

RESEARCH PAPER

Overexpression of *PwTUA1*, a pollen-specific tubulin gene, increases pollen tube elongation by altering the distribution of α -tubulin and promoting vesicle transport

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Abstract

Tubulin genes are intimately associated with cell division and cell elongation, which are central to plant secondary cell wall development. However, their roles in pollen tube polar growth remain elusive. Here, a *TUA1* gene from *Picea wilsonii*, which is specifically expressed in pollen, was isolated. Semi-quantitative RT-PCR analysis showed that the amount of *PwTUA1* transcript varied at each stage of growth of the pollen tube and was induced by calcium ions and boron. Transient expression analysis in *P. wilsonii* pollen indicated that *PwTUA1* improved pollen germination and pollen tube growth. The pollen of transgenic *Arabidopsis* overexpressing *PwTUA1* also showed a higher percentage of germination and faster growth than wild-type plants not only in optimal germination medium, but also in medium supplemented with elevated levels of exogenous calcium ions or boron. Immunofluorescence and electron microscopy showed α -tubulin to be enriched and more vesicles accumulated in the apex region in germinating transgenic *Arabidopsis* pollen compared with wild-type plants. These results demonstrate that *PwTUA1* up-regulated by calcium ions and boron contributes to pollen tube elongation by altering the distribution of α -tubulin and regulating the deposition of pollen cell wall components during the process of tube growth. The possible role of *PwTUA1* in microtubule dynamics and organization was discussed.

Key words: Microtubules, *Picea wilsonii*, pollen tube, *PwTUA1*, transgenic *Arabidopsis*, α -tubulin.

Introduction

Three classes of fibres comprise the cytoskeleton of eukaryotic cells: microtubules (MTs), intermediate filaments, and microfilaments (MFs) (Kost and Chua, 2002). As an important component of the cytoskeleton of eukaryotic cells, MTs are involved in many crucial functions during cellular processes (Mayer and Jurgens, 2002), such as the maintenance of cell shape, cell motility, intracellular transport, cell division, and expansion (Mayer and Jurgens, 2002). Unusual MT arrays are found in cells undergoing tip growth such as pollen tubes, root hairs, and other specialized cells such as sperm cells (Pierson and Cresti, 1992). MT arrays of pollen tubes are approximately parallel to the direction of growth

(Derksen *et al.*, 1985; Lancelle *et al.*, 1987; Raudaskoski *et al.*, 1987) and persist during pollen tube growth, with new tubulin continuously added at nucleation sites near the tube apex (Heslop-Harrison and Heslop-Harrison, 1988). Recent identification of pollen-specific MT motor kinesin (Cai *et al.*, 2000; Romagnoli *et al.*, 2003) and MT-associated protein (Huang *et al.*, 2007) indicates that MTs may play a role, with the participation of actin filaments, in directing vesicle and organelle transport and the deposition of pollen cell wall components, and maintaining the shape of the pollen tube during pollen tube growth (Cai *et al.*, 2005). However, the role of MTs in pollen development remains elusive (Oakley

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et al., 2007), and is poorly understood compared with the function of MFs.

The α -tubulin (TUA) and β -tubulin (TUB) proteins, identified as the primary components of MTs (Weisenberg *et al.*, 1968), are continually reassembling according to developmental cues and environmental signals (Wasteneys, 2004). Recent experimental evidence indicates that post-translational modifications of the tubulin building blocks contribute to the functional diversity of MTs (Hammond, 2008). In most plants, tubulins are encoded by multigene families, and tubulin genes are generally regarded as housekeeping genes. However, recent research shows that the function of individual tubulin genes is distinct within species, organs, and even cells, and the existence of pollen-specific tubulin genes has been demonstrated in several species (Carpenter *et al.*, 1992; Cheng *et al.*, 2001; Yoshikawa *et al.*, 2003; Oakley *et al.*, 2007), suggesting an important role in pollen tube growth. For example, *AtTUA1* is expressed preferentially in pollen (Carpenter *et al.*, 1992) among the six α -tubulin genes of *Arabidopsis thaliana* (Kopczak *et al.*, 1992), while the other five are expressed in vegetative tissues (Kopczak *et al.*, 1992; Abe *et al.*, 2004). Of the nine *Arabidopsis* β -tubulin genes, *AtTUB9* accumulates specifically in pollen (Carpenter *et al.*, 1992; Cheng *et al.*, 2001). Rice (*Oryza sativa*) also contains one pollen-specific isoform (*OsTUB8*) and seven other *TUB* genes expressed variably during development (Yoshikawa *et al.*, 2003). Eight α -tubulin and 20 β -tubulin genes are expressed in the woody perennial *Populus tremuloides* Michx., of which *PtTUA6* and *PtTUA8* are abundant only in pollen (Oakley *et al.*, 2007). However, further functional analysis of tubulin genes and proteins in plant pollen tubes is lacking.

Vegetative pollen tube growth occurs only at the apex of the cell through the fusion of vesicles to the apical plasma membrane. Simultaneously, an intricate MT array assembles in the vegetative cell, forming a network with actin, the endoplasmic reticulum, and the plasma membrane (Lancelle *et al.*, 1987). The system in conifers differs from that of angiosperms in the growth rate of the pollen tube, the tendency to ramify, and the absence of a tip-to-base zonation of organelles (Win *et al.*, 1996). To our knowledge, all of the pollen-specific tubulin genes identified are currently attributed to angiosperms, and the possible roles of these genes during pollen development have been mainly investigated in *Arabidopsis*. Gymnosperms, an economically important plant group, represent a major evolutionary divergence with respect to the development of the male gametophyte (Fernando *et al.*, 2005). Little information exists about tubulin genes in gymnosperms, and whether current theories regarding angiosperms are valid remains questionable.

As part of a long-term aim to elucidate the complex functions of MTs during gymnosperm pollen development, mainly focusing on the roles of individual tubulin gene products in different MT arrays, here the characterization of a pollen-specific TUA from *Picea wilsonii* is reported. Semi-quantitative RT-PCR analysis revealed that the expression of *PwTUA1* is up-regulated by calcium ions and boric acid

treatments during pollen tube growth. Ectopic expression of *PwTUA1* in *Arabidopsis* suggested that *PwTUA1* not only improved pollen germination and pollen tube growth even in suboptimal pollen tube germination media, but also altered the subcellular localization of α -tubulin and the ultrastructure of the pollen tube. In addition, the possible functions of *PwTUA1* are discussed.

Materials and methods

Plant material

Cones with mature pollen were collected in mid April 2007 from mature trees of *P. wilsonii* Mast. in the Beijing Botanical Garden of the Institute of Botany, the 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

Picea 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 medium for germination. The standard medium for pollen germination and tube growth contained 12% sucrose, 0.03% $\text{Ca}(\text{NO}_3)_2$, 0.01% H_3BO_3 , and 5 mM citrate-phosphate buffer, pH 5.8. Pollen grains were incubated in small dishes at $25\pm 1^{\circ}\text{C}$ in a saturated atmosphere (100% relative humidity) and 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 by the standard CTAB (cetyltrimethylammonium bromide) extraction and lithium chloride precipitation as described previously (Chang *et al.*, 1993). The quality and quantity of RNA were assessed by agarose gel electrophoresis and UV spectroscopy, respectively.

