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PHYSIOLOGY

Arabidopsis *HIT1*, a putative homolog of yeast tethering protein Vps53p, is required for pollen tube elongation

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ABSTRACT. The Arabidopsis *HIT1* gene encodes a protein that is homologous to the yeast tethering factor Vps53p, which is involved in retrograde vesicle trafficking from the endosome to the trans-Golgi network. Although the ethyl methanesulfonate mutagenized *hit1-1* allele can be maintained homozygously, T-DNA insertional *hit1-2* and *hit1-3* mutants can only be isolated as hemizygous lines. No heterozygous progeny were produced in outcrosses to wild-type plants using pollen from either *hit1-2* or *hit1-3* heterozygotes. The reciprocal cross using pollen from wild-type plants on either *hit1-2* or *hit1-3* mutants produced heterozygous and wild-type progeny. In reproductive tissues, *HIT1* promoter-driven GUS activity was detected only in mature pollen and elongated pollen tubes. *In vitro* pollen germination further showed that only half the pollen grains from *hit1-2* and *hit1-3* heterozygote plants produced normal pollen tubes. In contrast, the pollen tube length of pollen grains from the *hit1-1* mutant was reduced compared to that of the wild type. These results suggest that *HIT1* may govern a vesicle trafficking event that is required for pollen tube tip growth during male gametogenesis and that disruption of *HIT1* results in male specific transmission defect. Moreover, while the *hit1-1* mutant is partially functional leading to reduced pollen tube length, *hit1-2* and *hit1-3* are total-loss-of-function alleles.

Keywords: *Arabidopsis thaliana*; Pollen tube; Tip growth; Vesicle tethering factor; Yeast Vps53p protein.

Abbreviations: ***hit*,** heat-intolerant; **GARP,** Golgi-associated retrograde protein; **TGN,** trans-Golgi network; **VPS,** vesicular protein sorting.

INTRODUCTION

Pollen tube elongation is a crucial event in the sexual reproduction of flowering plants. It begins when pollen germinates on the stigma and then continues to penetrate the stigmatic tissue, style, and transmitting tract. Finally, the pollen tube reaches the ovule and delivers the genetic material for fertilization. Pollen tube elongation involves many specialized mechanisms that control growth and navigation in order for the pollen tube to reach the dedicated destination. These features make the pollen tube a unique system for the study of not only male fertility in plants, but also many other distinctive aspects of biology (Franklin-Tong, 1999; Edlund et al., 2004). One of them is that, while most plant cells expand through a diffuse growth mechanism, pollen tube elongation occurs only at the utmost apex of the tube through a polarized tip growth mechanism (Hepler et al., 2001).

Because the pollen tube's growth is restricted to the tube apex, many molecules must be translocated to the growing tip. For example, pectic polysaccharides are assembled in the Golgi apparatus, then transported in vesicles, and subsequently released at the growing tip through exocytosis (Sterling et al., 2001). This process is essential because pectin is a major component of the cell wall at the growing pollen tube tip (Ferguson et al., 1998). In addition to the cell wall materials, vesicles are responsible for carrying nascent membranes with embedded proteins and secretory molecules to the growing tube tip. Pollen tube growth also involves the recycling of excess membranes and uptake of female derived molecules through endocytosis (Derksen et al., 1995; Parton et al., 2001). It has been well characterized that an inverted cone-shaped region adjoining the pollen tube apex is devoid of organelles and almost exclusively occupied by vesicles (Franklin-Tong, 1999). These vesicles must retain their individual specificity so that each of them can find and fuse with its unique target membrane and deliver its cargo to its designated compartment during pollen tube tip growth.

Current models indicate that the recognition between a vesicle and a target membrane is initially mediated by tethering factors. Tethering factors can be generally

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divided into a group of extended coiled-coil proteins and a class of oligomeric complexes. These tethers act to establish physical links, over considerable distances, between two membranes that are due to fuse (Sztul and Lupashin, 2006). Despite the fact that many of these regulators have been identified in plants through genomic analysis, the question of which of them are essential and what biological roles they play during pollen tube tip growth has yet to be resolved. Only POK, a putative homolog of yeast Vps52p in the Golgi-associated retrograde protein (GARP) complex, and AtSEC8, a subunit of the putative exocyst complex, have recently been shown to regulate pollen tube growth in Arabidopsis (Lobstein et al., 2004; Cole et al., 2005).

