



# Characterization and functional analysis of pollen-specific *PwSWEET1* in *Picea wilsonii*

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**Abstract** SWEET transporters play a pivotal role in sugar transport in plants. However, their functions in pollen tube growth, especially in coniferous species remain unknown. Here, we used RT-qPCR to reveal that a *SWEET1* gene was specifically expressed in pollen and pollen tubes of *Picea wilsonii*. A pollen germination assay showed that *PwSWEET1* was induced by H<sub>3</sub>BO<sub>3</sub> but not by Ca<sup>2+</sup>. In a sugar specificity experiment, sucrose (Suc) and glucose (Glc) were effective sugars for pollen germination and pollen tube growth. *PwSWEET1* expression was induced most by Suc and Glc. Heterologous expression of *PwSWEET1* in yeast showed that PwSWEET1 can restore the glucose absorption in yeast strain EBY.VW4000, which has a hexose absorption defect, and the absorption of glucose is pH-independent. This evidence supports the involvement of PwSWEET1 in boron-dependent glucose transport in pollen germination and pollen tube growth of *Picea wilsonii*.

**Keywords** *PwSWEET1* · *Picea wilsonii* · Pollen tube · Glucose transporter

## Introduction

Sugars play essential roles as a main source of energy for many developmental processes in plants. Sugars are present mainly in the form of sucrose, glucose and fructose (Ap Rees 1994; Koch 1996). Sinks such as pollen grains, pollen tubes and the anther tapetum are usually completely isolated symplastically with no access to plasmodesmata (Buttner and Sauer 2000; McCormick 2004). The early developmental stage of pollen microspores and the subsequent pollen germination and pollen tube growth, however, absorb an enormous amount of nutrients from tapetal cells and female tissues, respectively (Ylstra et al. 1998; Ma 2005). Disruptions in carbohydrate supply or transport can irreversibly impair pollen development and pollen tube growth (Engelke et al. 2010; Ji et al. 2010). The large amounts of sugars and components required for these sinks must therefore be exported via the apoplast (Slewinski 2011), and photoassimilates can only be imported via specific transporters in the plasma membrane (Buttner and Sauer 2000). Chen et al. (2012) found that AtSWEET10-15 and OsSWEET11,-14 can transport sucrose across the plasma membrane to assist phloem loading in leaves and that they have lower transport activity for glucose. SWEETs may also function in carbohydrate transport during senescence; overexpression of AtSWEET15 can accelerate senescence, which may be related to carbohydrate supply in different organs (Seo et al. 2011).

SWEETs have been widely investigated in plants such as Arabidopsis, rice, and wheat (Yang et al. 2006; Guan et al. 2008; Song et al. 2009; Chen et al. 2010, 2012;

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Chardon et al. 2013; Guo et al. 2014; Xu et al. 2016) and have been divided into four subclades (Chen et al. 2010). Typical SWEETs proteins contain either seven transmembrane helices (TMs) or fewer than seven (Chen et al. 2010; Feng et al. 2015; Patil et al. 2015). They have also been reported to be involved in stress responses. In wild rice, infection with PXO99<sup>A</sup> (*Xanthomonas oryzae* pv. *oryzae*) can induce the expression of *OsSWEET11* to provide the bacteria with glucose (Chen 2014). In *Arabidopsis*, the pathogen *Botrytis cinerea* induces *AtSWEET4*, *-15* and *-17*, and infection by *Golovinomyces cichoracearum* leads to increased expression of *AtSWEET12* (Ferrari et al. 2007). SWEET responses to abiotic stress reported are related primarily to cold stress (Chardon et al. 2013; Klemens et al. 2013; Liu et al. 2016), and the cold stress response is always closely correlated with sugar accumulation (Ramirez et al. 2004; Ypsilos and Magan 2004; Ogawa et al. 2005).

Various researchers have raised awareness that sugar transporters are also involved in pollen development and pollen tube growth (Marger and Saier 1993; Ylstra et al. 1998; Johnson et al. 2006; Cheng et al. 2015). In cucumber, antisense suppression of *CsHT1* inhibits pollen germination and tube growth (Cheng et al. 2015). *Arabidopsis thaliana* mutants with a loss of *AtSUC1* function are impaired in pollen germination (Sivitz et al. 2008). Inhibition of *LeSUT2* in tomato leads to poorer germination and pollen tube growth, thus affecting fruit and seed development (Hackel et al. 2006). Also, several SWEETs related to pollen development have been reported. For example, pollen grains of *atsweet8* mutants of *A. thaliana* are aborted early in development (Guan et al. 2008). *OsSWEET11*-silenced rice plants have low pollen viability (Yang et al. 2006).