Amplification and cloning of the PwTUA1 gene

Degenerate primers were designed based on conserved regions of TUA sequences from *Pseudotsuga menziesii*, *Gossypium hirsutum*, *Oryza sativa*, and *A. thaliana* (Table 1). Total RNA was isolated from *P. wilsonii* pollen after incubation (0, 6, 12, 18, 24, and 36 h) using Trizol reagent (Gibco-BRL). Reverse transcription of the pooled RNA was carried out with oligo(dT) primers using M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. Subsequently, PCR was performed with 35 cycles of 94°C for 1 min, annealing at 68°C for 1 min, and extension at 72°C for 1 min in a Tgradient (Biometra). After sequencing the specific PCR fragment, 5'- and 3'-RACE

Table 1. The primers used in this study

Primer	Purpose	Primer sequence(5' → 3')
F1	Full-length cDNA	GATGCA(T/C)TTCA(T)ACACA(T/C)TTC(T)TTC(T)G
R1	Full-length cDNA	AGGGCA(G)GCA(G/C)AGA(G)TCCTCACG
AAP	5'-RACE	GGCCACGCGTCTGACTAGTACG(G) ₁₄
B ₂₆	3'-RACE	GACTCGAGTTCGACATCGA(T) ₁₈
R2	5'-RACE	GAACCGAGACCAGAACCAGTTCC
F2	3'-RACE	CTGGAACACACCGATGTGGC
PwTUA1F	Full-length cDNA	GGACGACGAGCTGAGCCCTTCG
PwTUA1R	Full-length cDNA	CAAAACACGCGTTTCATTTA
EF1- α 5	RT-PCR	AACTGGAGAAGGAACCCAAG
EF1- α 3	PT-PCR	AACGACCCAATGGAGGATAC
FW	RT-PCR	TGCATGATCTCCAATTCGAC
RW	PT-PCR	GCACTGTTCTCAACATGAAG

(rapid amplification of cDNA ends) were performed to achieve full-length cDNA using the Gibco-BRL kit (Gibco-BRL).

Expression analysis

RNA from each tissue was treated with DNase I to remove genomic DNA, and then the cDNA was synthesized. RT-PCR was carried out with 30 cycles of denaturation, annealing, and extension steps for each sample. Gene primer pairs were designed to anneal near the 3' end of each transcript [usually in the 3'-untranslated region (UTR)] to ensure primer specificity. An elongation factor- α gene (*EF1- α*) was used as a standard control in the RT-PCRs. The PCR experiment has been carried out at least three times. The forward and reverse primers are shown in Table 1.

Particle bombardment-mediated transient expression in pollen and determination of pollen germination and tube growth

Mature pollen grains collected from *P. wilsonii* plants as described in 'In vitro *P. wilsonii* pollen germination' were used for transient expression using a particle bombardment procedure. Microprojectile bombardment was performed using a helium-driven PDS-1000/He biolistic system (Bio-Rad, Hercules, CA, USA). Tungsten particles (1.1 μ m) were coated with plasmid DNA according to the manufacturer's recommendation (Bio-Rad) (Sanford *et al.*, 1993). The pollen grains were then incubated for 12 h or 24 h before observation under a fluorescence microscope (Olympus BX50); 100 tubes were counted and measured for each sample. 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 a fluorescence microscope (Olympus BX50). Fluorescence images used to analyse pollen tube length (using image analysis software: IMAGE) were taken with exposure times of <500 ms.

Arabidopsis transformation

The pBI121 binary vector containing *35S::PwTUA1* or *Lat52::GFP* was introduced into *Agrobacterium tumefaciens* strain GV3101 and the wild-type *Arabidopsis* plants were transformed by floral dipping (Clough and Bent, 1998). The transgenic plants were screened on MS medium containing 50 μ g ml⁻¹ kanamycin. T₀ transgenic *Arabidopsis* plants were identified by PCR to amplify the *PwTUA1* gene with specific primers. The corresponding T₁ transgenic seedlings that segregated at a ratio of ~3:1 (resistant:sensitive) were selected to propagate T₂ individuals, which were used for further analysis.

Arabidopsis pollen germination and tube growth measurement

Arabidopsis pollen grains were germinated *in vitro* and tube growth was measured using a modification of the method of Li *et al.* (1999).

Indirect immunofluorescence microscopy

For subcellular immunolocalization, the method described by Lin and Yang (1997) was used. Briefly, pollen tubes were incubated in fixative [2% paraformaldehyde in phosphate-buffered saline (PBS), comprising 10 mM phosphate buffer, pH 7.2, 138 mM NaCl, 2.7 mM KCl] for 1 h. After washing with PBS, pollen tubes were incubated at room temperature for 10 min in a solution of 1.5% cellulase R-10 and 1.2% macerozyme R-10 in 15 mM MES, pH 5.5, 400 mM mannitol, 5 mM CaCl₂, and 1 mg ml⁻¹ proteinase inhibitors (aprotinin, pepstatin A, chymostatin, and leupeptin; Calbiochem). After washing with PBS, pollen tubes were blocked with 3% bovine serum albumin (BSA) in PBS at room temperature for 1 h and then incubated at 4 °C overnight with purified anti- α -tubulin mouse monoclonal antibody (1:50 diluted with 1% BSA in PBS; Calbiochem). After washing in PBS containing 0.05% Triton X-100, pollen tubes were incubated with a secondary antibody (fluorescein isothiocyanate-conjugated, affinity-purified goat-mouse IgG) at 1:100 dilution in PBS containing 1% BSA for 1 h at 37 °C. As controls, pollen tubes were stained as above except for the omission of the primary antibody. Observations and photography were obtained with a confocal microscope (LSM 510 META, Zeiss).

Protein extraction and western blotting

Arabidopsis and *P. wilsonii* pollen grains were germinated *in vitro*. Proteins were extracted using the method of Poulter *et al.* (2008). Samples were measured using the Bio-Rad protein assay; equal amounts were loaded on a 12% SDS-polyacrylamide gel and separated by electrophoresis. For western blotting, the proteins were electrotransferred onto a nitrocellulose membrane (0.45 mm, Amersham Pharmacia Biotech Ltd). The membrane was blocked for 1 h at room temperature with 3% (w/v) BSA and 15% (v/v) calf serum in TRIS-buffered saline (TBS; 25 mM TRIS-HCl, pH 7.5,

137 mM NaCl, 5 mM KCl) and then incubated with gentle shaking for 2 h at room temperature in mouse anti- α -tubulin antibody diluted 1:500 (Calbiochem) in blocking buffer. After three washes of 5 min each in TBS, the membrane was incubated with alkaline phosphatase-conjugated secondary antibody raised against mouse IgG diluted 1:1000 in blocking buffer. The membrane was stained with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

Electron microscopy

For electron microscopy, pollen tubes were fixed for 2 h in 2.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.2) with 2% (w/v) sucrose. Samples were washed with 100 mM phosphate buffer, post-fixed in 2% osmium tetroxide for 2 h, dehydrated in an ethyl-alcohol series, and finally embedded in Spur resin. Semi-thin sections were cut and the pollen tubes were localized. Sections were cut using an LKB-V ultramicrotome, and examined with a JEM-1200 electron microscope (JEOL Ltd, Tokyo, Japan).

Results

Isolation and characterization of the cDNA clone encoding α -tubulin from P. wilsonii

The TUA1 cDNA fragment was obtained from *P. wilsonii* by RT-PCR using primers designed according to conserved regions of known plant TUAs. After extension by 5'- and 3'-RACE-PCR, the complete open reading frame (ORF) was obtained. This gene was designated as *PwTUA1* with

the GenBank accession number EU268195. The full-length cDNA sequence was 1724 bp and contained an ORF of 1356 bp that tentatively encoded a PwTUA1 protein of 451 amino acids with a calculated mol. wt of 49.6 kDa and an isoelectric point (pI) of 4.97. A 207 bp 3'-UTR followed the stop codon (TGA) and a 161 bp 5'-UTR was upstream from the start codon (ATG). A BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST>) and multialignment analysis revealed that PwTUA1 was highly related to other plant TUAs, sharing homology of 88.47% with AtTUA1, 97.78% with GhTUA1, 96.9% with OsTUA1, and 99.78% with PmTUA1 (Fig. 1). The predicted protein sequence contained a GTP nucleotide-binding site (GGGTGSG), which was conserved and necessary for the polymerization of α - β -tubulins (Kirschner and Mitchison, 1986). An MRECI domain, implicated in autoregulated post-transcriptional mechanisms (Yen et al., 1988a, b; Cleveland, 1989), was found at the N-terminus of PwTUA1. Phosphorylated residues were found at R79 and K336, and an acetylated residue at K40. PwTUA1 contained a C-terminal glutamic acid residue (referred to as an E-type), which was different from the more typical α -tubulins that contain a tyrosine at the C-terminus.