Previously, an ethyl methanesulfonate (EMS) mutagenized *hit1-1* (heat-intolerant) mutant, the growth of which had been inhibited by high temperatures, was isolated from Arabidopsis (Wu et al., 2000). The mutated gene was later identified to encode a homolog of the yeast tethering factor Vps53p (Lee et al., 2006). In yeast, Vps51p, Vps52p, Vps53p and Vps54p constitute a tetrameric GARP tethering complex. This complex interacts with a SNARE and a Rab GTPase to mediate retrograde vesicle trafficking from the endosome to the trans-Golgi network (TGN) (Conibear and Stevens, 2000; Siniossoglou and Pelham, 2001; Conibear et al., 2003). Although *hit1-1* mutant can be maintained homozygously, T-DNA insertional *hit1-2* and *hit1-3* mutants are like the T-DNA insertional *pok* mutant that can only be isolated as a hemizygous line. This finding suggests that *HIT1* may also be involved in pollen tube growth. Here we provide evidence to support this hypothesis.

MATERIALS AND METHODS

Plant materials and growth conditions

The wild-type *Arabidopsis thaliana* plants used in this study were in the Colombia-0 background. Seeds were obtained from the Lehle Seeds Company (Round Rock, TX, USA). The *hit1-1* mutant line was isolated from the F_2 progeny of plants mutagenized with EMS, as described (Wu et al., 2000). The T-DNA insertion alleles *hit1-2* (GABI_100C01) and *hit1-3* (SALK_047230) were obtained from the Max-Planck Institute, Cologne, Germany (Rosso et al., 2003) and the Arabidopsis Biological Resource Center at Ohio State University, Columbus, Ohio, respectively. Plants were either grown in soil or on Murashige Skoog (MS) salt agar plates at 23 ºC under a light cycle of 16 h light/8 h dark for both wild type and *hit1-1* mutant. For the selection of T-DNA insertion lines, seeds were pregerminated on MS-salt agar plates with sulfadiazine (*hit1-2*) or kanamycin (*hit1-3*) according to the suppliers' instructions.

Genotyping of T-DNA insertion *hit1* mutants

The predicted *HIT1* gene has 24 exons with translation start for the *HIT1* transcript located in the second exon, based on the most current sequence information from the Arabidopsis Information Resource (TAIR, http://www.Arabidopsis.org), the mutant supplier's data, and the open reading frame derived from cloned cDNA. The *hit1-2* allele has a T-DNA inserted in the eighth exon and *hit1-3* allele has a T-DNA inserted in the seventh intron (Figure 1a). Genotyping of the *hit1-2* lines was performed by PCR using the *HIT1*-complemented forward primer (5′-GCTGGAACGGATTTTATTTCTGG-3′) and the T-DNA left border complemented reverse primer (5′-CCCATT TGGACGTGAATGTAGACAC-3′) to amplify a 601 bp DNA fragment corresponding to the junction sequence for *hit1-2*. Control PCR assays amplified the 909 bp non-disrupted *HIT1* sequence using the same forward primer with the *HIT1*-complemented reverse primer (5′-ATTATTCCCGTGCCAAGTAGG-3′). A similar strategy was also applied to genotype the *hit1-3* lines with *HIT1*-complemented forward primer (5′-CCAAACCAGCTCA TTGTCATTTTG-3′), T-DNA left border complemented reverse primer (GCGTGGACCGCTTGCTGCAACT-3′), and *HIT1*-complemented reverse primer (5′-GCCTATACGGCACATGCCAAG-3′).

