L, Belles-Boix E, Gunl M, Pautot V. 2008. Interaction of *KNAT6* and *KNAT2* with *BREVIPEDICELLUS* and *PENNYWISE* in *Arabidopsis* inflorescences. *The Plant Cell* **20,** 888–900.

Rutjens B, Bao D, van Eck-Stouten E, Brand M, Smeekens S, Proveniers M. 2009. Shoot apical meristem function in Arabidopsis requires the combined activities of three BEL1-like homeodomain proteins. *The Plant Journal* **58,** 641–654.

Smith HM, Boschke I, Hake S. 2002. Selective interaction of plant homeodomain proteins mediates high DNA-binding affinity. *Proceedings of the National Academy of Sciences, USA* **99,** 9579–9584.

Smith HM, Campbell BC, Hake S. 2004. Competence to respond to floral inductive signals requires the homeobox genes *PENNYWISE* and *POUND-FOOLISH*. *Current Biology* **14,** 812–817.

Smith HM, Hake S. 2003. The interaction of two homeobox genes, *BREVIPEDICELLUS* and *PENNYWISE*, regulates internode patterning in the Arabidopsis inflorescence. *The Plant Cell* **15,** 1717–1727.

Venglat SP, Dumonceaux T, Rozwadowski K, Parnell L, Babic V, Keller W, Martienssen R, Selvaraj G, Datla R. 2002. The homeobox gene *BREVIPEDICELLUS* is a key regulator of inflorescence architecture in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **99,** 4730–4735.

Voinnet O, Rivas S, Mestre P, Baulcombe D. 2003. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *The Plant Journal* **33,** 949–956.

Wu G, Lin WC, Huang T, Poethig RS, Springer PS, Kerstetter RA. 2008. KANADI1 regulates adaxial–abaxial polarity in Arabidopsis by directly repressing the transcription of *ASYMMETRIC LEAVES2*. *Proceedings of the National Academy of Sciences, USA* **105,** 16392–16397.

Xu L, Xu Y, Dong A, Sun Y, Pi L, Xu Y, Huang H. 2003. Novel *as1* and *as2* defects in leaf adaxial–abaxial polarity reveal the requirement for *ASYMMETRIC LEAVES1* and *2* and *ERECTA* functions in specifying leaf adaxial identity. *Development* **130,** 4097–4107.