Phylogenetic analysis of TUAs

To elucidate the evolutionary relationships among TUA proteins, a phylogenetic tree was generated in which representative algal and plant full-length TUA proteins were analysed (Fig. 2). The bootstrap values for most nodes were low (data not shown) because of the high degree of sequence similarity. Plant TUAs make up two distinct

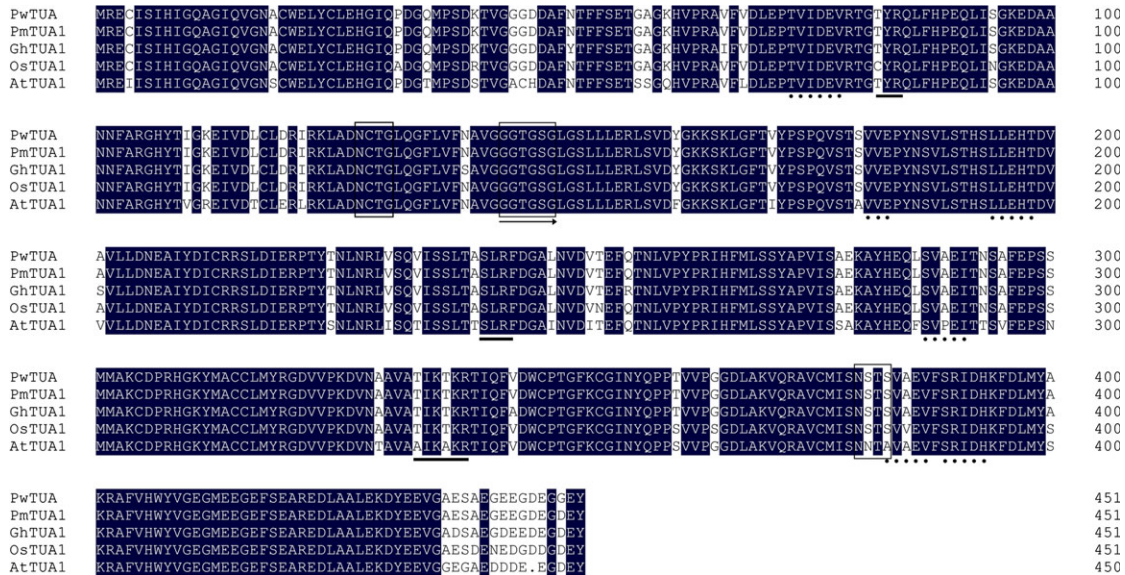


Fig. 1. Alignment of the deduced PwTUA1 protein sequence (EU268195) with other plant TUA1s. Amino acid sequences used in the analysis are from *Pseudotsuga menziesii* (PmTUA1, AAV92379), *Gossypium hirsutum* (GhTUA1, AY345603), *Oryza sativa* (OsTUA1, Os03g0726100), and *Arabidopsis thaliana* (AtTUA1, At1g64740). Identical amino acid residues in this alignment are shaded in black. Possible phosphorylation sites were obtained with a PROSITE motif search: boxed amino acids are N-glycosylation sites, underlined amino acids are protein kinase C phosphorylation sites, amino acids underlined with dotted lines are casein kinase II phosphorylation sites, and the arrow indicates tubulin subunits α , β , and γ signatures.

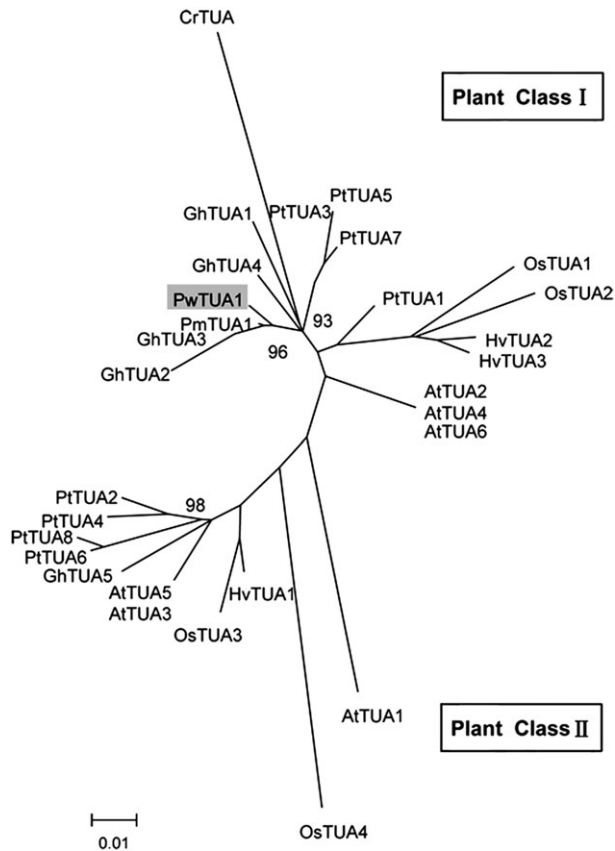


Fig. 2. Phylogenetic tree of *Picea wilsonii* α -tubulin 1 (*PwTUA1*) and previously characterized α -tubulins. A Neighbor-Joining tree based on the deduced amino acid sequences of the TUAs. *CrTUA* was used as an outgroup. This bootstrap consensus tree was based on 1000 replicates. Numbers on nodes are bootstrap values. The accession numbers in GenBank and sources of the proteins are as follows: *AtTUA1* (At1g64740), *AtTUA2* (At1g50010), *AtTUA3* (At5g19770), *AtTUA4* (At1g04820), *AtTUA5* (At5g19780), and *AtTUA6* (At4g14960) from *Arabidopsis thaliana*; *CrTUA* (AY182002) from *Chlamydomonas reinhardtii*; *GhTUA1* (AY345603), *GhTUA2* (AY345604), *GhTUA3* (AF106569), *GhTUA4* (AY345605), and *GhTUA5* (AF106571) from *Gossypium hirsutum*; *HvtTUA1* (X99623), *HvtTUA2* (Y08490), and *HvtTUA3* (AJ132399) from *Hordeum vulgare*; *OsTUA1* (Os03g0726100), *OsTUA2* (Os11g0247300), *OsTUA3* (Os07g0574800), and *OsTUA4* (Os03g0219300) from *Oryza sativa*; *PmTUA1* (AY832610) from *Pseudotsuga menziesii*; and *PtTUA1* (AY229881), *PtTUA2* (AY229882), *PtTUA3* (EF583813), *PtTUA4* (EF584828), *PtTUA5* (EF583814), *PtTUA6* (EF583816), and *PtTUA7* (EF584829) from *Populus tremuloides*.

classes. Although angiospermous TUAs exist in both class I and class II, the known gymnospermous TUAs are currently attributed to class I. Class I contains TUAs involved in secondary cell wall development, including *PtTUA1* originating from *Populus* xylem and fibre-specific *GhTUA2*, *GhTUA3*, and *GhTUA4* from cotton. Class I also includes a third distinct branch containing three *Arabidopsis* isoforms (*AtTUA2/4/6*). In class II, *AtTUA1* (Carpenter *et al.*, 1992), *PtTUA6*, and *PtTUA8* (Oakley *et al.*, 2007) were found to be preferentially expressed in pollen.