Preparations of the *HIT1* promoter construct

The promoter region of *HIT1* was amplified with gene-specific primers 5′-GTGAAGCTTGGCATCAAC

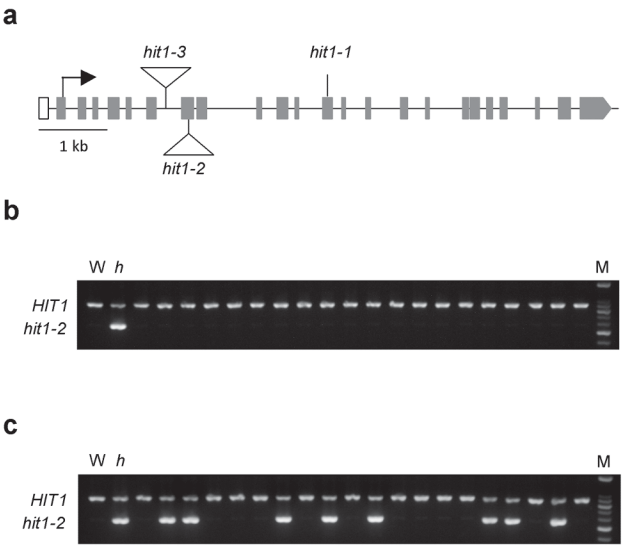

Figure 1. Insertional mutations affecting the *hit1* transmission is male specific. (a) Intron-exon structure of the *HIT1* gene. Solid black lines represent introns, and gray boxes represent exons. The positions of the point mutation in *hit1-1* and the T-DNA insertion in *hit1-2* and *hit1-3* are indicated, respectively. The predicted translation start is specified by an arrow; (b) Progeny from an outcross using *HIT1*/*hit1-2* heterozygote anthers to pollinate wild-type plants were genotyped by PCR using a set of three primers to produce distinct *HIT1* and *hit1-2* bandings. No heterozygous progeny were produced; (c) Heterozygous progeny were produced in an outcross to a *HIT1*/*hit1-2* heterozygote using pollen from a wild-type homozygote. *W*, wild type; *h*, *HIT1*/*hit1-2* heterozytote; *M*, molecular marker.

ACCATCATCTAAACA-3′ and 5′-GGCCTGCAGTTT GTATGATAAAACCAAAAATCA-3′ (*Hind*III and *Pst*I sites are underlined). The 2.4 kb amplified products were resolved by electrophoresis, gel purified, and cloned into pGEMT-Easy vector (Promega, Madison Wisconsin, USA). After sequence verification, the fragments were inserted upstream of the β-Glucuronidase (GUS) reporter gene followed by the nopaline synthase terminator in pCAMBIA1300 Ti-derived binary vector (CAMBIA, Canberra, Australia). *Agrobacterium tumefaciens* strain GV3101 was used to deliver this construct into wild-type plants by vacuum infiltration (Clough and Bent, 1998). Transgenic plants were selected on MS agar medium containing 25 μg/mL Hygromycin B for 14 days. Resistant T1 seedlings were transferred to soil and grown to maturity. Homozygous T2 lines were selected by screening their T3 progeny using resistance to Hygromycin B as the marker. T3 seeds derived from homozygous T2 lines were used for GUS assays.

Histochemical GUS assays

Agar grown whole seedlings were vacuum-infiltrated with 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc), 29 mM Na_2HPO_4, 21 mM NaH_2PO_4, and 0.1 % (v/v) Triton X-100 at pH 7.0 and then incubated at 37 °C to stain for GUS activity. After staining for 4 h, the samples were washed several times with 70% ethanol to remove the chlorophyll. For GUS assays on pollen grains, inflorescences were cut off and prefixed in chloroform: ethanol:water (3:6:1, v/v/v), 0.1% (v/v) Triton X-100 for 30 min followed by the vacuum-filtration and GUS staining procedures described above (Procissi et al., 2003). After staining, anthers at different stages were separated from flowers, and their contents were released using a needle under a dissecting microscope.

