

RESEARCH PAPER

# PwHAP5, a CCAAT-binding transcription factor, interacts with PwFKBP12 and plays a role in pollen tube growth orientation in *Picea wilsonii*

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## Abstract

The HAP complex occurs in many eukaryotic organisms and is involved in multiple physiological processes. Here it was found that in *Picea wilsonii*, HAP5 (*PwHAP5*), a putative CCAAT-binding transcription factor gene, is involved in pollen tube development and control of tube orientation. Quantitative real-time reverse transcription-PCR showed that *PwHAP5* transcripts were expressed strongly in germinating pollen and could be induced by Ca<sup>2+</sup>. Over-expression of *PwHAP5* in pollen altered pollen tube orientation, whereas the tube with *PwHAP5RNAi* showed normal growth without diminishing pollen tube growth. Furthermore, *PwFKBP12*, which encodes an FK506-binding protein (FKBP) was screened and a bimolecular fluorescence complementation assay performed to confirm the interaction of *PwHAP5* and *PwFKBP12* *in vivo*. Transient expression of *PwFKBP12* in pollen showed normal pollen tube growth, whereas the tube with *PwFKBP12RNAi* bent. The phenotype of overexpression of *HAP5* on pollen tube was restored by *FKBP12*. Altogether, our study supported the role of *HAP5* in pollen tube development and orientation regulation and identified *FKBP12* as a novel partner to interact with *HAP5* involved in the process.

**Key words:** Growth orientation, *Picea wilsonii*, pollen tube, PwFKBP12, PwHAP5.

## Introduction

The CCAAT box, one of the most ubiquitous promoter elements in eukaryote genomes (Bucher, 1990; Maity and de Crombrughe, 1998; Mantovani, 1999), occurs in 30% of all eukaryotic genes (Bucher, 1990; Wuarin *et al.*, 1990). It can be recognized by a highly specific heteromeric factor called the HAP (for histone- or haem-associated protein) complex, also known as CBF (for CCAAT-binding factor) or NF-Y (for nuclear factor Y) (Mantovani, 1999), which is an evolutionarily conserved transcription factor that occurs in a wide range of eukaryotes from yeast to humans. The HAP

complex in mammals and plants (known as NF-Y in mammals) includes three subunits: NF-YA (CBF-B or HAP2), NF-YB (CBF-A or HAP3), and NF-YC (CBF-C or HAP5), which are required for DNA binding of the complex and are sufficient for transcriptional activity (Maity and de Crombrughe, 1998; Mantovani, 1999). In yeast, the HAP complex is composed of four subunits: HAP2, HAP3, HAP4, and HAP5 (McNabb and Pinto, 2005). In contrast to yeast and mammals, in which a single gene generally encodes each subunit, plants have greatly expanded subunit classes. For

Abbreviations: ABRE, abscisic acid response element; BiFC, bimolecular fluorescence complementation; CHSA, chalcone synthase; CTAB, cetyltrimethylammonium bromide; EF1- $\alpha$ , elongation factor  $\alpha$ ; GFP, green fluorescent protein; HAP, histone (or haem)-associated protein; o-NPG, o-nitrophenyl  $\beta$ -D-galactopyranoside; ORF, open reading frame; RT, reverse transcription; X- $\alpha$ -Gal, 5-bromo-4-chloro-3-indoyl- $\alpha$ -D-galactoside; YFP, yellow fluorescent protein.

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example, in *Arabidopsis*, 10, 11, and 13 genes encode the HAP2, HAP3, and HAP5 subunits, respectively (Riechmann *et al.*, 2000), and rice has 10 *HAP2* genes, 11 *HAP3* genes, and 7 *HAP5* genes (Thirumurugan *et al.*, 2008). Thus, the large number of HAP2/HAP3/HAP5 heterotrimer combinations in plants provides the potential for the HAP complex to be recruited into a wide range of processes and play diverse roles in gene transcription in higher plants (Edwards *et al.*, 1998). However, relatively little is known about the biological function of the HAP complex in plants compared with its role in yeast and mammals, which has been extensively analysed (Pinkham and Guarente, 1985; Dang *et al.*, 1996; Mantovani, 1999).

A growing body of evidence indicates that individual plant HAP subunits function in numerous physiological processes, including embryogenesis and seed maturation (Lotan *et al.*, 1998; Kwong *et al.*, 2003; Lee *et al.*, 2003; Yazawa and Kamada, 2007; Yamamoto *et al.*, 2009), chloroplast biogenesis (Miyoshi *et al.*, 2003), meristem growth (Combier *et al.*, 2006), and stress responses (Nelson *et al.*, 2007; Liu and Howell, 2010). The first identified plant HAP gene, *LEAFY COTYLEDON1* (*LECI*), in *Arabidopsis* and its most closely related subunit, *LECI-LIKE* (*LIL*), which is similar to *AtHAP3* subunits, controls embryogenesis and seed maturation via interaction with ABA-response element (ABRE)-binding factor, *bZIP67* (Lotan *et al.*, 1998; Kwong *et al.*, 2003; Lee *et al.*, 2003; Yamamoto *et al.*, 2009). Similarly, *C-LECI* in carrot was shown to be a functional orthologue of *LECI* that regulates gene expression during carrot embryo development (Yazawa and Kamada, 2007). In particular, HAP subunits are involved in flowering regulation, and changes in member activities can influence flowering time (Ben-Naim *et al.*, 2006; Wenkel *et al.*, 2006; Cai *et al.*, 2007; Chen *et al.*, 2007; Kumimoto *et al.*, 2008). Wenkel *et al.* (2006) revealed that the CCT-domain protein CO forms a heterotrimeric CO–*AtHAP3*–*AtHAP5* complex and functions in the photoperiodic flowering pathway. *AtHAP3b* promotes flowering by enhancing the expression of key flowering time genes in the photoperiod-regulated flowering pathway, but is not involved in flowering promoted by gibberellin or vernalization (Cai *et al.*, 2007; Chen *et al.*, 2007; Kumimoto *et al.*, 2008). Subsequently, Wei *et al.* (2010) identified DTH8 in rice, which encodes a putative HAP3 subunit and appears to play an important role as a novel suppressor in the signal network of photoperiodic flowering, as well as regulation of plant height and yield potential (Wei *et al.*, 2010). Most recently, HAP5 subunits have been shown to be required for CONSTANS-mediated, photoperiod-dependent flowering and involved in the regulation of photosynthesis-related genes in plants (Kumimoto *et al.*, 2010). Despite the many reports concerning the roles of HAP subunits in flowering time regulation, whether they function in other plant sexual reproduction processes, such as pollen tube development, is not known.

During sexual reproduction in flowering plants, pollen grains land on the stigma of the pistil and germinate into

a cylindrical tube, which penetrates the stigma, grows through the transmitting tract, and orients towards the ovule to deliver the sperm cell for fertilization. Thus, pollen germination and tube growth are key steps in the success of fertilization. Tube growth and sperm delivery in conifers are fundamentally different from those in angiosperms (Singh, 1978). Pollen tubes of coniferous species, which represent a major evolutionary divergence in the development of the male gametophytes (Lazzaro *et al.*, 2005), with little information regarding this development available, grow more slowly than angiosperm pollen tubes; they also tend to ramify, and lack a tip-to-base zonation of organelles (Knuiman *et al.*, 1996), implying that the development and fertilization mechanisms of the tubes of the two types of plant differ.

In this study, a gene encoding the *HAP5* transcription factor in *Picea wilsonii* Mast. was isolated and characterized. The expression pattern of PwHAP5 was examined and it was demonstrated that PwHAP5 was induced by Ca<sup>2+</sup>, and that overexpression of PwHAP5 in pollen altered the orientation of pollen tube growth. Moreover, a novel HAP5-interacting protein, an FK506-binding protein (FKBP12), was identified, which interacts with the C-terminal, N-terminal, and full-length sequence of PwHAP5 *in vivo* to regulate pollen tube development. These results provide a novel function for this protein in the control of pollen tube orientation by interaction with PwHAP5.