The expression of *PwTUA1* is specific to pollen and is up-regulated by Ca^{2+} and boron

To obtain a profile of *PwTUA1* expression patterns, total RNA was isolated from needles, stems, and roots, as well as from germinating *P. wilsonii* pollen mixtures incubated at 6 h intervals, and transcript accumulation was examined by semi-quantitative RT-PCR using gene-specific primers (Table 1). The RW primer was designed based on the 3'-UTR downstream from the stop codon, as this region was hypervariable. The results showed that *PwTUA1* was expressed specifically in germinating pollen but was not detected in needles, stems, and roots (Fig. 3A). RT-PCR was next used to examine the expression of *PwTUA1* during pollen development. As shown in Fig. 3B, the expression of *PwTUA1* in pollen was first detected at 6 h after incubation (just germinated); it was abundant during the middle developmental stages (12, 18, and 24 h after incubation) and then continued weakly at later stages (30 h and 36 h after incubation).

Boron was reported to have effects on pollen grain germination and pollen tube growth in *Picea meyeri* (Wang *et al.*, 2003) and several angiosperm species (Dickinson, 1978). It was speculated that boron acts as an essential element in the induction or repression of *PwTUA1* expression. Gymnosperm pollen germination and tube growth were previously reported to be severely inhibited by 0.1% H_3BO_3 (Wang *et al.*, 2003) and 0.1% $CaCl_2$ in the medium (Hao *et al.*, 2005), respectively. Given that Ca^{2+} has been appreciated for many years as an essential requirement for pollen tube growth (Franklin-Tong, 1999), the level of *PwTUA1* expression was evaluated during different pollen tube developmental stages in boron-stressed (H_3BO_3 concentration, 0.1%) and Ca^{2+} -stressed medium (Ca^{2+} concentration, 0.1%). As shown in Fig. 3C, *PwTUA1* was effectively induced by boron and Ca^{2+} during all phases tested. These results indicated that *PwTUA1* was a boron/ Ca^{2+} -responsive gene that may reasonably be expected to contribute adaptively to pollen development including pollen germination, pollen tube growth, and orientation.

Transient expression of PwTUA1 improves pollen germination and pollen tube growth in P. wilsonii

Microprojectile bombardment was performed to investigate the role of *PwTUA1* in pollen germination and pollen tube growth. Considering that green fluorescence protein (GFP) fused to α -tubulin could affect the polymerization of α -tubulin and β -tubulin directly, *PwTUA1* 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 growth rate between the samples transformed with GFP and those left untreated (Fig. 4A, B), suggesting that neither the microprojectile bombardment itself nor the GFP had any significant effect on pollen germination and pollen tube growth. GFP expression acted as an indicator of co-transformed pollen tubes as it had previously been established that the level of transgene expression is linearly

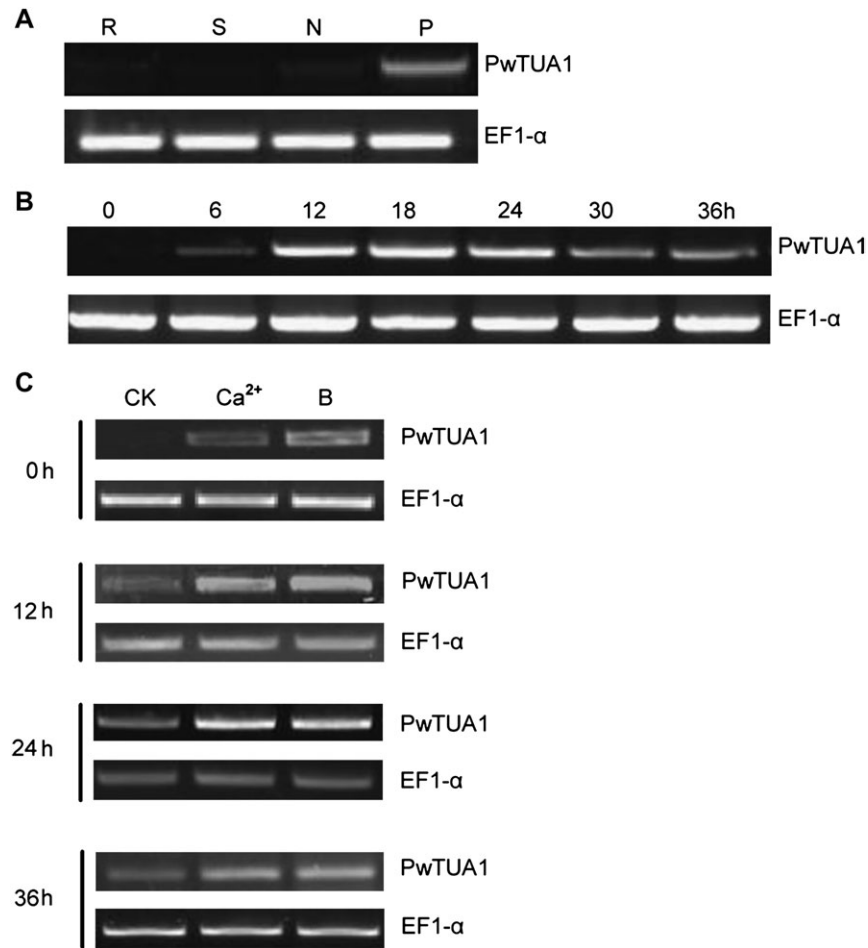


Fig. 3. Expression of *PwTUA1* in different tissues of *Picea wilsonii*. (A) Tissue-specific expression of *PwTUA1* in *P. wilsonii*. Total RNA was isolated from needles (N), stems (S), roots (R), and pollen (P) (incubated for 0, 6, 12, 18, and 24 h). (B) Expression of *PwTUA1* in pollen at different growth stages. (C) *PwTUA1* gene expression induced by 0.1% (w/v) Ca^{2+} and 0.1% (w/v) H_3BO_3 at different time points.

correlated with the amount of input DNA (Chen *et al.*, 2002). All pollen grains were co-bombarded with 5 μ g of *Lat52::GFP* as a marker for transformation and to assess for comparable transgene expression in all samples. Less than 5 μ g of experimental chimeric gene was used in the samples, and *Lat52- β -glucuronidase* (*GUS*) DNA was added to make up to 10 μ g of total DNA used for every transformation. This ensured that equal amounts of DNA were coated onto the tungsten particles and introduced into the pollen samples. The data presented in Fig. 4A and B show that increasing amounts of *Lat52::PwTUA1* introduced into these pollen tubes were associated with gradually increasing pollen germination percentages and pollen tube growth. The length of pollen tubes growing for 24 h was increased by the overexpression of *PwTUA1*: the average length was $239.1 \pm 24 \mu$ m ($n \geq 100$) at lower *PwTUA1* concentrations and $328.7 \pm 33 \mu$ m at higher *PwTUA1* concentrations, which was significantly longer than the tubes transformed with *GFP* ($197.6 \pm 20 \mu$ m) (Fig. 4C). No significant difference was seen in the pollen tube length at 12 h compared with 24 h, indicating that expression of *PwTUA1* exerted its greatest effect in the first 12 h (Fig. 4B). Although

the percentage germination of each sample at 12 h was lower than that at 24 h (Fig. 4A), as pollen germination is related to the time of incubation in *P. wilsonii*, the percentage pollen germination increased significantly with increasing amounts of *Lat52::PwTUA1*. These results showed that overexpression of *PwTUA1* could improve pollen germination and accelerate pollen tube growth in *P. wilsonii*.