In vitro pollen germination

The *in vitro* pollen germination assay was adapted from previous reports (Li et al., 1999; Fan et al., 2001; Johnson-Brousseau and McCormick, 2004). The medium used for pollen germination was prepared using double distilled water and contained 18% (w/v) sucrose, 0.01% (w/v) boric acid, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)$, and 1% (w/v) agar at pH 7. After heating at 100°C for 2 min, the medium was allowed to solidify on glass slides, forming a 1-mm thick layer. Newly dehisced anthers were detached from flowers and carefully rubbed onto the surface of the germinating medium to transfer the pollen grains. Following the application of pollen, the slides were immediately put inside a box with water in the bottom to maintain the relative humidity at 100%. The entire assembly was then transferred to a growth chamber at 25°C with constant illumination and incubated for 12 h before examination under a light microscope.

RESULTS

Disruption of the *HIT1* gene causing a male-specific transmission defect

The *hit1-2* and *hit1-3* alleles can only be isolated as hemizygous lines, and the siliques produced from the heterozygous parental plants were fully filled with viable seeds, suggesting that disruption of *HIT1* gene by T-DNA insertion may result in a male-specific transmission defect (Lee et al., 2006). To verify that the transmission defect was indeed male specific, outcrosses were performed using pollen from *hit1-2* heterozygotes to pollinate the stigma of wild-type plants. PCR-based genotyping revealed that no heterozygous progeny were produced (Figure 1b). Outcrosses were also made using pollen from wild-type plants to pollinate *hit1-2* heterozygotes, and the PCR-based genotyping revealed that both wild-type and heterozygous progeny were produced (Figure 1c). Similar results were also found when testing *hit1-3* alleles (Table 1), confirming that the transmission defect is specifically associated with male gametophyte. While T-DNA insertion in the *hit1-2* and *hit1-3* alleles showed a strong male transmission defect, the single base substituted allele

Table 1. Inheritance of mutant *HIT1* alleles.

	No. of Progeny tested[a]	Genotypes of progeny[b]			x^2	*P*
		+/+	+/m	m/m		
Natural self cross of *HIT1* heterozygotes						
		25%	50%	25%	*Expected*	
HIT1/*hit1*-2	425	54%	46%	0%	251.8	≤0.001
HIT1/*hit1*-3	124	53%	47%	0%	70.8	≤0.001
Outcross of *HIT1* heterozygotes						
		50%	50%	--	*Expected*	
HIT1/hit1-2 (♂) × WT (♀)	66	100%	0%	--	66.0	≤0.001
HIT1/hit1-3 (♂) × WT (♀)	58	100%	0%	--	58.0	≤0.001
WT (♂) × *HIT1/hit1-2* (♀)	66	55%	45%	--	0.545	NS[c]
WT (♂) × *HIT1/hit1-3* (♀)	54	54%	46%	--	0.296	NS[c]

[a]Samples are from three or more individual crosses; [b]+, *HIT1*; m, mutant *hit1* allele; [C]NS, Not significantly different.

hit1-1 was maintained homozygously. These findings suggest that *hit1-2* and *hit1-3* are null alleles while *hit1-1* is a partially functional allele.

The *HIT1* promoter is predominately active in the male gametophyte

The expression pattern of *HIT1* was determined in plants expressing the β-glucuronidase reporter gene under the control of the putative *HIT1* promoter, a 2.4 kb DNA fragment upstream of the *HIT1* coding region. In 10-day-old plantlets, the blue staining was restricted to the root with the apex region showing enhanced intensity (Figure 2a). In mature plants, GUS staining was observed in particular reproductive tissues at certain stages of flower development. More specifically, blue coloration was detected in the anthers after floral stage 11. At this time, the petal height has exceeded that of the short stamens, stigmatic papillae begin to appear, and meiosis of microspore mother cell is complete, as previously defined (Figure 2b-c) (Smyth et al., 1990; Sanders et al., 1999). The blue color appears in the anthers as the filaments continue to elongate rapidly through floral stage 12. GUS staining begins to appear in the stigmatic region of the style at floral stages 13 and 14, as the flower opens and the long anthers extend to the stigma for pollen release. After pollination, the style elongates, and the stigma extends above long anthers, the landmark of floral stage 15. At this stage, the blue coloration was still apparent in the stigmatic region but had faded away in the anthers. The absence of staining was due to the evacuation of pollen grains, causing the anthers to appear translucent.