Due to species differences and the complexities of the physiological processes, the role of sugar transporters in pollen tube growth has remained poorly understood (Cheng et al. 2015). Compared with angiosperm pollen tubes, coniferous pollen tubes grow more slowly and tend to ramify with a lack of a tip-to-base organelle zonation (Yu et al. 2009). Therefore, angiosperm pollen tubes are considered to be another evolutionary divergence of the male gametophytes (Lazzaro 2005); however, little information regarding this development is available. Here, we used RT-qPCR to show that the gene *SWEET1* from *Picea wilsonii* Mast. is specifically expressed in pollen and pollen tubes. Suc and Glc are the effective sugars for pollen germination of *P. wilsonii* and induce *PwSWEET1* expression. Heterologous expression in a yeast strain defective in hexose absorption showed that *PwSWEET1* can restore the ability to absorb hexose.

## Materials and methods

### Plant materials

Pollen grains, cones and seeds were collected in 2014 from mature trees of *P. wilsonii* in the Beijing Botanical Garden, Chinese Academy of Sciences. Needles, stems and young-needles were collected from trees on the campus of Beijing Forestry University. Pollen grains were dried overnight at room temperature, then stored at  $-80^{\circ}\text{C}$  until use. Seedlings were cultivated for 8 weeks in a greenhouse at  $25^{\circ}\text{C}$  with 16 h light/8 h dark.

### In vitro pollen germination

Pollen grains stored at  $-80^{\circ}\text{C}$  were revived by transferring to  $4^{\circ}\text{C}$  for 24 h, then at room temperature for 2 h. The revived pollen grains were then cultured in different media. The standard liquid medium for pollen germination and tube growth was composed of 12% (w/v) sucrose, 0.03% (w/v)  $\text{Ca}(\text{NO}_3)_2$ , 0.01% (w/v)  $\text{H}_3\text{BO}_3$ , and 5 mM citrate-phosphate buffer (pH 5.8). The effect of B and Ca on expression of *PwSWEET1* was assessed using 0.1%  $\text{Ca}^{2+}$  or 0.1%  $\text{H}_3\text{BO}_3$  in place of the standard concentrations in the standard liquid medium, then quantifying expression after 12 h and 24 h. In the sugar specificity experiment, 3%, 8%, 12% and 18% (w/v) of a sugar (Suc, Glc, Gal, Mal, Fru, or Man) was added to the medium, then pollen tubes were examined after 24 h. Pollen grains were incubated in small dishes at  $25^{\circ}\text{C}$ , 120 rpm/min in the dark and sampled at 6, 12, 18, 24, 30 and 36 h.

### Cloning of *PwSWEET1* cDNA

The shotgun method was used to obtain the full length sequence of *PwSWEET1* cDNA based on the *P. wilsonii* cDNA library constructed in our previous study (Zhang et al. 2012). Universal primers of pDONR222 vector 5'-GGT AAC GCC AGG GTT TTC C-3' (M13F) and 5'-CAG GAA ACA GCT ATG ACC-3' (M13R) were used.

### Bioinformatics analyses

Nucleotide and amino acid sequences were analyzed using DNAMAN software. The homologous amino acid sequences of *PwSWEET1* in *Arabidopsis* were searched from The *Arabidopsis* Information Resource (<http://www.arabidopsis.org/>). Amino acid sequences were aligned using Clustal X, and the phylogenetic tree was constructed using MEGA5.0 software. The TMHMM v. 2.0 server (<http://www.cbs.dtu.dk/services/TMHMM/>) was used to predict transmembrane domains.

## Stress and hormone treatments of *P. wilsonii* seedlings

The seedlings described in the Plant Materials section were used in this experiment. For salt stress, the roots of seedlings were treated with 200 mM NaCl for 1, 3, 6 and 12 h. For cold stress and heat stress, the seedlings were respectively treated in refrigerator at 4 °C for 1, 6 and 12 h and in water bath at 42 °C for 1, 3, 6 and 12 h. For drought stress, the seedlings were exposed to air without watering for 1, 3, 6, 12 h. For osmotic stress, the roots of seedlings were treated with 20 mM H<sub>2</sub>O<sub>2</sub> for 1, 3, 6 and 12 h. For ABA treatment and MeJA treatment, the roots of seedlings were respectively treated with 100 μM ABA for 3, 6 and 12 h and 100 μM MeJA for 1, 3, 6 and 12 h. The control group (0 h) was cultivated in a greenhouse at 25 °C with 16 h light/8 h dark. The treated seedlings were then frozen in liquid nitrogen and stored at −80 °C until RNA was extracted. All of experiments were done three times.