PwTUA1 overexpression accelerates pollen tube growth and early germination in transgenic *Arabidopsis*

To investigate the function of *PwTUA1* during pollen development, this gene was ectopically expressed in *Arabidopsis* pollen exclusively by using the pollen-specific promoter *Lat52*. The development of transgenic *Arabidopsis* pollen was consistent with the results observed for the transformed *P. wilsonii* pollen grains. Both pollen germination and pollen tube growth were enhanced in the transgenic lines when compared with wild-type *Arabidopsis*, even at the non-optimal boron or Ca^{2+} concentrations present in the culture medium (Figs 5, 6A). Pollen expressing the *PwTUA1* gene exhibited earlier germination than wild-type

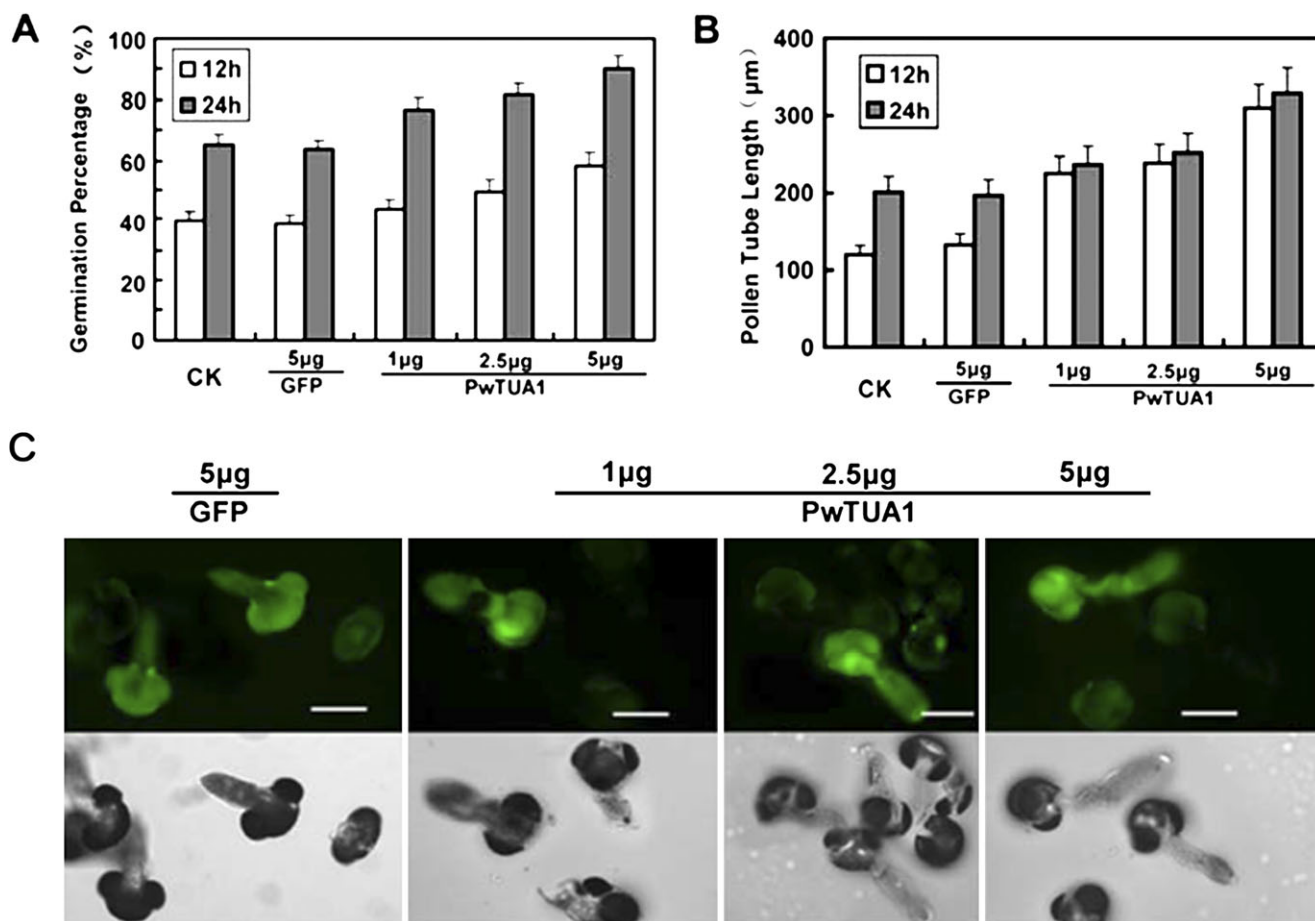


Fig. 4. Transient expression of *PwTUA1* improves *Picea wilsonii* pollen germination and pollen tube growth. (A) The effect of *PwTUA1* on pollen germination using microprojectile bombardment. After 12 h and 24 h of incubation, the percentage germination of pollen bombarded with the *GFP*-only construct showed no significant difference compared with the untreated control pollen ($P < 0.05$). Significant differences were observed in the percentage of germination of pollen bombarded with 2.5 µg and 5 µg of the *PwTUA1* gene construct compared with untreated pollen or *GFP*-transformed pollen ($P < 0.05$). Error bars indicate averages from the number of pollen tubes measured ($n \geq 81$). (B) The effect of *PwTUA1* on pollen tube length using microprojectile bombardment. After 12 h and 24 h of incubation, no significant differences were detected in the length of pollen tubes bombarded with *GFP* compared with untreated pollen tubes ($P = 0.47$). Significant differences were seen in the length of pollen tubes bombarded with 5 µg of *PwTUA1* compared with untreated pollen or *GFP*-transformed pollen ($P < 0.05$). Error bars indicate averages from the number of pollen tubes measured ($n \geq 65$). (C) Microscopic analysis of pollen tube co-expression 24 h after gene transfer of different doses of *PwTUA1* with *GUS* and *GFP*. Upper panel: fluorescence images. Lower panel: transmitted light images. The data were obtained from three independent experiments, and every condition was tested three times. Bars = 100 µm.

pollen exposed to 0.1% H_3BO_3 (w/v), but resulted in the same germination rate as observed for wild-type pollen. However, pollen germination of the transgenic and wild-type plants was severely and completely inhibited in 1% H_3BO_3 (data not shown). Furthermore, the pollen tube growth rate of the wild-type plants showed a significant decrease compared with that of transgenic plants. The mean growth rate of wild-type plant pollen tubes was $16.7 \mu\text{m h}^{-1}$ compared with $35.6 \mu\text{m h}^{-1}$ for pollen tubes of transgenic plants cultured in medium containing 0.1% H_3BO_3 (Fig. 6A). In the above medium, the concentration of Ca^{2+} was 0.2 mM.