## Materials and methods

### Plant material

Cones with mature pollen were collected in mid-April 2008 from mature *P. wilsonii* Mast. trees in the Beijing Botanical Garden, Institute of Botany, Chinese Academy of Sciences, and were dried overnight at room temperature. The dry pollen was stored at –80 °C until use.

### *In vitro* *P. wilsonii* pollen germination

*P. wilsonii* pollen grains stored at –80 °C were resuscitated by transfer to 4 °C for 12 h, and then to room temperature for another 2 h. The resuscitated pollen was cultured in standard liquid pollen germination and tube growth medium [12% sucrose, 0.03% Ca(NO<sub>3</sub>)<sub>2</sub>, 0.01% H<sub>3</sub>BO<sub>3</sub>, and 5 mM citrate-phosphate buffer, pH 5.8]. Pollen grains were incubated in small dishes at 25±1 °C in a saturated atmosphere (100% relative humidity) and experimentally sampled at 6, 12, 18, 24, 30, and 36 h after germination.

### RNA extraction

For RNA isolation, the plant tissues were harvested separately, frozen in liquid nitrogen, and stored at –80 °C until use. Total RNA from germinating pollen was isolated using Trizol reagent (Gibco-BRL, Grand Island, NY, USA) according to the manufacturer's instructions. Total RNA from other tissues was extracted using standard cetyltrimethylammonium bromide (CTAB) extraction and lithium chloride precipitation as described previously (Chang *et al.*, 1993). The quality and quantity of RNA was assessed by agarose gel electrophoresis and UV spectroscopy, respectively.

### cDNA library construction and screening

Poly(A) RNA (0.5 µg) isolated from *P. wilsonii* pollen incubated in Ca<sup>2+</sup>-stressed medium (0.1% Ca<sup>2+</sup> concentration) for 12 h was used to synthesize first-strand cDNA, which was then amplified via long-distance PCR according to the manufacturer's protocol (SMART™ cDNA Library Construction Kit; Clontech, Palo Alto, CA, USA). The original cDNA library, estimated to contain 5 × 10<sup>5</sup> independent recombinants, was amplified with *Escherichia coli* XL-Blue before screening. The cDNA library was screened by differential hybridization (one hybridization with a Ca<sup>2+</sup>-untreated pollen cDNA probe and another with a Ca<sup>2+</sup>-treated pollen cDNA probe) as described previously (Wu *et al.*, 2004). One cDNA clone, *PwHAP5*, is described in this study.

### RT-PCR and quantitative real-time RT-PCR

Total RNA extracted from each tissue was treated with DNase I to remove genomic DNA, after which the cDNA was synthesized. Reverse transcription (RT) reactions were performed using M-MLV reverse transcriptase (Promega, Madison, WI, USA) with oligo(dT) primers. Each sample was assayed in triplicate. The *PwHAP5* gene was amplified using the gene-specific primers 5'-ATCAGCAG-CAGCCACAA-3' and 5'-ATCCTCGTCTGCCTTCAT-3'. An elongation factor  $\alpha$  gene (*EF1- $\alpha$* ) was amplified as an internal control in the RT-PCRs, and the primer sequence used was as previously described (Yu *et al.*, 2009).

For quantitative RT-PCR analysis, SYBR Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan) and Chromo 4 Real-Time PCR Detector (Bio-Rad, Hercules, CA, USA) were used. Quantitative real-time RT-PCR analysis of *PwHAP5* was performed using the specific primers of the *PwHAP5* gene, 5'-GGTAACCAGAT-GAGGGAG-3' and 5'-TCTTGTCTCCTCGGTGT-3'. *EF1- $\alpha$*  mRNA expression was amplified as an internal control for real-time analysis, using the specific primers 5'-AACTGGAGAAGGAACC-CAAG-3' and 5'-AACGACCAATGGAGGATAC-3'. At least two different biological replicates were used for all RT-PCR determinations, which were performed with 30 cycles of denaturation, annealing, and extension steps for each sample. Quantitative PCR experiments were repeated independently three times.

### Determination of RNAi specificity

In the experiments reported here, the specificity of RNAi was assured using a modification of the method of Miki and Shimamoto (2004). To test transient suppression of gene expression caused by direct introduction of the RNAi vector into *P. wilsonii* pollen, the pFGCLat52-GFP vector, which carries a 300-bp fragment of the green fluorescent protein (*GFP*) gene, was generated. The GFP-RNAi construct, *Lat52-GFP*, as a target gene and *Lat52-CHERRY* as a control for bombardment efficiency were co-bombarded. The results shown in Supplementary Fig. S1 (available at JXB online) indicate that the bombarded *Lat52-GFP* gave sufficient expression of GFP protein, which was evidenced by the green fluorescence in the pollen. When the GFP-RNAi construct was co-bombarded with *Lat52-GFP*, strong silencing of the green fluorescence was observed, while no difference in the expression of the control *CHERRY* gene was observed (Supplementary Fig. S1 at JXB online). These results suggested that the RNAi vector based on pFGCLat52 was useful for transient suppression of gene function in *P. wilsonii* pollen. Meanwhile, a 386-bp fragment for *PwHAP5RNAi* and a 294-bp fragment for *PwFKBP12RNAi*, which were conserved at a very low level among members of the PwHAP5 family and PwFKBP family, respectively, were used in the experiment in order that the sequences used as RNAi trigger had enough specificity.

### RNAi vector constructs

To achieve the objective of pollen-specific expression, the 35S promoter in pFGC5941 was replaced by the *Lat52* promoter to

form the pFGCLat52 vector. To construct the *PwHAP5RNAi* and *PwFKBP12RNAi* vector, two fragments (post-blast) were obtained. The first was a 386-bp fragment, conservative to *PwHAP5* and amplified from cDNA of *P. wilsonii* pollen using the primer pair: 5'-**TCTAGAGGCGCGCCTGATCTCTGC-AGAGGCACC-3'** (bold italics represent the *XbaI-AscI* site) and 5'-**GGATCCATTTAAATTACGTCCTCAAAGAACCC-3'** (bold italics represent the *BamHI-SwaI* site). The second was a 294-bp fragment conservative to *PwFKBP12* amplified from cDNA of *P. wilsonii* pollen using primer pair 5'-**TCTAGAGGCGCG-CCTGAGGATCTGTCTCAGA-3'** (bold italics represent the *XbaI-AscI* site) and 5'-**GGATCCATTTAAATGTTTGCTGG-CAATATC-3'** (bold italics represent the *BamHI-SwaI* site). The fragments were digested with *SwaI* and *AscI* and inserted on the left side (5' end) of the chalcone synthase intron from *Petunia hybrida* in pFGCLat52, and further digested with *BamHI* and *XbaI* and inserted on the right side (3' end) of the chalcone synthase intron, thus completing the two RNAi vectors.

### Particle bombardment-mediated transient expression in pollen

Mature *P. wilsonii* pollen grains were used in a transient expression experiment, using the particle bombardment procedure described in 'In vitro *P. wilsonii* pollen germination', above. Microprojectile bombardment was performed using the helium-driven PDS-1000-He biolistic system (Bio-Rad). Tungsten particles (1.1 µm) were coated with plasmid DNA according to the manufacturer's recommendation (Bio-Rad; Sanford *et al.*, 1993). The pollen grains were incubated for 12 h or 24 h before observation under a confocal microscope (LSM 510 META; Zeiss), with 100 tubes of each sample counted and measured. Pollen grains were considered to be germinated when the pollen tube length was greater than the diameter of the pollen grain (Rodriguez-Riano and Dafni, 2000). Fluorescence and transmitted light images were recorded using the fluorescence microscope.