## Quantitative RT-PCR

*PwSWEET1* expression was quantified using SYBR GREEN SuperReal Premix (Tiangen, Beijing) with a StepOnePlus Real-Time PCR System (ABI, USA) and specific primers for *PwSWEET1* (5'-GTGCGTTGTGGGTGTTAT-3' and 5'-TCGTTTTTGTCCCTACAG-3'). *EF1-α* was amplified as an internal control using specific primers 5'-AACTGGAGAA GGAACCCAAG-3' and 5'-AACGACCCAATGGAG GATAC-3' (Yu et al. 2011). The RT-qPCR was performed with 30–33 cycles of 95 °C denaturation, 55 °C annealing and 72 °C extension and done independently three times.

## Functional characterization of *PwSWEET1* by heterologous expression in yeast

To test the biochemical properties of *PwSWEET1*, we constructed the plasmid combined with the CDS domain of *PwSWEET1* in the yeast expression vector pDR196 (Fan et al. 2009). The primers was designed based on appropriate restriction sites (5'-SmaI, 3'-SalI) with forward primer: 5'-TCCCCCGGA TGGCGAATACGGATACT-3' and reverse primer: 5'-TTCCGCGCCGCTATGGCCGACGTCGACT CACGGAATTCCATTCCTATC-3'. The PCR products were digested simultaneously with SmaI and SalI to combine with digested vector pDR196 to form the recombinant plasmid pDR196/*PwSWEET1*, and the construct was confirmed by PCR. The hexose transporter-deficient yeast strain EBY.VW4000 (Wieczorke et al. 1999) was transformed with pDR196/*PwSWEET1*, and a transformation with empty vector pDR196 was used as a control. The yeast uptake test was done as described by Chen

et al. (2010) and Cheng et al. (2015). Cells were allowed to grow in liquid SD (lacking ura) supplemented with 2% (w/v) maltose as the sole carbon source and harvested at OD<sub>600</sub> 0.6. Different dilutions of the cell suspension were dropped on solid SD (lacking ura) (pH 5.0) supplemented with 2% (w/v) maltose or Glc as the sole carbon source at different pHs. Cells were incubated 2–5 d at 29 °C in the dark before photography.

## Results

### Characterization of the cDNA clone for *SWEET* from *P. wilsonii*

The 1237-nucleotide *PwSWEET1* cDNA encodes a 261-amino-acid polypeptide with an ATG initiation codon at position 100 and a termination codon at position 887 (TGA). To examine the evolutionary relationship among *PwSWEET1*, *AtSWEETs* and *OsSWEETs*, a phylogenetic analysis based on the amino acid sequences was conducted. *PwSWEET1* was highly homologous with *AtSWEET4*, -5, -6 and -7 and *OsSWEET4*, -5, -6a, -6b, -7a, -7b, -7c, -7d and -7e, which belong to clade 2 in the *SWEET* superfamily (Fig. 1).

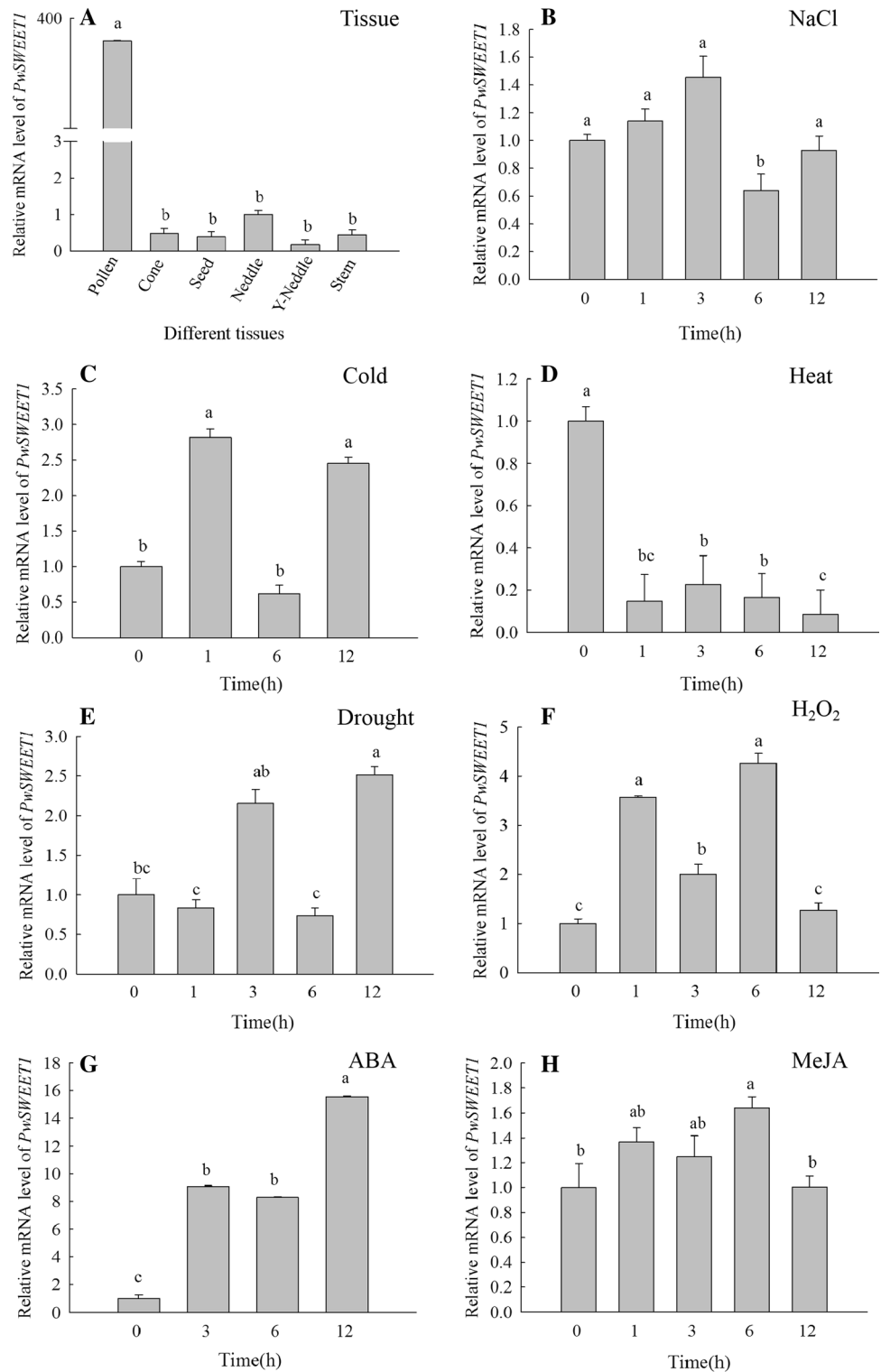
### Expression of *PwSWEET1* in different tissues and in response to stresses and hormones