The effect of varying Ca^{2+} influxes on pollen tube growth of the transgenic plants was further determined. Pollen tubes were grown on medium containing various amounts

of Ca^{2+} . Optimal growth of wild-type *Arabidopsis* pollen tubes required nearly 2 mM extracellular Ca^{2+} (Li *et al.*, 1999). Pollen from wild-type plants and transgenic plants homozygous for the *PwTUA1* gene was germinated on medium containing 0, 2, 10, or 20 mM Ca^{2+} . The percentage of pollen germination and tube elongation rates of transgenic plants were higher than those of wild-type plants when extracellular Ca^{2+} levels increased from 0 mM to 20 mM (Fig. 6B, C). The pollen tubes of wild-type plants showed significantly slower growth rates than transgenic plants when the extracellular Ca^{2+} concentrations were < 10 mM (Fig. 6C). Note that when 0.1 mM EGTA (a chelating agent with a high affinity for Ca^{2+}) was added into the medium, almost none of the wild-type pollen germinated. In contrast, the mean pollen germination

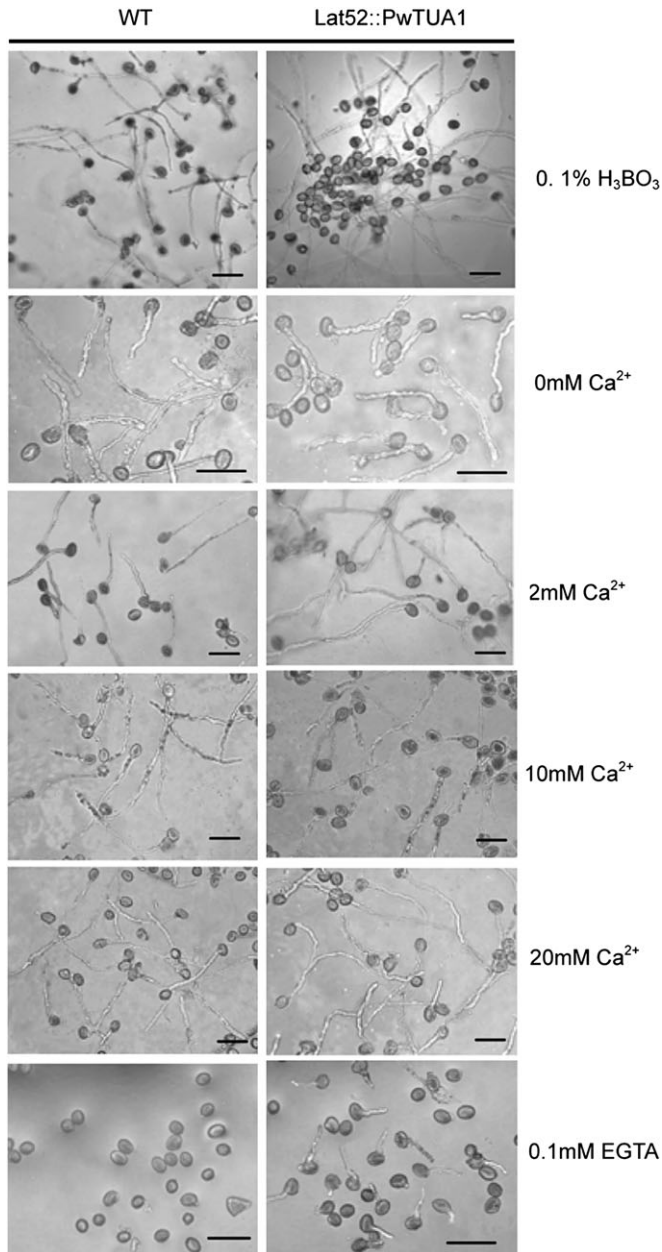


Fig. 5. Pollen tubes of wild-type (WT) and transgenic *Arabidopsis* cultured in different media. Pollen from wild-type *Arabidopsis* and transformants was germinated on medium containing 0.1% H_3BO_3 , different concentrations of Ca^{2+} , and 0.1 mM EGTA for 6 h and examined under a light microscope. Bars=50 μm .

percentage of the transgenic plants reached 21% (Fig. 5). These observations suggest a general and important role for *PwTUA1* in the regulation of pollen development.

PwTUA1 overexpression changes the subcellular localization of the α -tubulin protein in *Arabidopsis* pollen tubes

The subcellular localization of α -tubulin in pollen tubes of wild-type and transgenic *Arabidopsis* was investigated using anti- α -tubulin serum and indirect immunofluorescence microscopy. A series of experiments conducted at different

growth stages showed that the α -tubulin protein existed throughout the tubes of wild-type *Arabidopsis*, excluding the tip or apical region (Fig. 7A). However, highly intense fluorescence was observed in the apical region of transgenic *Arabidopsis* pollen tubes, and the fluorescence in the pollen grains and pollen tubes exhibited decreasing intensities compared with that observed in the tube tips (Fig. 7A) in accordance with the observations in *P. wilsonii* pollen tubes (data not shown) as well as the findings reported for pollen tubes in several other gymnosperm species (Lazzaro, 1999). Note that the distribution of MTs in transgenic *Arabidopsis* pollen tubes was very different from that in angiospermous pollen tubes (Pierson and Cresti, 1992; Li et al., 1997), where MTs are absent in the tip regions. Moreover, no fluorescence was found in any of the controls not treated with the antiserum or pre-immune serum controls, indicating that the antiserum was specific and that non-specific labelling was negligible.

PwTUA1 overexpression affects the ultrastructure of pollen tubes in transgenic *Arabidopsis*

As the *PwTUA1* gene not only improved pollen germination and tube growth, but also altered the distribution of α -tubulin protein in transgenic *Arabidopsis*, the ultrastructure of the pollen grains and tubes was investigated. Note that the pollen tube wall of transgenic *Arabidopsis* was only slightly detectable (Fig. 8F), while the wall of wild-type *Arabidopsis* was much thicker (Fig. 8E). Additionally, many short, solitary cytoplasmic MTs were observed in the tip-most portion of the transgenic pollen tubes where the vesicles accumulated (Fig. 8D), but were rarely observed in the tip region of wild-type pollen tubes (Fig. 8C). It was also determined that more lipid bodies were in the wild-type pollen tube apex than in transgenic pollen, while many more vacuoles were in the transgenic pollen tube apex compared with wild-type plants (Fig. 8C, D).

Discussion

Controversy exists regarding the role of MTs during pollen tube growth. MTs have been suggested not to be strictly required for tube extension because the depolymerization of MTs by specific inhibitors did not inhibit the rate of pollen tube growth to the same extent (Mascarenhas, 1993; Åström et al., 1995). In contrast, MTs have been reported to be essential in imposing the typical tubular shape and directionality upon expansion in *Pinus* (Terasaka and Niitsu, 1994). However, research on the function of MTs has focused on protein depolymerization rather than gene transcriptional regulation during pollen germination and tube growth.

PwTUA1 is a pollen-specific gene and induced by Ca^{2+} and boron

In the present study, a full-length α -tubulin cDNA was obtained from *P. wilsonii* pollen using PCR and RACE.