### Y2H assays

The full-length coding region (F), plus a fragment carrying residues 1–77 (N77) and another carrying residues 92–221 (C130) of *PwHAP5*, were fused to the plasmid pGBKT7 and transformed into *Saccharomyces cerevisiae* strain AH109 with the empty pGADT7-Rec vector. The transformants of all three fragments plus the empty pGADT7-Rec vector can grow on SD/-Trp-Leu-His-Ade medium. F, N77, and C130 were used as bait to screen a *P. wilsonii* pollen cDNA library constructed in the pGADT7-Rec vector. The cDNA library was transformed into yeast strain AH109 with F, N77, and C130. Y2H assays were performed according to standard methods (Chien *et al.*, 1991). Positive clones were selected on SD/-Trp-Leu-His-Ade medium. After confirmation using the 5-bromo-4-chloro-3-indoyl- $\alpha$ -D-galactoside (X- $\alpha$ -Gal) test and retransformation, the inserts were sequenced. In addition, pGADT7-Rec and pGADT7-PwFKBP12 were transformed into yeast strain AH109, with the empty pGBDT7 vector as control. The expression of the third reporter gene *lacZ* was followed by measuring at OD<sub>420</sub> the accumulation of the product metabolized by  $\beta$ -galactosidase, with *o*-nitrophenyl  $\beta$ -D-galactopyranoside (*o*-NPG; Sigma, St Louis, MO, USA) as substrate.

### Bimolecular fluorescence complementation (BiFC) assays

BiFC assays were performed as described by Guo *et al.* (2009). cDNA without a termination codon encoding *PwHAP5* was cloned into pSPYNE-35S, and the cDNAs encoding *PwFKBP12* were cloned into pSPYCE-35S. Both the cDNAs encoding *PwTUA1* (*P. wilsonii*  $\alpha$ -tubulin protein) in pSPYCE-35S and the empty vector 35S-pSPYCE were used as negative controls, and the bZIP63-pSPYNE-35S and bZIP63-pSPYCE-35S vectors were used as positive controls (Walter *et al.*, 2004). Thus, the plasmids pUC-SPYNE-*PwHAP5* and pUC-SPYCE-*PwFKBP12* were expressed

as PwHAP5–yellow fluorescent protein (YFP)<sub>N</sub> and PwFKBP12–YFP<sub>C</sub> fusion proteins. These vectors were introduced into *Agrobacterium tumefaciens* strain GV3101. For infiltration of *Nicotiana benthamiana*, the P19 protein of tomato bushy stunt virus was used to suppress gene silencing. The *A. tumefaciens* strains were grown overnight in YEB media containing appropriate antibiotic selections. Cells were pelleted at 4000 g, resuspended in infiltration medium (10 mM MgCl<sub>2</sub>, 10 mM MES, 150 mM acetosyringone), and incubated for at least 2 h at room temperature. Co-infiltration of *A. tumefaciens* strains containing the BiFC constructs and the P19 silencing plasmid was carried out at an OD<sub>600</sub> of 0.7:0.7:1.0. Resuspended cells were infiltrated into leaves of 4-week-old *N. benthamiana* plants as described previously (Voinnet *et al.*, 2003; Walter *et al.*, 2004). After 2 d, epidermal cell layers of tobacco leaves were assayed for fluorescence under a fluorescence microscope (BX51 model 7.3; Olympus). These data clearly indicated both that PwFKBP12 is an interaction partner of PwHAP5 *in vivo* and that the bimolecular interaction takes place in the cytoplasm.

## Results

### *Isolation and characterization of the cDNA clone encoding HAP5 from P. wilsonii*

The putative PwHAP5 cDNA clone was isolated from a *P. wilsonii* subtractive cDNA library of pollen after a 12-h incubation in Ca<sup>2+</sup>-stressed medium (0.1% Ca<sup>2+</sup> concentration) via differential hybridization screening to identify genes involved in pollen development. The resulting 988-nucleotide PwHAP5 cDNA, with an ATG initiation codon at position 64 and a termination codon at position 667 (TGA), contained an open reading frame (ORF) encoding a 202-amino-acid polypeptide with a calculated molecular weight of 22.44 kDa and a predicted pI of 6.20. The full cDNA sequence was submitted to GenBank as accession number EU579453.1. Like the majority of HAP5 (NF-YC) proteins, the PwHAP5 protein contained the required conservative amino acids. The predicted PwHAP5 amino acid sequence contained a fragment (~53 residues) with strong similarity to the conserved domain of HAP5 subunits in other organisms (Fig. 1A). Of these 53 residues, 10 were absolutely conserved among all HAP5 subunits. The protein also contained NF-YA/NF-YB (HAP2/HAP3) interaction domains, as well as a DNA-binding domain similar to previously characterized NF-YCs (Yazawa and Kamada, 2007).

To examine evolutionary relationships among HAP5 proteins, a phylogenetic analysis based on the amino acid sequences of the conserved domains was conducted. This showed that PwHAP5 and AtNF-YC3/9, which are required for CONSTANS-mediated, photoperiod-dependent flowering in *Arabidopsis* (Kumimoto *et al.*, 2010), and OsHAP5A/B were in the same clade and were highly conserved (Fig. 1B).

### *Expression pattern of PwHAP5 in different tissues and germinating pollen at different developmental stages*

To obtain the profile of PwHAP5 expression patterns, total RNA was isolated from needles, stems, roots, and from incubated germinating *P. wilsonii* pollen mixtures at 6-h

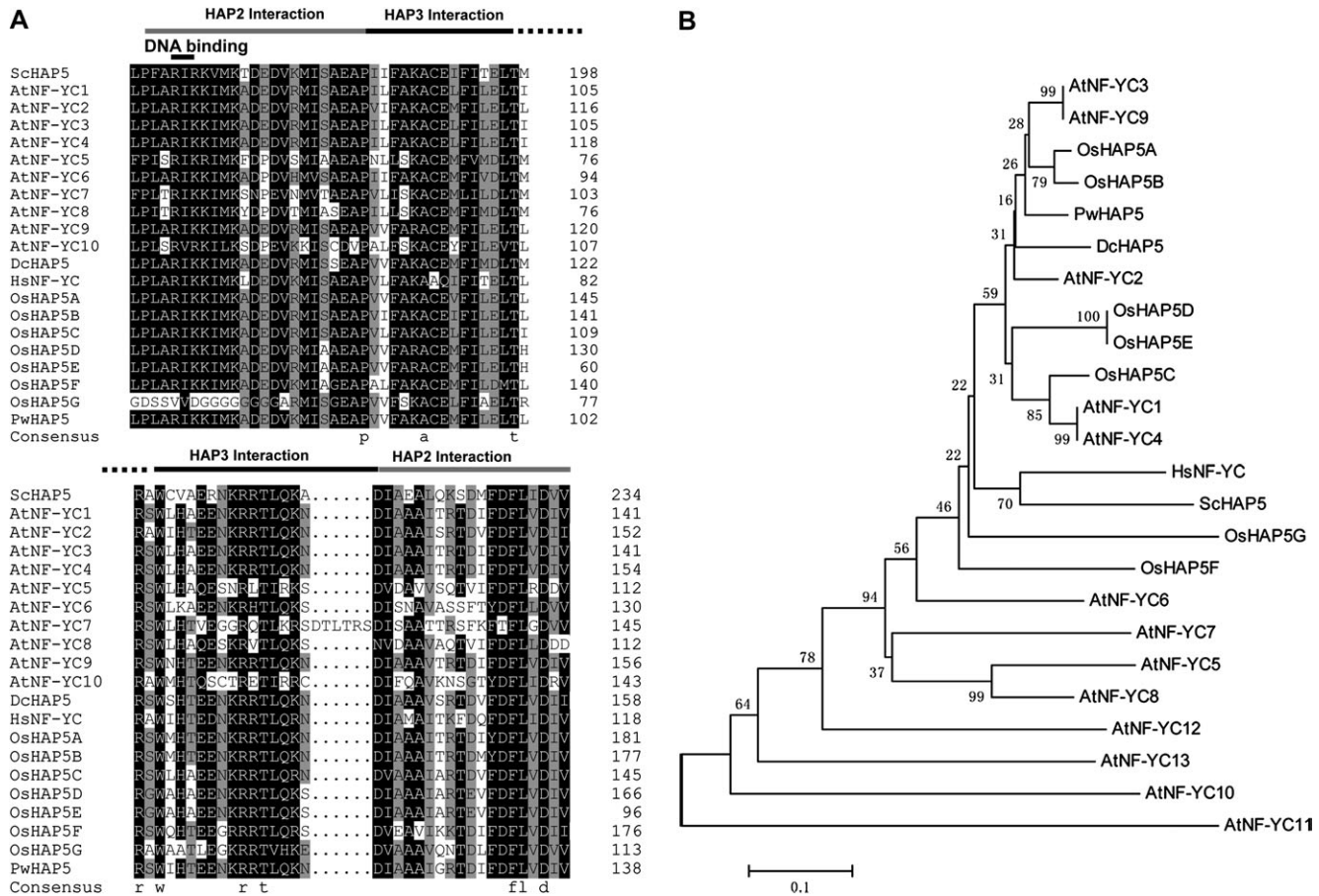
intervals and transcript accumulation examined using RT-PCR and quantitative real-time RT-PCR analyses. PwHAP5 transcripts were expressed strongly in needles, germinating pollen, and stems, but less in roots (Fig. 2A). Among the various tissues, needles had the highest PwHAP5 transcription level. PwHAP5 expression was further examined during pollen development. As shown in Fig. 2B, PwHAP5 expression was first detected in pollen 6 h post-incubation (germination only). It increased gradually, reaching a maximum 18 h post-incubation. Transcription levels remained at this same high level during the late stages (24, 30, and 36 h post-incubation). The PwHAP5 expression level in boron-stressed (0.1% H<sub>3</sub>BO<sub>3</sub> concentration) and Ca<sup>2+</sup>-stressed medium (0.1% Ca<sup>2+</sup> concentration) was also analysed during various pollen tube developmental stages. PwHAP5 was induced by Ca<sup>2+</sup>, but not by boron, during all of the tested stages (Fig. 2C).