As determined by RT-qPCR, *PwSWEET1* was extensively expressed in the different organs and tissues, but pollen grains had the highest transcript level, up to 390-fold higher than in the needles and other tissues (Fig. 2a). Expression was also relatively high in needles, suggesting *PwSWEET1* is probably involved in responses to stress and hormones (Fig. 2a). To further explore this involvement, we treated 8-week-old seedlings with salt (NaCl), cold, heat, drought, H<sub>2</sub>O<sub>2</sub>, ABA and MeJA (Fig. 2). Under cold treatment (4 °C), *PwSWEET1* expression increased and reached a maximum of 2.8-fold at 1 h, then declined at 6 h and increased again at 12 h (Fig. 2c). In contrast, the transcript level of *PwSWEET1* decreased greatly at 1 h and declined to 0.08-fold during heat treatment (42 °C) (Fig. 2d). Drought treatment induced *PwSWEET1* expression at 3 h to 2.1-fold and 12 h to 2.5-fold (Fig. 2e). Similarly, expression was also enhanced under H<sub>2</sub>O<sub>2</sub> (20 mM) treatment (Fig. 2f) and greatly increased in response to ABA (100 μM) (Fig. 2g) up to 15-fold at 12 h. Nevertheless, transcript levels did not obviously change during the salt (200 mM) (Fig. 2b) and MeJA (100 μM) treatments (Fig. 2h). Thus, we speculate that *PwSWEET1* very likely participates in ABA signal transduction and multiple stress responses.



