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

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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 HOMEBOX 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 hp mutant exhibits defective inflorescence architecture, with downward-pointing pedicels (Douglas et al., 2002; Venglat et al., 2002). Consistent with these abnormal hp 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 hp 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 hp background fully rescue the pedicel phenotype caused by the hp 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 HOMEBOX GENES* (*ATH1*) gene, which plays an important role in the *KNAT2* pathway, regulates pedicel development.

*ATH1* is a member of the *BELL*-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 *cryptochrome* (*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 *F2* 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 (M1) were planted in soil, and plants with impaired *iso-2D* phenotypes were identified in the *M2* generation. Mapping of the suppressor locus was performed by analysis of an *F2* population from a cross between one of the suppressor mutants and the polymorphic Landsberg *erecta* (*Ler*) plants. The suppressor locus was mapped to the proximal arm of chromosome 4, between two simple sequence length polymorphism (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 *F2* 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′-CCTCCAAACCGTTTTCTC-3′ and 5′-TTTA TGCATTTGTGCTCCTCTCA-3′ for *ATH1*; 5′-CTTTGGAGCT CGACAACA-3′ and 5′-TAATGCAACTCCCACCAC-3′ for *BP*; 5′-GAACTCGCTACCGCTTTGTCCTC-3′ and 5′-ATCCGGCATGTGCTTCTTGTG-3′ for *KNAT2*; 5′-CGAGTICAGACAGAAAG CTC-3′ and 5′-GAGATCTTACTACGAGACG-3′ for *KNAT4*; 5′-CTC CGCCGCTGAAAATCTGTTG-3′ and 5′-GGTTCCGCTACT-3′ for *KNAT6*; 5′-ATGAAAGAGAGACACCAA GCTGG-3′ and 5′-GGGGCGGTCTAATCTGCAA-3′ for *ASI*; 5′-ATGCACTTCTTCTCCACAAAC-3′ and 5′-AGACGCCAT CAGTACGC-3′ for *AS2*; and 5′-TGCCATTCA(t/c)ACCTTCTCA A-3′ and 5′-CCACCAC(t/g/a/t)ACGACAAAGTT-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′-agatctATGGATAGAATGTTGTTCC-3′ and 5′-gtagcta CTCGGTAAAGAATGTTCTTT-3′ for *KNAT2*; 5′-agatc tATGGATAGAATGTTGCTCTTCT-3′ and 5′-gtagctTCGCC TGAAGAATGTTCTTT-3′ for *KNAT6*; and 5′-atcggct tatactTTGAAATGTTCTTT-3′ for *AS2*; and 5′-GCCATCA(t/c)ACCTTCTCA A-3′ and 5′-CCACCAC(t/g/a/t)ACGACAAAGTT-3′ for *ACTIN*.
control of a 35S promoter and with a 3' in-frame fusion to sequences encoding yellow fluorescent protein (YFP), using the following primers: 5'-gtgcttagctATGGGAAACAAACAAAGA-3' and 5'-ttctagagctcTTATGCGGACTTGGCTATC-3' for AS2; 5'-agctgctgATGGGAAACAAACAAAGA-3' and 5'-gtgcttagctATGGGAAACAAACAAAGA-3' for KNAT2; and 5'-agctgctgATGGGAAACAAACAAAGA-3' and 5'-gtgcttagctATGGGAAACAAACAAAGA-3' for KNAT6. Fragments of YFP were truncated at residue 155 (designated YN and YC) as previously described (Kerpola, 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 downw ard-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 downw ard-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 arabi dopsis thaliana homebox gene 5 (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 athl-1 mutant (Proveniers et al., 2007; Gomez-Mena and Sablowski, 2008), and all F1 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 athl-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 athl-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 athl-4 iso-2D double mutant (Fig. 1E). To confirm further that removal of the ATH1 gene can rescue the pedicel phenotypes of AS2 overexpression, athl-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 athl-4 as2-5D (Fig. 1G) and p35S:AS2/athl-4 (data not shown) plants displayed the rescued pedicels, similar to athl-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 athl-4 iso-2D and athl-4 as2-5D, the up-curved 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 athl-4 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 athl-4 iso-2D inflorescence. Expression of BP and several other related genes was thus analysed in iso-2D single and athl-4 iso-2D double mutants by qRT-PCR. Surprisingly, although the downward-pointing pedicel phenotypes of iso-2D were suppressed in the athl-4 iso-2D plants, the BP expression remained at a very low level in athl-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 athl-4 iso-2D compared with those in the wild type. As controls, while the AS2 transcript level was elevated in both iso-2D and athl-4 iso-2D, the AS1, ATH1, and KNAT4 levels in iso-2D and athl-4 iso-2D showed no significant changes compared with those in the wild type (Fig. 2). To confirm further that athl-4 iso-2D does not affect BP expression, a pBP:GUS transgenic line was crossed to iso-2D and athl-4 iso-2D, respectively, to generate the isogenic pBP:GUS/iso-2D and
**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 F1 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

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**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) 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 as2-5D**. Bars=1 cm in C–G, and 0.1 cm in H–L.
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
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. 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. Bars¼1 cm in A–I.
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 fulfill 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 hp 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 hp triple mutants, removal of KNAT2 is equivalent to removal of ATH1, and the KNAT6 transcripts must increase due to hp 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
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. 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.
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).

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