### *The effects of PwHAP5 on pollen tube growth*

To further examine the function of the PwHAP5 gene in pollen tube development, an overexpression vector and an RNAi expression vector of PwHAP5 were constructed (Fig. 3A). The vectors were used to transiently transform *P. wilsonii* pollen by microprojectile bombardment. A reporter gene encoding GFP was fused to PwHAP5 to visualize transiently transformed pollen. The *Lat52* promoter was used to drive the pollen-specific expression of transgenes in these studies (Twell *et al.*, 1990). Considering that GFP fused to HAP5RNAi vector directly could affect gene silencing, PwHAP5RNAi was transiently co-expressed under the pollen-specific promoter *Lat52* (Twell *et al.*, 1990) with *Lat52::GFP* as an indicator. No significant difference was observed in pollen germination percentage and tube length between the samples transformed with fusion proteins and those left untreated (Fig. 3B) suggesting that neither the microprojectile bombardment itself nor the fusion proteins had any significant effect on pollen germination and pollen tube length. The orientation of *P. wilsonii* pollen tube growth was altered when PwHAP5 was overexpressed in the pollen tube. However, the pollen tube with PwHAP5RNAi showed normal growth (Fig. 3C). This phenotype was observed in most of the transformed pollen tubes although some tip bending was occasionally observed in untreated pollen tubes after prolonged culture (Table 1).

### *PwHAP5 protein interacts with PwFKBP12*

Y2H screening was performed to identify proteins that interact with PwHAP5. The PwHAP5 protein contained HAP2/HAP3 interaction domains and a DNA-binding domain. To examine which domain is crucial to the function of PwHAP5 and its interaction with other proteins, PwHAP5 was split into two fragments. The bait was constructed by fusing 77 N-terminal amino acids (N), 130 C-terminal amino acids (C), and full-length PwHAP5 (H) to the pGBDKT7 vector to screen the cDNA library from *P. wilsonii* pollen. A total of 6×10<sup>6</sup> colonies were screened



**Fig. 1.** HAP5 gene of *P. wilsonii*. (A) Alignment of the HAP5 proteins, sequences correspond to the conserved regions in HAP5 proteins across various lineages. Dc, *Daucus carota*; Hs, *Homo sapiens*; Os, *Oryza sativa*; Sc, *Saccharomyces cerevisiae*. Note that the HAP2 interaction domain extends across two separate regions. The DNA-binding domain in HAP5 consists of the two amino acids AR (found in most HAP5 homologues). (B) Phylogenetic tree of *P. wilsonii* HAP5 (PwHAP5) and other HAP5 proteins previously characterized. A neighbor-joining tree based on the deduced amino acid sequences of the conserved domains in HAP5s. This bootstrap consensus tree was based on 1000 replicates. Numbers on nodes are bootstrap values. The accession numbers in GenBank and sources of the protein are as follows: AtNF-YC1 (At3g48590), AtNF-YC2 (At1g56170), AtNF-YC3 (At1g54830), AtNF-YC4 (At5g63470), AtNF-YC5 (At5g50490), AtNF-YC6 (At5g50480), AtNF-YC7 (At5g50470), AtNF-YC8 (At5g27910), AtNF-YC9 (At1g08970), AtNF-YC10 (At1g07980), AtNF-YC11 (At3g12480), AtNF-YC12 (At5g38140), AtNF-YC13 (At5g43250) from *Arabidopsis thaliana*; DcHAP5 (AB104612) from *D. carota*; HsNF-YC (U78774) from *H. sapiens*; OsHAP5A (AB288041), OsHAP5B (AB288042), OsHAP5C (AB288043), OsHAP5D (AB288044), OsHAP5E (AB288045), OsHAP5F (AB288046), OsHAP5G (AB288047) from *O. sativa*; ScHAP5 (U19932) from *S. cerevisiae*.

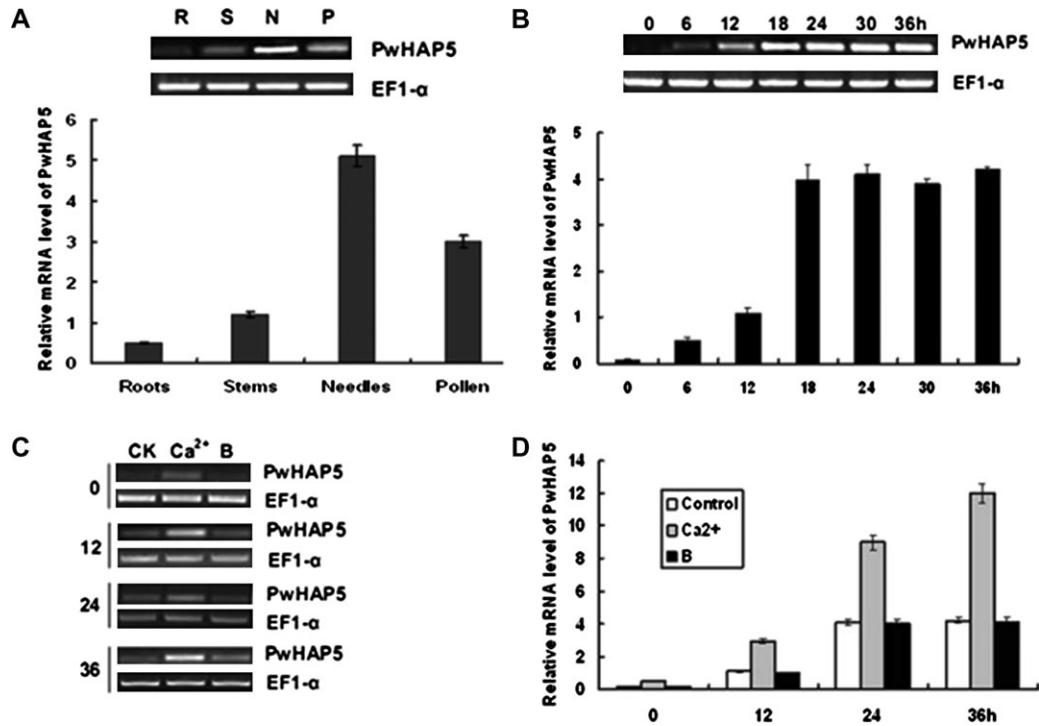
and 35 positive clones corresponding to eight cDNAs were identified (data not shown). Among the eight clones, the 5-15/3-11 clone was highly homologous to AtFKBP12 (FK506-binding protein) in *Arabidopsis*, and it was named PwFKBP12. The full cDNA sequence of PwFKBP12 was submitted to GenBank under accession number GQ5140630. As shown in Fig. 4A, PwFKBP12 conserves three of the five residues with strongest influence over catalytic activity in mammalian FKBP12 (DeCenzo *et al.*, 1996; Tradler *et al.*, 1997), as well as a cysteine pair (Cys26 and Cys80) that is unique to the plant FKBP12 isoforms and was vital for interaction with calcineurin *in vitro* (Xu *et al.*, 1998).