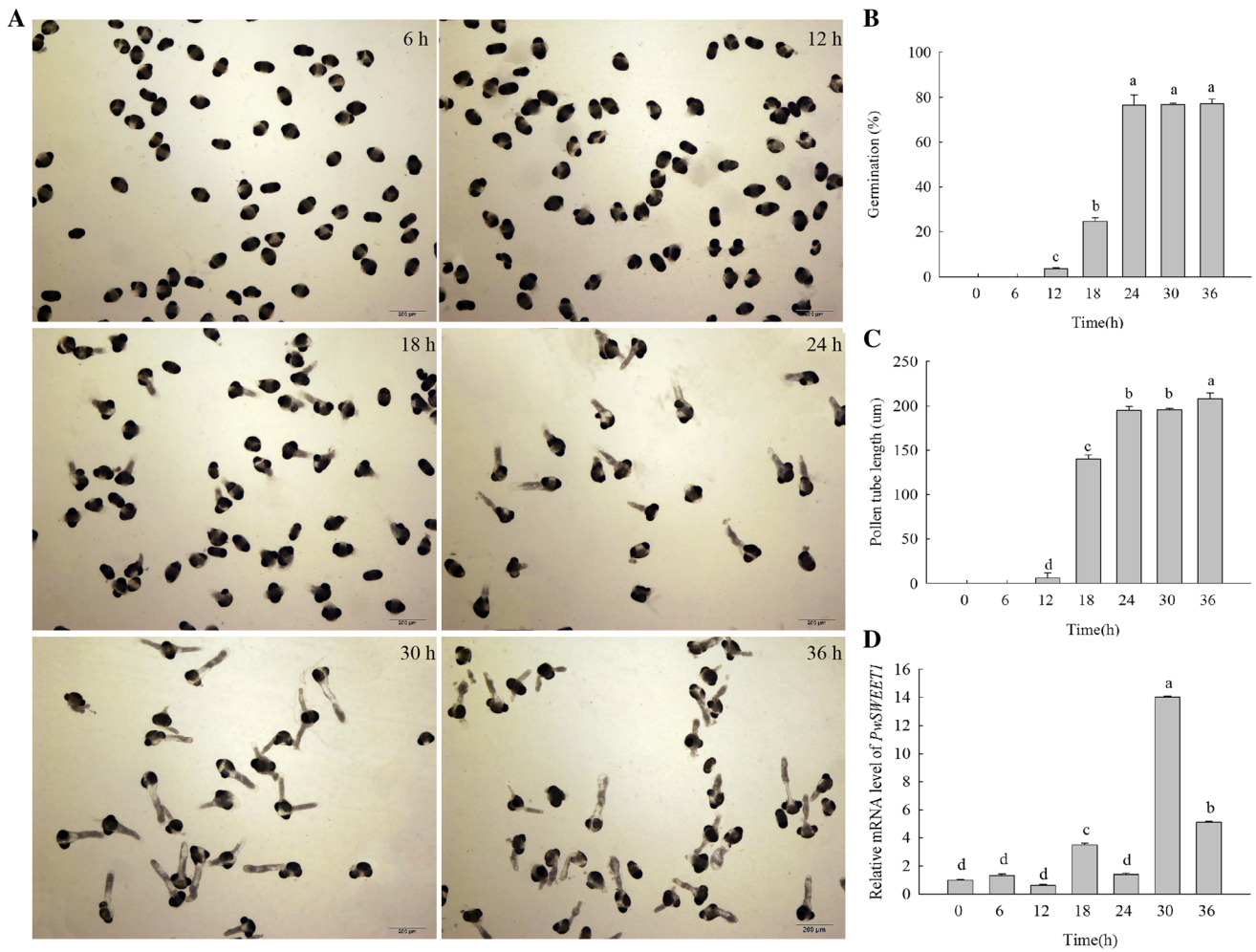


**Fig. 2** Expression profiles of *PwSWEET1* in different tissues and responses to stresses and hormones in 8-week-old *Picea wilsonii* seedlings. **a** Different tissues, **b** salt stress, **c** cold stress, **d** heat stress, **e** drought stress, **f** H<sub>2</sub>O<sub>2</sub> stress, **g** ABA, **h** MeJA

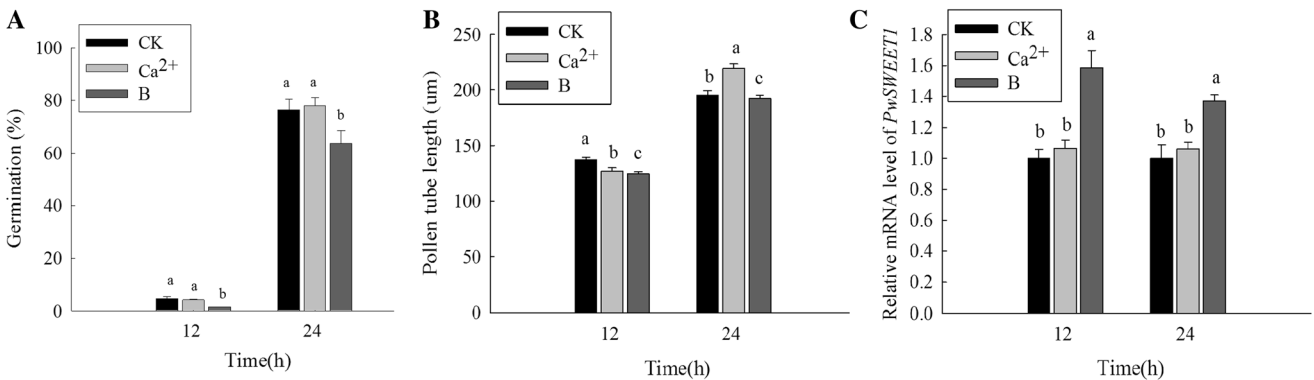


0.1% (w/v) Ca<sup>2+</sup> and 0.1% (w/v) H<sub>3</sub>BO<sub>3</sub>. As shown in Fig. 4a and b, the percentage germination and pollen tube elongation were both repressed in the boron-amended medium after 12 and 24 h. However, the tubes were significantly longer in the Ca<sup>2+</sup>-amended medium after 24 h,

and germination did not differ significantly from the control, despite the fact that 0.1% (w/v) Ca<sup>2+</sup> induced double-tipped tubes to fourfold compared to the control (Fig. S1A). *PwSWEET1* expression was induced by boron, but not by Ca<sup>2+</sup> after 12 and 24 h (Fig. 4c).