To better define the *HIT1* promoter activities, GUS stained tissues were analyzed microscopically. In the root apex, enhanced GUS activity was observed in the meristem and elongation zones. In contrast, GUS activity was not detected in the root cap (Figure 3a). Microscopic observation also revealed that the blue coloration was clearly detectable in the pollen grains within the anthers at stages 12 and 13, but not at stage 11 (Figure 3b-d). Further examination showed that GUS activity was only expressed in matured pollen grains, but not in tetrad or unicellular microspores (Figure 3e-g). Although the blue coloration in the pollen grains became lighter after pollination, possibly caused by the exit of cytoplasm, strong GUS staining occurred in the pollen tubes as they extended along the papillae cells toward the style (Figure 3h). These results indicate that the blue coloration observed in the pollinated stigma came from pollen tubes, not the papillae cells.

Mutations in *HIT1* affect pollen tube elongation

To examine if mutation of the *HIT1* gene can result in abnormal pollen tube growth, pollen germination experiments were performed *in vitro*. Thirty pollen grains from each of three flowers per plant were randomly selected for observation. After 12 h of incubation, the length of each pollen tube was measured. As shown in Figure 4, most of the wild-type pollen tubes exceed 300 μm in length, resulting in a single peak in the distribution curve. For the *hit1-1* mutant, none of the pollen tubes exceeded 350 μm. Their lengths were mainly between 50 μm and 250 μm, making a central plateau in the distribution curve. For the *HIT1*/*hit1-2* heterozygous plants, roughly half of the pollen tubes were shorter than 50 μm and half were longer than 300 μm, creating two peaks at the opposite sides of the distribution curve. These peaks may correspond to the *hit1-2* and the *HIT1*