Protein interactions between N/C/H and PwFKBP12 were further confirmed by analysing growth on selective medium, followed by measuring true  $\beta$ -galactosidase activity. Growth of the N-PwFKBP12, C-PwFKBP12, and

H-PwFKBP12 combinations, but no growth of the control combinations was observed (Fig. 4B).  $\beta$ -Galactosidase activities of the N/C/H fusion proteins were nearly 20 times higher than those of the controls (Fig. 4C), indicating specific interaction between PwHAP5 and PwFKBP12.

*In vivo detection of the interaction between PwHAP5 and PwFKBP12*

Next a BiFC assay was performed (Walter *et al.*, 2004) in a tobacco transient expression system (Voinnet *et al.*, 2003) to confirm the interaction of PwHAP5 and PwFKBP12 *in vivo*. PwFKBP12 was fused with YFP<sub>C</sub> (SPYCE), and the full length (H) of the PwHAP5 protein was fused with YFP<sub>N</sub> (SPYNE). Fluorescence from YFP in transgenic tobacco epidermis transformed with PwHAP5(H)-YFP<sub>N</sub> and PwFKBP12-YFP<sub>C</sub> was observed throughout the



**Fig. 2.** Expression of *PwHAP5* in different tissues and in developing pollen tubes of *P. wilsonii*. (A) Tissue-specific expression of *PwHAP5* in *P. wilsonii*. Total RNA was isolated from needles, stems, roots, and pollen (incubated after 0, 6, 12, 18, and 24 h). Above the graph is shown semi-quantitative RT-PCR analysis of expression of *PwHAP5*. The *EF1-α* gene was amplified as an internal control. The graph shows quantification of *PwHAP5* expression. Quantitative real-time RT-PCR was performed using *PwHAP5*-specific primers. Column heights represent expression levels relative to the *EF1-α* gene. The values are means  $\pm$ SD ( $n = 3$ ) from three independent experiments. (B) Expression of *PwHAP5* in pollen at different development stages (incubated after 0, 6, 12, 18, 24, 30, and 36 h). Above the graph is shown semi-quantitative RT-PCR analysis of expression of *PwHAP5*; the graph represents the quantification of *PwHAP5* expression. The control and values are as in (A). (C) Semi-quantitative RT-PCR analysis of expression of *PwHAP5* in pollen 0, 12, 24, and 36 h after incubation, induced by 0.1% (w/v) Ca<sup>2+</sup> or 0.1% (w/v) H<sub>3</sub>BO<sub>3</sub>. (D) Quantification of *PwHAP5* expression in *P. wilsonii* pollen at the same intervals after incubation as in (C). The control and values are as in (A).

cytoplasm, but not in the nucleus (Fig. 5). The same results were obtained in transgenic tobacco epidermis transformed with *PwHAP5* N77 or C130 and *PwFKBP12*-YFPc (Fig. 5). The negative controls (empty vectors or *PwTUA1*-YFP<sub>C</sub>) produced no or only background fluorescence and the positive control (YFP<sub>N</sub>-bZIP63/bZIP63-YFP<sub>C</sub>) produced fluorescence only in the nucleus, showing the specificity of the BiFC assays.

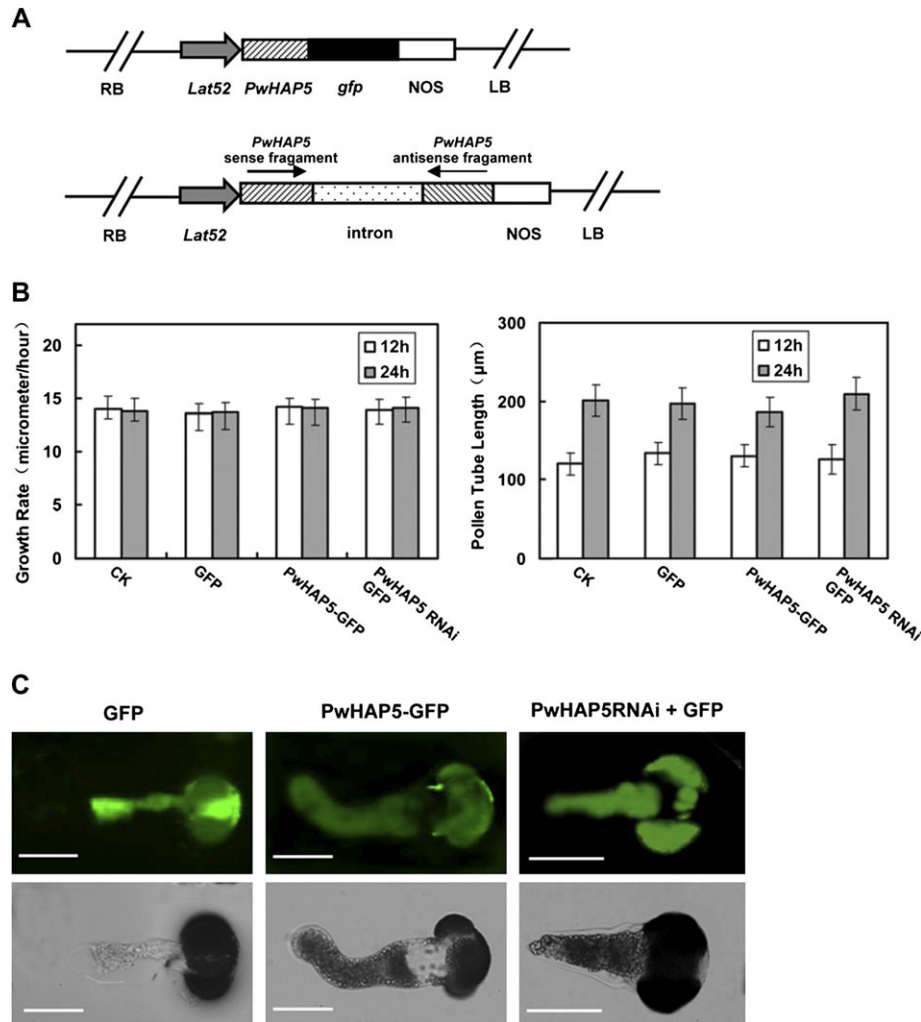
#### The effect of *PwHAP5* and *PwFKBP12* on pollen tube growth