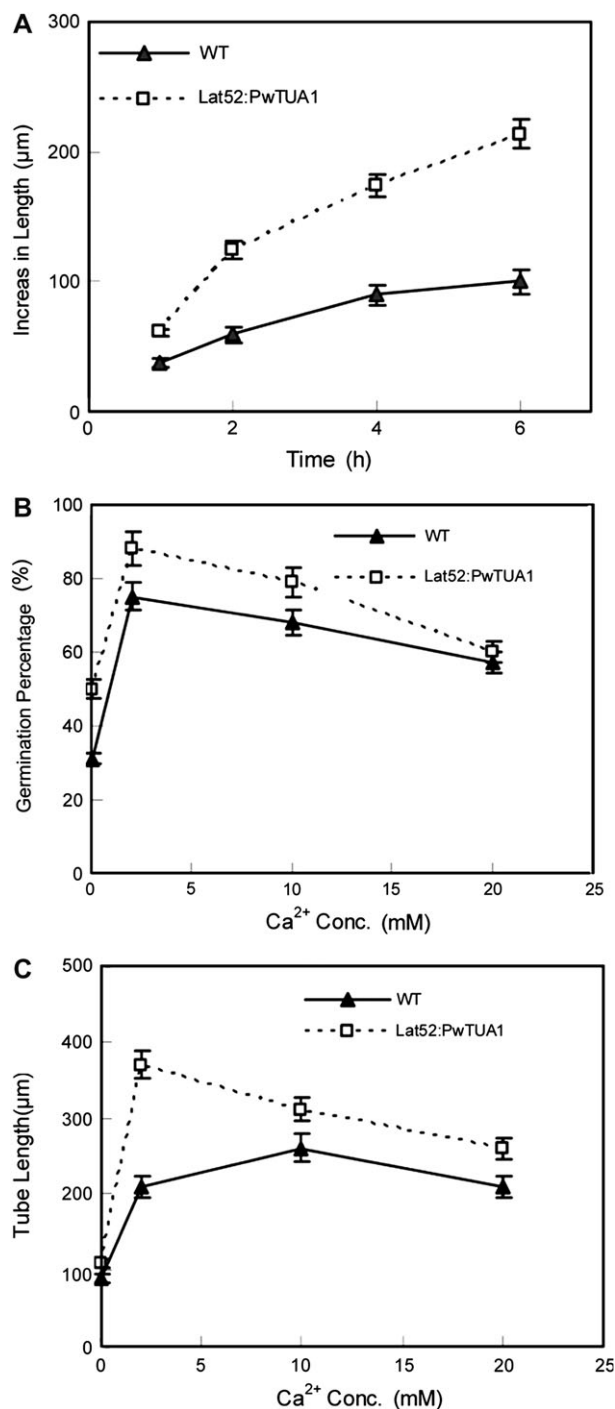


Fig. 6. Effects of *PwTUA1* overexpression on plant pollen germination and pollen tube growth exposed to different concentrations of boron and Ca^{2+} . (A) Comparison of tube growth of wild-type (WT) and transgenic plant pollen cultured in medium with 0.1% H_3BO_3 for 6 h. Only the pollen tubes of germinated pollen grains were measured. (B) *Arabidopsis* pollen was incubated in the germination medium with the indicated amounts of Ca^{2+} . After 6 h of incubation, the percentage of germinating pollen grains was determined. To establish the pollen germination percentage, the percentage germination of pollen grains in at least 10 aliquots per treatment was determined and experiments were repeated three times. (C) Statistical analysis of pollen tube length after 6 h of incubation. Only the pollen tubes of germi-

Amino acid sequence alignment showed that the deduced *PwTUA1* protein had high identity with other previously characterized plant α -tubulins. In addition, important evolutionarily and functionally related structural features were conserved (Fig. 1). Note that the C-terminal glutamate residue of *PwTUA1* is thought to be involved in the

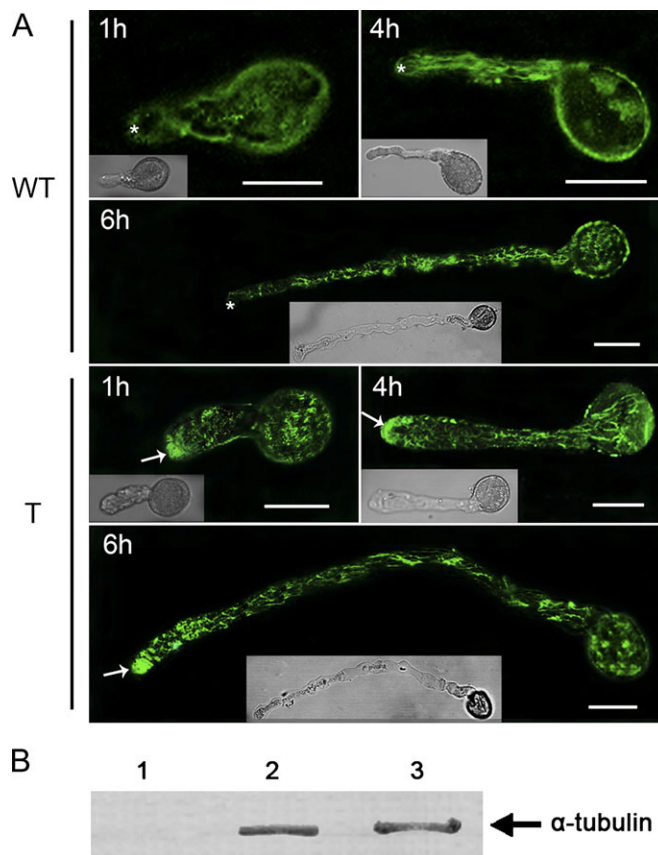


Fig. 7. Subcellular localization of the α -tubulin protein in *Arabidopsis* pollen tubes. *In vitro* cultured pollen tubes were fixed, incubated with anti- α -tubulin antiserum, and detected with goat anti-mouse secondary antibody as described in the Materials and methods. The fluorescence of stained pollen tubes was observed under a confocal microscope. (A) Fluorescence from immunolabelling of *Arabidopsis* pollen tubes harvested at 1, 4, and 6 h after germination. WT indicates pollen of wild-type *Arabidopsis*; T indicates pollen of transgenic *Arabidopsis*. The transgenic tubes tip (arrows) were strongly labelled with antibody for α -tubulin, whereas wild-type pollen tubes showed a much weaker signal in the apical region (asterisks). These data were obtained from three independent experiments, and every condition was tested three times. Bars=20 μm . (B) Western blot of extracts (equal loading of samples) from *Arabidopsis* pollen tubes (lane 2) and *P. wilsonii* pollen tubes (lane 3) probed with anti- α -tubulin antibody. Lane 1 is the blank control.

nated pollen grains were measured. Average lengths of >100 tubes from each line are shown. The experiments were repeated three times, and consistent results were obtained in all repeat experiments.