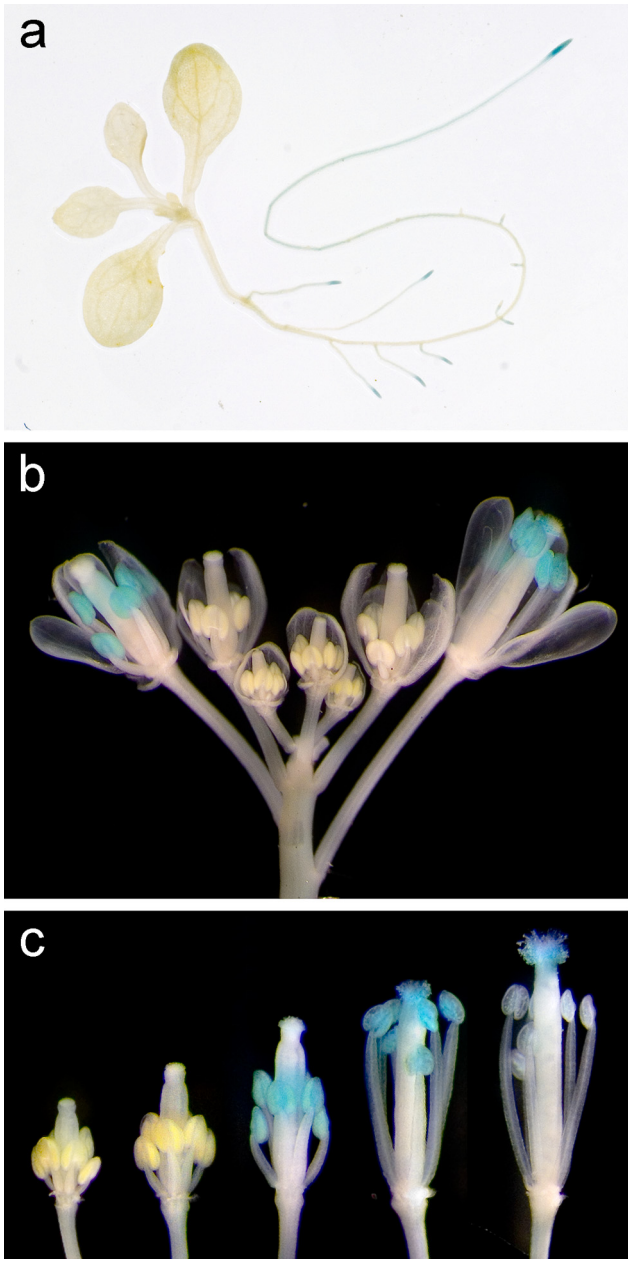

Figure 2. GUS assay of transgenic Arabidopsis plants harboring *HIT1* promoter-driven GUS reporter gene. (a) GUS staining on a 10-day-old plantlet; (b) GUS staining on an inflorescence. Blue signals are observed in the anthers of late flowers; (c) A series of five flower buds on an inflorescence. The detached flower buds were stained with X-Gluc solution, then the sepals and petals were removed for photographic documentation.