To further clarify the function of the association of *PwHAP5* and *PwFKBP12* in pollen tube growth, RNAi and overexpression vectors of *PwHAP5* and *PwFKBP12* fused to CHERRY and GFP, respectively, were constructed. The overexpression and RNAi vectors were used to transform *P. wilsonii* pollen transiently alone or in combination via microprojectile bombardment. Considering that GFP/CHERRY fused to *PwHAP5RNAi*/*PwFKBP12RNAi* vector directly could affect gene silencing, *PwHAP5RNAi*/*PwFKBP12RNAi* was transiently co-expressed under the pollen-specific promoter *Lat52* (Twell et al., 1990) with *Lat52::GFP/CHERRY* as an indicator. As

expected, the pollen tube overexpressing *PwHAP5* or with *PwFKBP12RNAi* showed bent growth (Fig. 6, Table 1), which was consistent with that observed in Fig. 3. However, neither the transient expression of *PwFKBP12* alone nor the co-expression of *PwHAP5* and *PwFKBP12* in pollen altered pollen tube growth orientation and the pollen tubes showed normal growth (Figs 3, 6). Furthermore, the pollen tube with *PwHAP5RNAi* (whether *PwFKBP12* overexpression or RNAi) also showed normal growth (Figs 3, 6). No significant difference was observed in the pollen germination percentage or tube growth rate between samples transformed with GFP or CHERRY and those left untreated, suggesting that neither microprojectile bombardment itself nor GFP or CHERRY had a significant effect on pollen germination and pollen tube growth in *P. wilsonii* (Fig. 3; Yu et al., 2009).

## Discussion

Plant sexual reproduction is a key determinant of crop yield, and although the *HAP* gene family and its orthologues have been shown to be implicated in flowering time regulation, knowledge of the function of plant *HAP* genes



**Fig. 3.** Transient expression of *PwHAP5* alters the orientation of *P. wilsonii* pollen tube growth. (A) Diagram of plant expression vectors. The pollen-specific expression promoter *Lat52* was used to drive the expression of *PwHAP5* in pollen tube. (B) The effect of *PwHAP5* on pollen germination and tube length using microprojectile bombardment. After 12 h and 24 h of incubation, the percentage germination and tube length of pollen bombarded with the *GFP*-only, *PwHAP5* overexpression, or RNAi construct showed no significant difference compared with the untreated control pollen ( $P < 0.05$ ). Error bars indicate averages from the number of pollen tubes measured ( $n \geq 50$ ). (C) Transient expression of *PwHAP5* in *P. wilsonii* pollen tube growth. *PwHAP5*-*GFP* represents overexpression of *PwHAP5* in *P. wilsonii* pollen tube. *PwHAP5*RNAi+*GFP* represents interference of *PwHAP5*. *GFP* was used as control. The data were obtained from three independent experiments, and every condition was tested three times. Bars 20  $\mu\text{m}$ .

in pollen tube growth is limited. In this report, strong evidence is provided that *HAP5*, which encodes a CCAAT-binding transcription factor and interacts with FKBP12 in cytoplasm, is involved in pollen tube growth.

*PwHAP5* is highly expressed in germinating pollen and involved in pollen tube growth

This study confirms that *PwHAP5*, an orthologous *P. wilsonii* transcription factor, has an important role in pollen tube development and orientation regulation. This represents a novel function of *HAP5* in the *HAP* plant gene family. The protein identified contained NF-YA/NF-YC (*HAP2*/*HAP5*) interaction domains, as well as a DNA-binding domain similar to previously characterized NF-YCs (Yazawa and Kamada, 2007). *PwHAP5* was highly

conserved with *AtNF-YC3/9* and *OsHAP5A/B* and in the same clade (Fig. 1B). RT-PCR and quantitative real-time RT-PCR analyses showed that *PwHAP5* is highly expressed in needle and germinating pollen and maintains a high level of expression throughout pollen tube development (Fig. 2). Furthermore, it was shown that the direction of tube growth is altered by particle bombardment-mediated transient overexpression of *PwHAP5*. However, the pollen tube with *PwHAP5*RNAi showed normal growth, without diminishing pollen tube growth (Fig. 3). It is noteworthy that neither overexpression nor RNAi of *PwHAP5* influences pollen germination percentage and tube growth rate (Fig. 3). This is in contrast to *PwTUAI*, a pollen-specific tubulin gene cloned from *P. wilsonii* pollen, overexpression of which greatly increases pollen germination percentage and pollen tube elongation (Yu *et al.*, 2009). These results

**Table 1.** Phenotype analysis of transformed pollen tubes

Transformed vectors	Bent	Unbent	Bent/unbent ratio
CK	1	49	1 : 49
GFP	1	48	1 : 48
PwHAP5-GFP	46	4	11.5 : 1
PwHAP5 (RNAi)+GFP	2	48	1 : 24
PwHAP5-CHERRY	45	4	11.25 : 1
PwFKBP12(RNAi)+GFP			
PwFKBP12-GFP	2	51	1 : 25.5
PwHAP5-CHERRY	2	47	1 : 23.5
PwFKBP12-GFP			
PwHAP5(RNAi)+CHERRY	1	48	1 : 48
PwFKBP12-GFP			
PwFKBP12(RNAi)+GFP	48	2	24 : 1
PwHAP5(RNAi)+CHERRY	0	50	
PwFKBP12(RNAi)+GFP			

extend the functions of HAP5, which is implicated not only in the control of flowering time (Kumimoto *et al.*, 2010), but also pollen tube growth orientation regulation.

Interestingly, distinct from the phenotype of overexpressed ROP GTPases, which act as key molecular switches controlling tip growth in *Arabidopsis* pollen tubes previously reported (Li *et al.*, 1999), in which pollen tubes ballooned or were furcated by the ectopic accumulation of ROP proteins, overexpression of *PwHAP5* altered only the direction of pollen tube growth (Fig. 3), suggesting a more pivotal role of HAP5 in pollen tube growth orientation regulation than in tube polarity maintenance compared with ROP GTPases.

#### *PwFKBP12 interacts with PwHAP5 to regulate pollen tube growth orientation*

FKBPs, which are receptors of the immunosuppressive drug FK506, are referred to as immunophilins and are found in all living organisms (Geisler and Bailly, 2007). Although historically linked to immunosuppression and proline bond rotation, the physiological importance of FKBPs far surpasses FK506 binding and general protein folding, extending to cellular processes, including signal transduction, chloroplast function, DNA transcription, protein trafficking, apoptosis, and fertility (Heitman *et al.*, 1992; Luan *et al.*, 1994; Gopalan *et al.*, 2004; Kang *et al.*, 2008; Meiri *et al.*, 2010).

In this study, *PwFKBP12* was screened via a Y2H system using *PwHAP5* as bait (Fig. 4). Yeast cell growth and  $\beta$ -galactosidase activity were not observed in the control combinations of the pGADT7-Rec vector (AD) with the pGBDKT7 vector (BD) and PwTUA1, suggesting specific interaction between *PwHAP5* and *PwFKBP12*. Unfortunately, HAP2 or HAP3 subunit cannot be screened via a Y2H system using the full length, N-terminus, or C-terminus of *PwHAP5* as bait. The possibility cannot be excluded that the accumulation of *PwHAP2* or *PwHAP3* was less compared with that of *PwFKBP12* during the stage of *P. wilsonii* pollen adopted in this study. The function of