**Fig. 3** Morphology and *PwSWEET1* expression in germinating pollen of *P. willsonii* over time. **a** Light micrographs of pollen grains and pollen tubes, **b** percentage germination, **c** pollen tube lengths, **d** relative transcript levels of *PwSWEET1*



**Fig. 4** Percentage germination, pollen tube elongation and *PwSWEET1* expression after 12 and 24 h incubation in liquid medium amended with either of 0.1% (w/v) Ca<sup>2+</sup> and 0.1% (w/v) H<sub>3</sub>BO<sub>3</sub> (B). **a** Percentage germination. **b** Pollen tube lengths. **c** *PwSWEET1* transcript levels in pollen. CK: control incubated in standard liquid medium

## Sugar specificity and *PwSWEET1* expression in pollen grains in different carbon sources

For pollen germination and tube growth in vitro, it tends to have a lower rate of double-tipped tubes when every sugar was at its best concentration (Fig. S1B). The best concentration for Suc, Glc, Gal, Mal and Fru was, respectively, 12% (w/v), 8% (w/v), 3% (w/v), 12% (w/v) and 3% (w/v), and Suc, Glc, and Mal yielded the best germination and longest pollen tubes (Fig. 5a, b). No pollen germination or tube growth occurred in medium containing mannose at any concentration we applied (not shown). Sucrose induced the highest expression of *PwSWEET1*, followed by Glc (Fig. 5c), suggesting that *PwSWEET1* mainly transports sucrose and glucose.

## Heterologous expression of *PwSWEET1* in yeast

We tested whether *PwSWEET1* is also a functional sugar transporter by transforming yeast strain EBY.VW4000 (Wieczorke et al. 1999), defective in hexose absorption, with the pDR196/*PwSWEET1* fusion vector. All hexose transporter family genes (*Hxt1-17*) and five other transporter genes (*Gal2*, *Stl1*, *Agt1*, *Mph2*, *Mph3*) have been deleted in mutant strain EBY.VW4000, so the strain cannot grow on monosaccharides but can grow on maltose. Drop test indicated that EBY.VW4000 transformed either with pDR196/*PwSWEET1* or the empty vector pDR196 can grow well on 2% (w/v) Mal, whereas only pDR196/*PwSWEET1* transformants can grow on 2% (w/v) Glc (Fig. 6a).

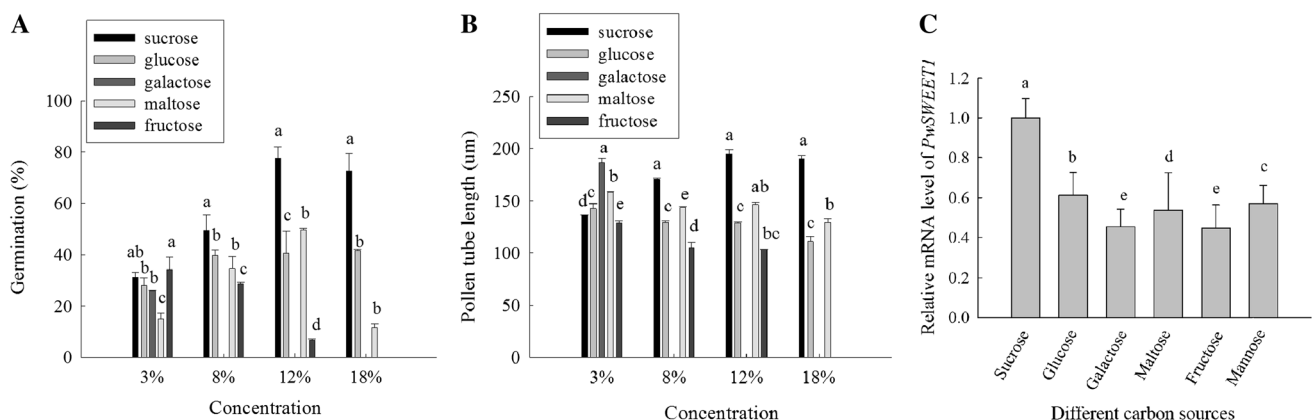
To identify whether *PwSWEET1* Glc transport is pH-dependent, we tested Glc uptake by EBY.VW4000 transformed with pDR196/*PwSWEET1* in medium supplemented with 2% (w/v) Mal or 2% (w/v) Glc at pH 4.0, 5.0, 6.0 and 7.0. The transformants grew well at each pH in

medium containing either maltose or glucose, implying that *PwSWEET1* is a pH-independent glucose transporter.