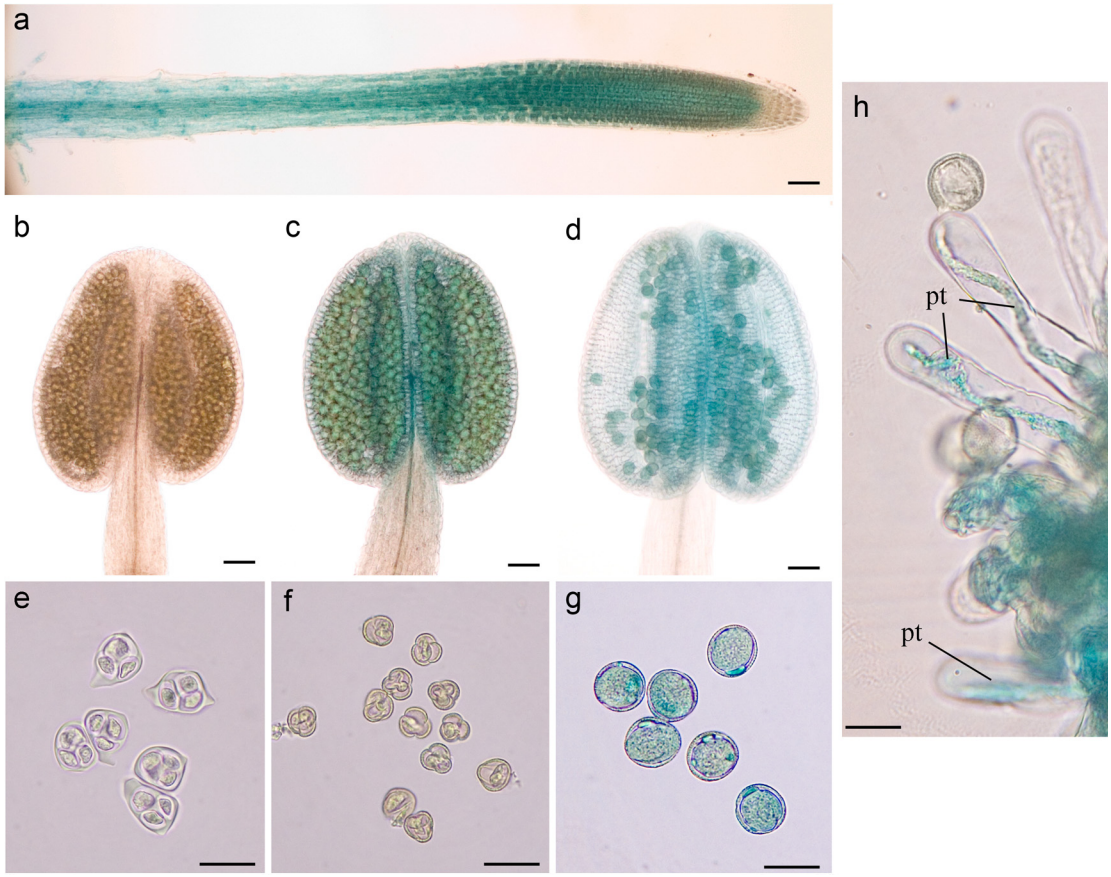

Figure 3. Microscopic observation of GUS activity in transgenic Arabidopsis plants harboring *HIT1* promoter-driven GUS reporter gene. (a) The root apex region from a 10-day-old plantlet; (b, c, d) Anthers from flowers at floral stages 11 (b); 12 (c), and 13 (d); (e) Tetrads; (f) Uninucleate microspore; (g) Mature pollen grains; (h) The stigmatic region of a self-pollinated transgenic plant, showing blue-colored pollen tubes along papillae cells. The pollen grains may become detached from the pollen tubes during sample preparation. Some pollen tubes and papillae cells are out of focus. *pt*, pollen tube. Scare bars: (a, b, c, d) 50 μm; (e, f, g, h) 25 μm.

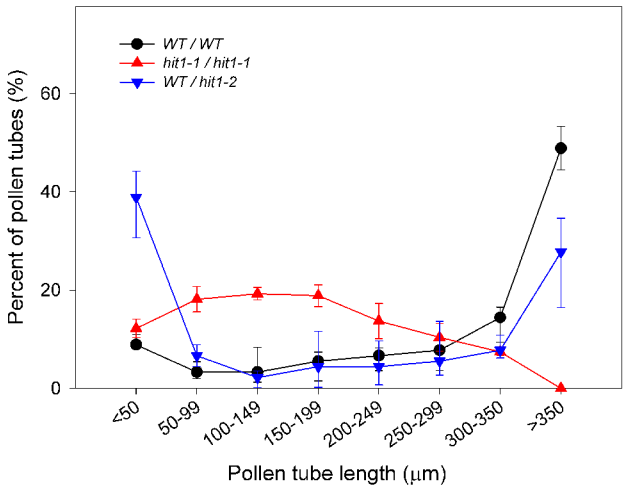

Figure 4. The distribution of pollen tube lengths *in vitro*. Three flowers from each plant were picked, and 30 pollen grains from each flower were randomly chosen for observation for a total of 90 pollen grains per plant. Three plants from each line were used to calculate the standard deviations of the mean percent of pollen tubes at each length for each genotype using SigmaPlot 2000 software (SPSS Inc., Chicago, IL).

allele, respectively, in the haploid pollen grains (Figure 4). Similar results were found when the pollen grains from *HIT1*/*hit1-3* heterozygous plants were examined (data not shown). Together, these data indicate that *HIT1* is crucial for pollen tube growth. Disruption of *HIT1* by T-DNA insertion restricts pollen tube elongation while *hit1-1* is a partially functional allele resulting in a reduction of pollen tube length.

DISCUSSION

Vesicle tethering factors are one of the major components conferring the specificity of membrane fusion, possibly at the earliest stage, in the course of vesicle trafficking. Tethering factors can be large multi-subunit complexes, and at least seven complexes have been proposed to play roles in vesicle tethering at distinct trafficking steps (Whyte and Munro, 2002). To date, yeast and animal systems have provided the basis of our understanding of vesicle tethering. Many homologs of these tethering factors were identified in plants through genomic analysis, but their biological roles remain unknown (Jürgens and Geldner, 2002; Elias et al., 2003;

Latijnhouwers et al., 2005). Only very recently, functional characterizations of the plant genes *AtSEC8* and *POK* by mutant analysis have been reported (Lobstein et al., 2004; Cole et al., 2005). Each of these genes, however, encodes a putative subunit of different tethering complexes. *HIT1* and *POK*, on the other hand, encode putative subunits of the same tethering complex. Therefore, the *hit1* mutants are valuable for both functional studies and for providing a new approach to dissecting the multifarious vesicle tethering in plants.