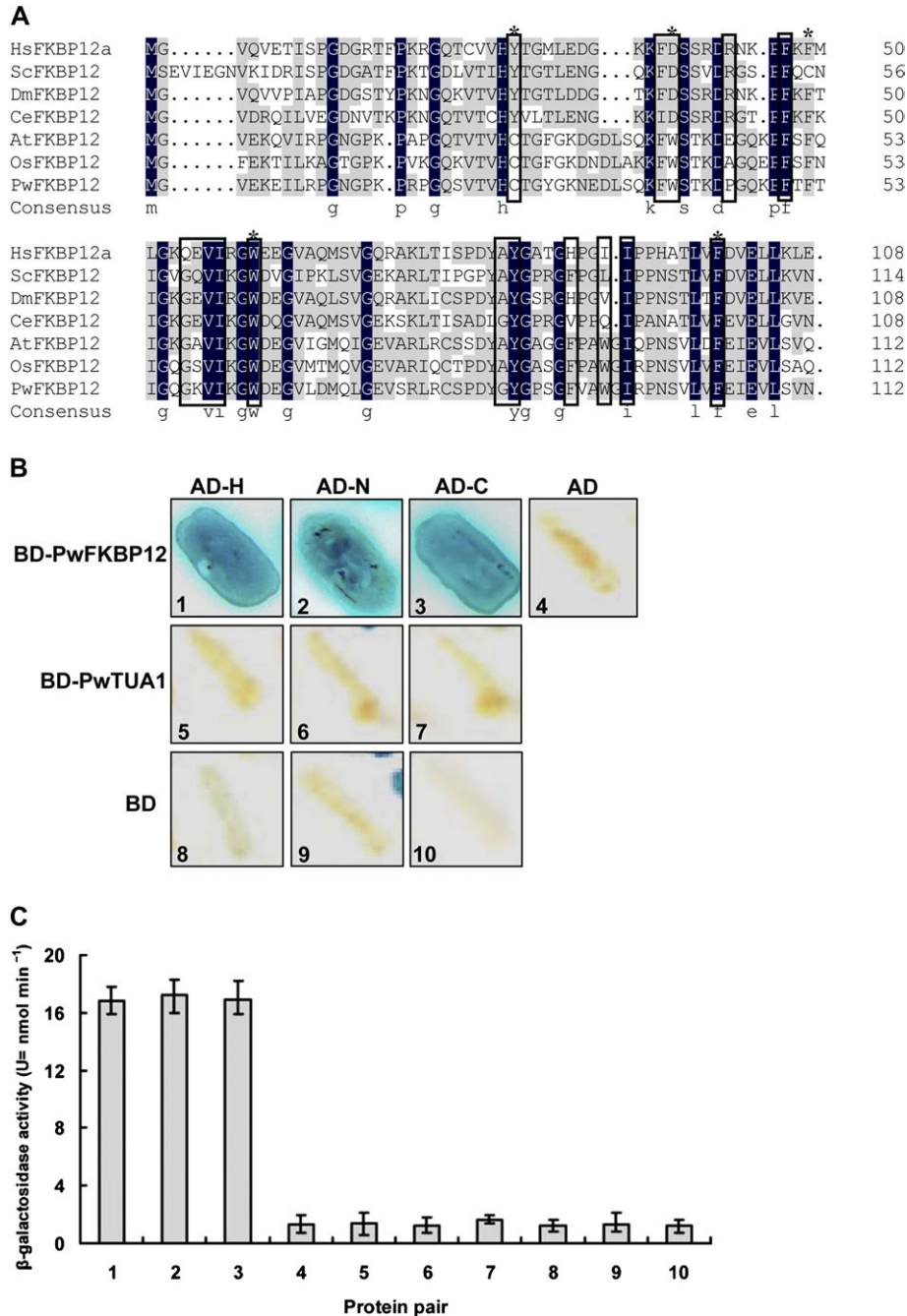
*PwFKBP12* interaction with *PwHAP5* in pollen tube development was investigated further. Transient expression of *PwFKBP12* in pollen showed normal pollen tube growth whether *PwHAP5* overexpression or RNAi or not, in contrast to the alteration of pollen tube growth direction by *PwHAP5* overexpression (Figs 3, 6). The pollen tube with *PwFKBP12RNAi* bent, whereas *PwHAP5RNAi* alone has normal growth. Importantly, the overexpression phenotype of *HAP5* in pollen tubes is restored by *FKBP12* overexpression, as shown by the normal growth observed when *PwHAP5* and *PwFKBP12* were co-expressed in pollen (Fig. 6). These data provide clear evidence that *PwFKBP12* interacts with *PwHAP5* to regulate pollen tube growth orientation. Notably, neither *PwFKBP12* or *PwHAP5* overexpression or RNAi nor the co-expression or the RNAi of the two genes affected the pollen germination percentage or pollen tube growth rate (data not shown).

The BiFC assay in a tobacco transient expression system demonstrated that the interaction of *PwHAP5* and *PwFKBP12* *in vivo* occurred mainly in the cytoplasm (Fig. 5). *Arabidopsis* FKBP12 was reported to be localized in the cytoplasm (Geisler and Bailly, 2007). This study also supports the localization of *PwFKBP12* in the cytoplasm (Fig. 6). However, the localization of different HAP subunits may differ in different cells or physiological processes. Frontini *et al.* (2004) reported that NF-YA (HAP2) and NF-YB (HAP3) are nuclear proteins, whereas NF-YC (HAP5) localizes to both cytoplasmic and nuclear compartments and its nuclear localization is determined by the interaction with its heterodimerization partner NF-YB. Interestingly, the compartmentalization of NF-YC transcription factors is a dynamic process and regulated under different physiological processes (Frontini *et al.*, 2004; Liu and Howell, 2010). Kahle *et al.* (2005) found that only the NF-YB/NF-YC (HAP3/HAP5) dimer, but not the monomeric components, are recognized by importin 13 and are imported into the nucleus. In the present study, the HAP3 homologue could not be obtained by Y2H screening using *PwHAP5* (H/N/C) as bait, which possibly explains in part why fluorescence representing *PwHAP5* was not observed in the nucleus. However, localization of HAP5 in the nucleus is not excluded.

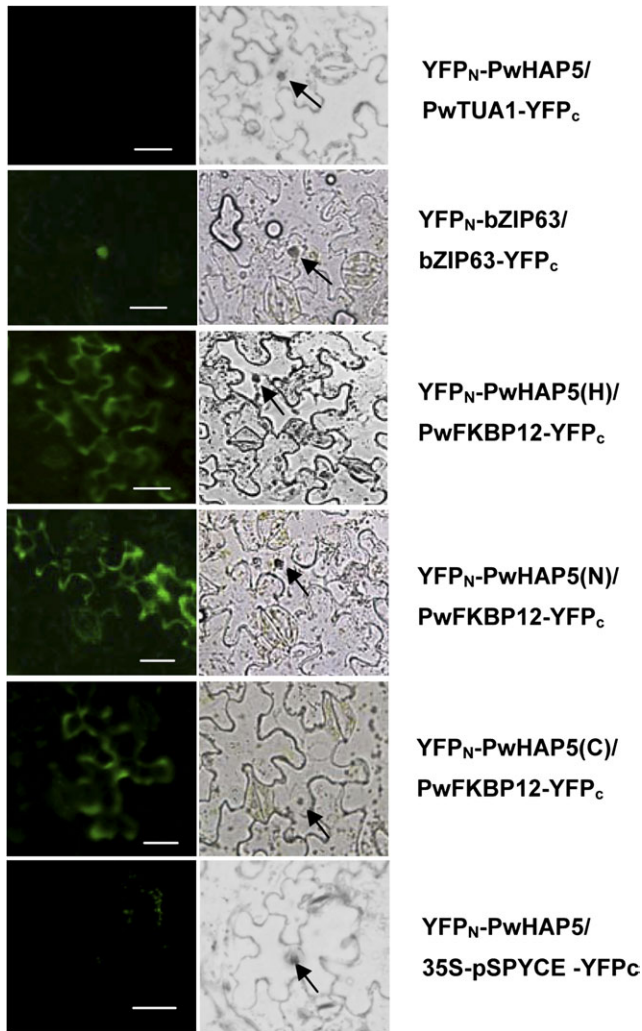
#### *Potential mechanisms of FKBP12 interaction with HAP5 in pollen tube growth*