## Discussion and conclusions

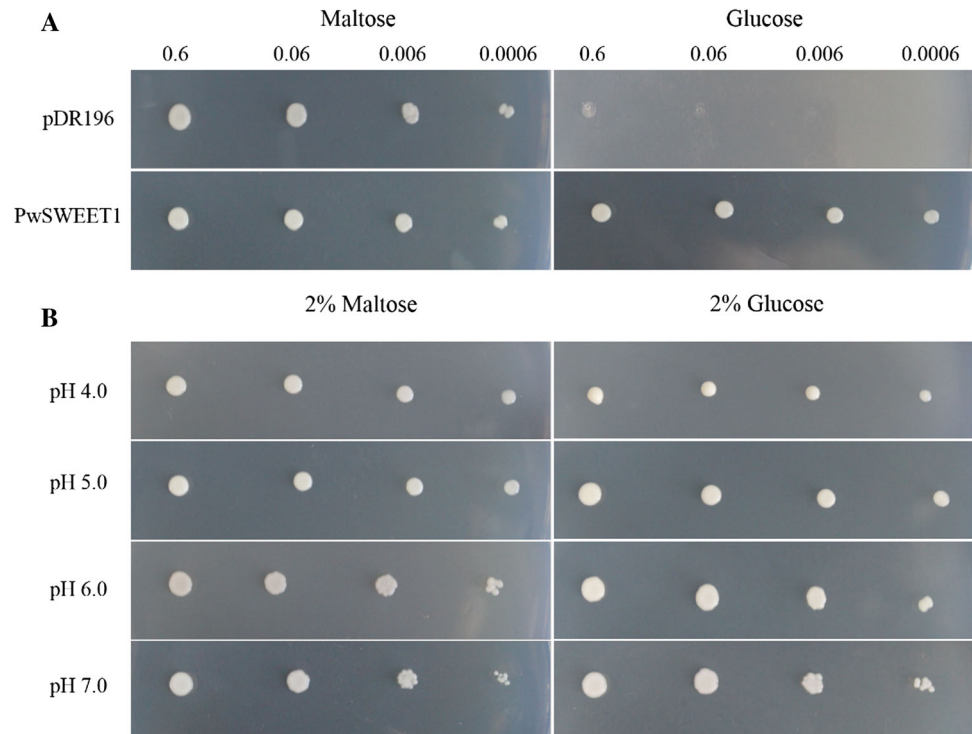
### *SWEET1* in *P. wilsonii* is highly expressed in pollen

*SWEET* gene family is widespread in eukaryotes and involved in many physiological processes and stress responses. In plants, it has a role in the nutrition of growing pollen tubes, senescence, phloem loading and plant-pathogen interactions (Chen et al. 2010, 2012; Seo et al. 2011; Klemens et al. 2013; Sun et al. 2013). *SWEET* genes have been well studied in Arabidopsis and rice (Yang et al. 2006; Guan et al. 2008; Song et al. 2009; Chen et al. 2010, 2012; Chardon et al. 2013; Guo et al. 2014). However, its role in pollen germination and tube growth in plant, especially in coniferous species, has needed further study. Here, we described the functionally characterized *PwSWEET1*, a highly expressed gene in pollen in *P. wilsonii*. Phylogenetic analysis based on the amino acid sequences showed that *PwSWEET1* was highly homologous with the four AtSWEET proteins and nine OsSWEET proteins. AtSWEET4, -5 and -7 function in glucose transport, and AtSWEET5 is pollen-specific (Engel et al. 2005; Chen et al. 2010). AtSWEET4 also mediates fructose transport during plant growth and development (Liu et al. 2016), and another member of clade 2 from Arabidopsis, AtSWEET8, which is also homologous to *PwSWEET1*, is a pollen-specific glucose transporter on the plasma membrane (Chen et al. 2010). In rice, OsSWEET5 encodes a galactose transporter (Zhou et al. 2014). The transmembrane helix (TM) prediction for *PwSWEET1* and AtSWEET4, -5, -6, and -7 showed that *PwSWEET1* and AtSWEET6 had 6 TMs, and the rest had 7 TMs (Fig. 1, Fig. S2).