Male gametophyte development occurs through a series of sequential steps, and disrupting any of these steps can result in male-specific transmission defects. Whether *HIT1* would affect pollen development at earlier developmental events, such as mitotic cytokinesis, is uncertain. However, because the *HIT1* promoter-GUS fusion activity appeared in the mature pollen grains and the growing pollen tubes, it was thought that one function of the *HIT1* was to facilitate the initiation and maintenance of the polarized growth of pollen tubes. The fact that *HIT1* is a homolog of known tethering factors provided additional support for this idea. The reduction in pollen tube length produced by pollen from *hit1-1* plants also lends credence to this hypothesis. Additionally, male gametophytes with reduced pollen tube length like those harboring *hit1-1* allele can theoretically cause segregation distortion, due to competition with wild-type gametophytes. In fact, the analysis of F2 progeny from a cross between the wild type and a *hit1-1* mutant revealed a 4:1 ratio of wild-type to mutant phenotype, instead of the standard 3:1 ratio (Wu et al., 2000).

In plants, the elongation of root hair also employs a tip growth mechanism. Since *hit1-2* and *hit1-3* can only be isolated as hemizygous lines, it is not possible to study the effects of these two T-DNA insertion alleles on root hair development. However, the *hit1-1* mutant did exhibit reduced root hair length (Wang and Wu, unpublished data), suggesting that HIT1 may have a general role in polar growth.

In yeast, Vps51p, Vps52p, Vps53p, and Vps54p constitute the newly discovered tetrameric GARP tethering complex (Conibear and Stevens, 2000; Siniossoglou and Pelham, 2001; Conibear et al., 2003). In addition to HIT1, which is homologous to Vps53p, Arabidopsis genes *POK* and *AtVps54* (At1g71270 and At4g19490, respectively) were recently identified to encode potential homologs of yeast Vps52p and Vps54p (Lobstein et al., 2004; Latijnhouwers et al., 2005). Nevertheless, *HIT1* and *POK* promoter-driven GUS staining showed different expression patterns in the reproductive tissues. While *HIT1* expression was specifically in the haploid male gametophyte, the expression of *POK* can be sporophytic and female oriented, as blue staining was also observed in ovules (Lobstein et al., 2004). The differences in GUS expression between the *HIT1* and *POK* constructs may have due to the fact that the GUS gene in the *POK* study was fused with the first ten exons of the *POK*, and the GUS gene for *HIT1* in the present study was placed directly downstream of the *HIT1* 5′ untranslated region. Alternatively, a gene encoding a homolog of Vps51p has not been detected in Arabidopsis, and considering the complexity in cellular organization and secretory proteins in plants, it has been proposed that the vesicle-trafficking machinery may have evolved independently in plants (Jürgens and Geldner, 2002).

The *hit1-1* mutant was originally identified by its susceptibility to heat and osmotic stress (Wu et al., 2000). This susceptibility made it clear that *HIT1* is not only important in the male gametophyte, but also in somatic cells. Vesicle trafficking has been proposed to be essential in plant responses to stress (Levine, 2002). This process is required for the removal of damaged membranes and the delivery of the new ones to their target sites. Indeed, if *HIT1* is involved in vesicle tethering events, the partially functional *hit1-1* allele would lead to inefficient vesicle trafficking, explaining the observed stress sensitive phenotypes in addition to reduced pollen tube length. Furthermore, a low level expression of *HIT1* was detected in other vegetative tissues by RNA gel blot and reverse transcription-PCR (Lobstein et al., 2004; Lee et al., 2006). Microarray analysis on the other hand showed that the expression of *HIT1* is low and lacks tissue specificity (Schmid et al., 2005). Although the data in this report shows that the *HIT1* promoter-driven GUS activity was primarily detected in the root of young plantlets, a trace amount of GUS activity was detected in soil-grown rosette leaves (Wang and Wu, unpublished data). The difference in the expression pattern may be because of different experimental methods. Taken as a whole, a single tethering protein homolog may govern many important cellular events, in both gametophytic and sporophytic cells, affecting plant growth in different ways at various developmental stages. Further characterization and analyses of the *hit1* mutants and the function of *HIT1* should provide new insights into the importance and the diverse roles of tethering factors in plants.

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