Some FKBPs are reported to be related to  $Ca^{2+}$  (Timerman *et al.*, 1993, 1995) and involved in sexual reproduction in plants (Kurek *et al.*, 2002). Kurek *et al.* (2002) found that wheat FKBP73 could bind calmodulin via the CaM-binding domain and that the deletion of the CaM-binding and TPR domains resulted in male-sterile plants, suggesting a role for FKBP73 in the plant sexual reproduction process. This study uncovered a novel function of FKBP12 in pollen tube development interaction with HAP5 in the control of pollen tube growth direction. In mammals, FKBP12 is tightly bound to the calcium release channel/ryanodine receptor of skeletal muscle terminal cisternae of the sarcoplasmic reticulum (Timerman *et al.*, 1993, 1995). Kurek *et al.* (2002)



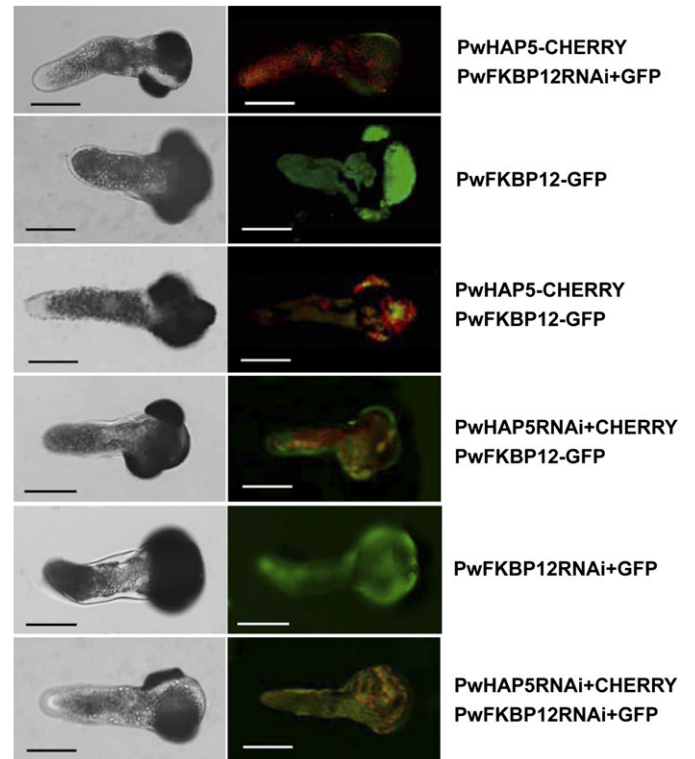


**Fig. 4.** Interaction of PwHAP5 with FKBP12. (A) Amino acid alignment of FKBP12 isoforms from different species. *H. sapiens* (Hs; GenBank protein accession number AAI19733), *S. cerevisiae* (Sc; AAA03564), *Drosophila melanogaster* (Dm; AAF57582), *Caenorhabditis elegans* (Ce; CAA22330), *A. thaliana* (At; AAB57847), and *O. sativa* (Os; NP\_001048188.1) aligned with *P. wilsonii* (Pw). Asterisks above residues denote residues that are important for peptidyl prolyl cis/trans isomerase activity in mammalian FKBP12 (DeCenzo *et al.*, 1996; Tradler *et al.*, 1997). Residues in boxes are involved in FK506 and/or rapamycin binding in mammalian FKBP12 (Van Duyne *et al.*, 1993). (B) The interactions were assayed in the GAL4 (a regulator of galactose-induced genes) Y2H system to re-examine the interactions of full-length PwHAP5 (H), N77 (N), and C130 (C) proteins with the pGBDKT7 vector alone (BD) and with PwFKBP12. As a control, combinations of the pGADT7-Rec vector (AD) with the pGBDKT7 vector (BD) and PwTUA1 are also shown. Yeast cells expressing AD-H, AD-N, AD-C, or AD alone and each of BD fusion proteins or BD alone were grown on medium-selective plates (SD/-Leu-Trp-His-Ade). The blue represents growth and interaction between PwHAP5 and PwFKBP. (C) Liquid  $\beta$ -galactosidase assay using *o*-NPG as a substrate.  $\beta$ -galactosidase activity is expressed in U (=nmol min<sup>-1</sup>). The values displayed are the average  $\beta$ -galactosidase activities for three individual double transformants, with standard deviations indicated by error bars.



**Fig. 5.** *In vivo* interaction of PwHAP5 with PwFKBP in the BiFC system. BiFC in *A. tumefaciens*-infiltrated tobacco (*N. benthamiana*) leaves was visualized by laser confocal microscopy. The laser-scanning confocal microscopy images show fluorescence (indicated by YFP) and merged images of double transformed tobacco leaves with the YFP<sub>N</sub>-PwHAP5 (full-length PwHAP5; H), N77 (N), and C130 (C), respectively) and PwFKBP12-YFP<sub>C</sub> fusions (YFP<sub>N</sub>-PwHAP5/ PwFKBP12-YFP<sub>C</sub>). PwHAP5 interacts with PwFKBP12 in the cytoplasm, and does not interact with the negative control PwTUA1 or empty vector. The YFP fluorescence of the positive control vectors, bZIP63-pSPYNE-35S and bZIP63-pSPYCE-35S, was detected only in the nucleus. Arrows indicate the nucleus. Bar 25  $\mu$ m.

showed that FKBP12-FK56 complexes can bind to and inhibit the activity of calcineurin, a Ca<sup>2+</sup> or calmodulin-dependent protein phosphatase. *PwHAP5* was induced by Ca<sup>2+</sup>, but not by boron, during pollen tube development (Fig. 2C), suggesting that *PwHAP5* is a Ca<sup>2+</sup>-responsive gene. Since Ca<sup>2+</sup> is a key factor for pollen tube growth and maintenance of polar growth (Li *et al.*, 1999), it is likely that PwFKBP12 is involved in the plant sexual reproduction process via interaction with the Ca<sup>2+</sup>-responsive gene



**Fig. 6.** Transient expression of *PwHAP5* or *PwFKBP12* in *P. wilsonii* pollen tube. The overexpression vectors *PwHAP5* or *PwFKBP12* fused to CHERRY or GFP, respectively, were constructed and expressed transiently alone or used to co-transform *P. wilsonii* pollen by microprojectile bombardment. The pollen-specific expression promoter *Lat52* was used to drive the expression of *PwHAP5* or *PwFKBP12* in pollen tube. Microscopic analysis of pollen tube expression was performed 24 h after gene transfer of *PwFKBP12* and *PwHAP5* in pollen. Transient expression of *PwHAP5* alone affects *P. wilsonii* pollen tube growth orientation, whereas both transient expression of *PwFKBP12* and co-expression of *PwHAP5* and *PwFKBP12* do not. Left panel: transmitted light images. Right panel: fluorescence images. The yellow fluorescence is the overlay of GFP and CHERRY proteins. The data were obtained from three independent experiments, and each condition was tested three times. Bars, 20  $\mu$ m.

*HAP5* or probably modulating Ca<sup>2+</sup> or Ca<sup>2+</sup>-related proteins or enzymes involved in pollen tube development and guidance in plants. Meanwhile, considering that FKBP12 may function as protein-folding chaperones, the possibility cannot be excluded that PwFKBP12 binds to other proteins to function in the process. Nevertheless, the hypothesis and the accurate signalling pathway related to HAP5 and FKBP12 involved in pollen tube development and orientation need detailed investigation in the future. However, the identification of *PwHAP5* and *PwFKBP12* in *P. wilsonii* presents an opportunity to identify downstream genes regulated by HAP5 mediating pollen tube guidance and fertilization, since pollen tube growth direction in the ovary determines ovule targeting and sperm delivery to the female gametophyte.

## Supplementary data

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1.** Transient suppression of *GFP* gene expression in *P. wilsonii* pollen by the pFGCLat52-based vector.

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