**Fig. 5** Effect of different carbon sources on pollen germination, tube elongation and *PwSWEET1* expression in vitro. **a** Germination. **b** Pollen tube lengths. **c** *PwSWEET1* expression

**Fig. 6** Functional characterization of PwSWEET1 in yeast. **a** PwSWEET1 complemented glucose uptake in hexose transporter-deficient yeast EBY.VW4000 but pDR196 empty vector did not. **b** Complementation of glucose uptake deficiency of EBY.VW4000 by PwSWEET1 at different pHs



*AtSWEET5* is expressed in mature, hydrated and germinating pollen (Engel et al. 2005). In the present tissue-specific assay, *PwSWEET1* was shown to be pollen-specific, with levels in the pollen hundreds of times higher than in the cones, needles, stems and seeds (Fig. 2). *PwSWEET1* expression was greatly induced at fast-growing stages of pollen tube development (Fig. 3) and enhanced further pollen grains exposed to 0.1%  $H_3BO_3$  for 12 and 24 h (Fig. 4). These results suggest that *PwSWEET1* is very important in pollen germination and pollen tube growth and induced by boron.

### PwSWEET1 is a pH-independent glucose transporter

In vitro pollen germination and pollen tube growth require sugar as an energy source, but different species require different sugars. Suc is the typical carbon source for pollen grains of most species (Okusaka and Hirastuka 2009; Cheng et al. 2015); however, many species use other sugars. Tobacco and *A. thaliana* pollen prefer Suc, whereas *Petunia* pollen can use Suc, Glc and Fru; cucumber pollen can use Suc, Glc and Gal, pear pollen uses Suc and Glc (Cheng et al. 2015; Lemoine et al. 1999; Okusaka and Hirastuka 2009; Scholz-Starke et al. 2003; Ylstra et al. 1998). Here, we found that *P. wilsonii* pollen tubes can grow on Suc, Glc and Mal at appropriate concentrations, but Suc was the most effective (Fig. 5).

*PwSWEET1* expression also differs when pollen grains are incubated with different carbon sources; expression was highest with sucrose, followed by glucose (Fig. 5), suggesting SWEET1 differs in its ability to transport different types of sugars. The heterologous expression assay showed that PwSWEET1 functions in glucose transport and complemented glucose uptake deficiency of EBY.VW4000 (Fig. 6), similar to the homologous glucose transporters *AtSWEET4*, -5 and -7 (Chen et al. 2010). These data suggest that the structure is evolutionarily conserved, that SWEETs have diverse functions, and that the homologous genes in the SWEET family among different plant species have similar conserved functions and sugar preferences. Further, Glc uptake by yeast strain EBY.VW4000 transformed with pDR196/*PwSWEET1* in medium supplemented with Mal or Glc at different pHs showed that transformants grew well at the four pHs either in Mal or Glc medium, proving that PwSWEET1 is a pH-independent glucose transporter. Although PwSWEET1 can transport glucose, we cannot preclude that it can also transport sucrose based on the function of homologs and the induction of PwSWEET1 by Suc and Gluc. It is not hard to imagine that there is an association among the preference of *P. wilsonii* pollen for Glc, the glucose transport ability of PwSWEET1 in yeast combination with the induction of *PwSWEET1* expression by glucose and sucrose, suggesting that *PwSWEET1* is mainly probably responsible for Glu and Suc transport in pollen germination and pollen tube growth.



## Possible function of the glucose transporter

The RT-qPCR analysis showed that *PwSWEET1* expression is enhanced under cold (4 °C), ABA, drought, and H<sub>2</sub>O<sub>2</sub> treatment, but decreased by the heat treatment (Fig. 2). In contrast, NaCl and MeJA treatment caused no obvious changes, suggesting *PwSWEET1* is involved in multiple abiotic stresses. Previous studies showed that cold treatment at 4 °C can induce the accumulation of soluble sugars in plants and that sugar transporter genes can be regulated to balance the sugar content and ensure normal plant growth (Wormit et al. 2006; Klemens et al. 2013; Guo et al. 2014). For instance, after a cold treatment, overexpression of *AtSWEET17* leads to decreased fructose accumulation (Chardon et al. 2013), and expression of *AtSWEET16* is repressed (Klemens et al. 2013). Here we speculate that the accumulation of soluble sugars regulated by sugar transporters in plants also positively contributes to plant response to other such stresses as ABA, drought, and H<sub>2</sub>O<sub>2</sub>. Considering that *PwSWEET1* is specifically expressed in pollen and functions as a glucose transporter, suggesting *PwSWEET1* plays an important role in sugar supply during the reproductive period and may take part in balancing sugar allocation in plants to ensure good growth since the accumulation of sugars can cause rapid changes in metabolism (Heineke et al. 1994). In fact, the *AtSWEET5* gene, which is highly homologous to *PwSWEET1* is localized specifically in the vegetative cell of pollen and may supply the generative cell with sugar (Engel et al. 2005).

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