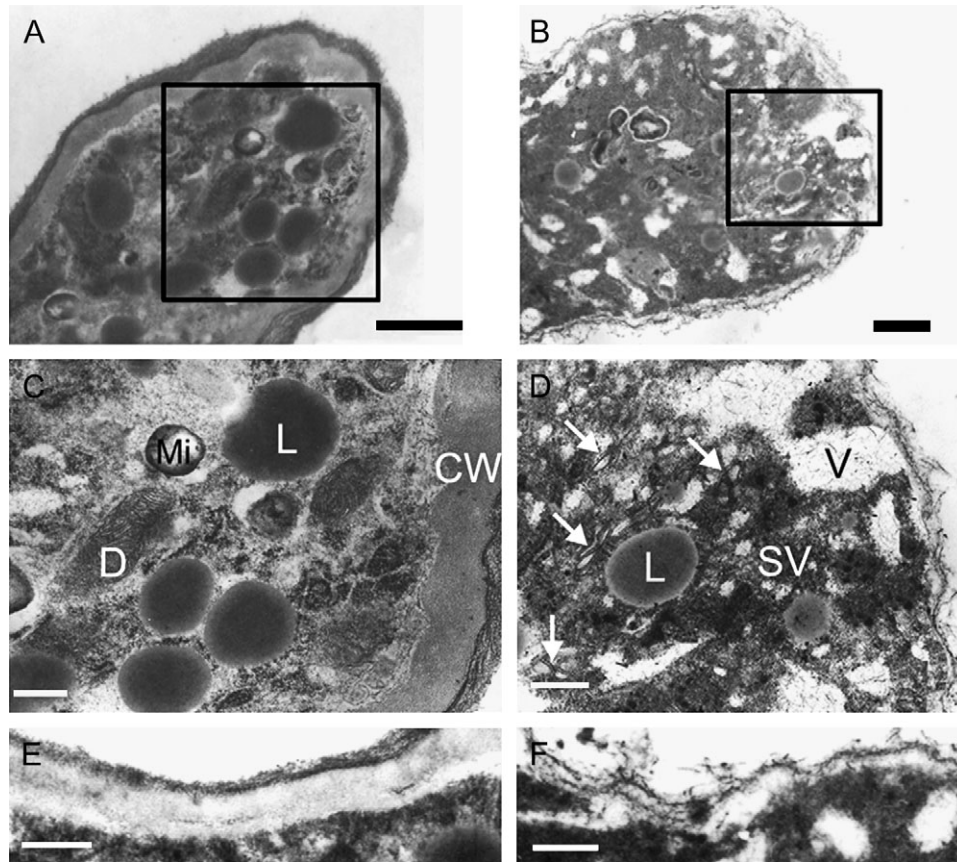


Fig. 8. Effects of *PwTUA1* on the ultrastructure of pollen tubes in transgenic *Arabidopsis*. Pollen from wild-type and transgenic plants was cultured *in vitro* for 6 h. Bars=0.2 μm (A, B, E, F) or 0.5 μm (C, D). (A) Tip region of wild-type pollen tube showing organelles at the tip. (B) Tip region of a pollen tube expressing *PwTUA1*; MTs (arrowhead) appeared and the number of vacuoles and secretory vesicles increased. (C and D) Magnified images of boxed sections in A and B. (E) Pollen tube wall of wild-type *Arabidopsis*. (F) Pollen tube wall of transgenic *Arabidopsis*. For *Arabidopsis* expressing *PwTUA1*, analyses of two independent transgenic lines showed a similar ultrastructure of pollen tubes. Data from one transgenic line were used. CW, cell wall; Mi, mitochondria; D, dictyosome; SV, secretory vesicle; V, vacuole; L, lipid body.

detyrosination reaction, which is a required event in animal cell proliferation (Phung *et al.*, 2006). This is in contrast to the angiospermous pollen-specific and pollen-preferential TUAs that contain a typical C-terminal tyrosine (Oakley *et al.*, 2007). Furthermore, analysis of the phylogenetic tree shows that *PwTUA1* is more closely related to the α -tubulins involved in secondary cell wall development than to the α -tubulins from other angiospermous species that are expressed in pollen preferentially. However, in contrast to observations that *AtTUA1* exhibited low levels of expression in other tissues (Carpenter *et al.*, 1992) and that *PtTUA6/8* was detected in roots in addition to pollen (Oakley *et al.*, 2007), the research demonstrated that *PwTUA1* was expressed specifically in pollen only (Fig. 3A).

It has been demonstrated that boron and Ca^{2+} are essential for pollen germination and tube growth (Dickinson, 1978; Franklin-Tong, 1999; Wang *et al.*, 2003). The present results showed that *PwTUA1* was up-regulated by Ca^{2+} /boron (Fig. 3C), and *PwTUA1* overexpression improved pollen germination and tube growth both in *P. wilsonii* and in *Arabidopsis* (Figs 4–6). It is hypothesized that boron and Ca^{2+} promote pollen germination and tube growth through

up-regulating the expression of *PwTUA1*. However, pollen germination and tube growth did not show consistent promotion with increasing concentrations of Ca^{2+} or boron (data not shown). This is due to the complicated signalling pathways regulated by Ca^{2+} /boron to promote pollen germination and tube growth. As one of these multiple complex pathways, up-regulated expression of *PwTUA1* by Ca^{2+} /boron just plays a part in this process.

PwTUA1 contributes to pollen tube growth based on altering the distribution of α -tubulin and ultrastructure of pollen tubes

By indirect immunofluorescence and electron microscopy, it was confirmed that a relationship exists between the cellular structure of germinating pollen and *PwTUA1* expression. In most angiosperms, a common feature of all pollen tubes is the absence of MTs from the actual tip region and the short and randomly oriented MTs in the subapical region (Raudaskoski *et al.*, 2001). In contrast, gymnosperm MTs have been reported to be enriched at the elongating tip, where they form an array beneath the plasma membrane

that is perpendicular to the direction of tube growth (Lazzaro, 1999). Overexpression of the *PwTUA1* gene alters the distribution of α -tubulin in germinating transgenic *Arabidopsis* pollen, which is enriched in the apex region instead of being absent altogether (Fig. 7). With the occurrence of short, solitary cytoplasmic MTs near the pollen tube apex region in transgenic *Arabidopsis* plants (Fig. 8), the existence of α -tubulin in the tube tip may contribute to the distribution and structural stability of pollen tube MTs in concert with microtubule-associated proteins (MAPs), actin filaments, or other protein factors.

In most plant species, the pollen tube cell wall consists of two layers, the inner sheath of callose and outer coating containing mainly pectin with cellulose and hemicellulose. The pollen tube grows exclusively at its tip, where the newly synthesized cell wall is continually forming (Taylor and Hepler, 1997; Krichevsky *et al.*, 2007). The digestion of pectin at the apex is sufficient to destabilize the cell wall, with the result that the turgor was able to expand the wall (Parre and Geitmann, 2005). In the present study, the cell wall of the tip region mainly composed of pectin was infrequently observed in the apex of transgenic pollen tubes (Fig. 8). This result was consistent with the observation that the earliest visible cell wall alteration upon emergence of the pollen tube is a loss of pectic material (Taylor and Hepler, 1997). Additionally, MTs contribute to the deposition of pollen cell wall components (Cai *et al.*, 2005) and, in conifers, the MTs at the tip may orient the deposition of cellulose microfibrils (Lazzaro, 1999). Taken together, it is suggested that *PwTUA1* plays a crucial role in regulating the deposition of pollen cell wall components in such a manner that it was able to promote pollen germination and tube growth.

In *Nicotiana tabacum* (Derksen *et al.*, 1995) and *Lilium longiflorum* pollen tubes (Lancelle *et al.*, 1997), the cell wall of the tip extremity has been demonstrated to be slightly thicker than the cell wall below the tip region, and the former region shows slow expansion, whereas the maximum growth rate occurs in the thin wall layer region below the tip extremity (Raudaskoski *et al.*, 2001). Visualization studies (Fig. 8) show directly that the pollen tube wall of transgenic *Arabidopsis* plants is thinner than that of the *Arabidopsis* plants not expressing *PwTUA1*. Considering that *PwTUA1* improves pollen tube growth in transgenic *Arabidopsis*, it is postulated that fast elongation preferentially takes place in regions where the tube wall is thinner. The balance between intracellular turgor pressure (hydrostatic pressure, reflected by the cell volume) and cell wall loosening is a critical factor driving pollen tube growth (Zonia *et al.*, 2006). The thinner pollen tube cell wall observed in *Arabidopsis* plants overexpressing *PwTUA1* suggests that cell wall rigidity of transgenic pollen tubes may decrease compared with wild-type *Arabidopsis*. Thus, the transgenic *Arabidopsis* pollen tubes displayed a faster growth rate than wild-type pollen tubes *in vitro*.

Pollen tubes grow by the deposition of cell wall components at the tip (Derksen *et al.*, 1995; Taylor and Hepler, 1997), and these cell wall components are carried by

vesicles, which are transported to the tip via highly active cytoplasmic streaming and incorporated into a zone of elongation in the apical dome of the pollen tube tip (Franklin-Tong, 1999). In gymnosperms, the cell wall may develop in the terminal 20 nm but may be mature in older parts of the tube (Lazzaro, 1999). Thus, the tube wall is not always thick in the region where cell wall components deposit. Within the transgenic *Arabidopsis*, vesicles accumulated in the tip region of pollen tubes were frequently observed. In contrast, empty compartments were hardly ever detected in the wild-type *Arabidopsis* tube tip. These results suggest that the contribution of *PwTUA1* to pollen tube growth is supported by the presence of vesicles localized in the tip of transgenic *Arabidopsis* pollen tubes.

Taken together, all these observations suggest that *PwTUA1* expression plays an important role in maintaining rapid pollen tube growth. However, at present, the mechanism underlying *PwTUA1* gene involvement in the structure of MTs cannot be explained. To address the question conclusively, more experimental evidence is needed.

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