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SciVerse ScienceDirect
Journal of Genetics and Genomics 39 (2012) 81−92
JGG

ORIGINAL RESEARCH

Arabidopsis AtVPS15 Plays Essential Roles in Pollen Germination Possibly by Interacting with AtVPS34

Wei-Ying Wang [a], Li Zhang [a], Shufan Xing [a], Zhiqiang Ma [a], Jingjing Liu [a], Hongya Gu [a,b], Genji Qin [a,*], Li-Jia Qu [a,b,*]

[a] *State Key Laboratory of Protein and Plant Gene Research, College of Life Sciences, Peking University, Beijing 100871, China*
[b] *The National Plant Gene Research Center (Beijing), Beijing 100101, China*

Received 29 December 2011; revised 4 January 2012; accepted 5 January 2012
Available online 14 January 2012

ABSTRACT

VPS15 protein is a component of the phosphatidylinositol 3-kinase complex which plays a pivotal role in the development of yeast and mammalian cells. The knowledge about the function of its homologue in plants remains limited. Here we report that AtVPS15, a homologue of yeast VPS15p in *Arabidopsis*, plays an essential role in pollen germination. Homozygous T-DNA insertion mutants of *AtVPS15* could not be obtained from the progenies of self-pollinated heterozygous mutants. Reciprocal crosses between *atvps15* mutants and wild-type *Arabidopsis* revealed that the T-DNA insertion was not able to be transmitted by male gametophytes. DAPI staining, Alexander's stain and scanning electron microscopic analysis showed that *atvps15* heterozygous plants produced pollen grains that were morphologically indistinguishable from wild-type pollen, whereas *in vitro* germination experiments revealed that germination of the pollen grains was defective. GUS staining analysis of transgenic plants expressing the GUS reporter gene driven by the *AtVPS15* promoter showed that *AtVPS15* was mainly expressed in pollen grains. Finally, DUALmembrane yeast two-hybrid analysis demonstrated that AtVPS15 might interact directly with AtVPS34. These results suggest that *AtVPS15* is very important for pollen germination, possibly through modulation of the activity of PI3-kinase.

KEYWORDS: *Arabidopsis*; AtVPS15; Pollen germination; PI3-kinase

1. INTRODUCTION

Proteins must be transported from the site of synthesis to the site of function in eukaryotic cells. A large number of proteins involved in vacuolar protein sorting were identified by selection of vacuolar-defective mutants in the yeast *Saccharomyces cerevisiae*. Vacuolar protein sorting (*vps*) defective mutants cannot deliver hydrolases to vacuoles correctly (Bankaitis et al., 1986; Rothman and Stevens, 1986; Robinson et al., 1988). The *VPS15p* gene is essential for vacuolar protein sorting processes, and several vacuolar hydrolases are not delivered to the vacuole in yeast *vps15p* loss-of-function mutants (Robinson et al., 1988). VPS15p is related to VPS34p (Stack et al., 1993; Budovskaya et al., 2002). The *VPS34p* gene encodes an 875-amino acid protein that shows phosphatidylinositol 3-kinase (PI3K) activity *in vitro* (Schu et al., 1993). VPS34p is able to produce PI3P by specifically phosphorylating phosphatidylinositol (Schu et al., 1993). Both *vps34p* and *vps15p* mutants display defects in protein sorting and growth in yeast (Stack et al., 1993). VPS15p, VPS34p and VPS30p form a complex that regulates protein trafficking in yeast cells (Kihara et al., 2001a; Stein et al., 2005; Funderburk et al., 2010; Thoresen et al., 2010).

* Corresponding authors. Tel: +86 10 62752409, fax: +86 10 6275 3319 (G. Qin); Tel: +86 10 62753018, fax: +86 10 6275 3319 (L.-J. Qu).
E-mail addresses: qingenji@pku.edu.cn (G. Qin); qulj@pku.edu.cn (L.-J. Qu).

doi:10.1016/j.jgg.2012.01.002

Pollen germination is a rapid event during pollination, and is critical for successful sexual reproduction in plants. In *Arabidopsis*, pollen germination starts with hydration regulated by aquaporins, which are long-chain lipids and pollen-coat proteins (Preuss et al., 1993; Ikeda et al., 1997; Chrispeels et al., 1999; Mayfield and Preuss, 2000). When pollen hydration is complete, Ca^{2+} flows into the pollen grain and forms a cytoplasmic calcium gradient beneath the site of germination, which is essential for pollen-tube tip growth (Obermeyer and Weisenseel, 1991; Rathore et al., 1991; Pierson et al., 1994; Franklin-Tong et al., 1996). Meanwhile, callose is deposited at the pore where the pollen tube emerges, which aids pollen tube extrusion (Johnson and McCormick, 2001). Many pollen germination and pollen tube growth mutants have been identified recently. The *male gametophyte defective*2 (*mgp*2) mutant shows inhibited pollen germination (Deng et al., 2010). FIMBRIN5 (FIM5) is needed in actin cytoskeleton organization in pollen grains and pollen tubes. Loss-of-function of FIM5 causes delayed pollen germination and inhibition of pollen tube growth (Wu et al., 2010). *Arabidopsis* VILLIN5 is necessary for normal pollen tube growth owing to its binding actin filaments (Zhang et al., 2010). Recently, the homologues of yeast *VPS34p* and *VPS30p* have been shown to be essential for pollen germination in *Arabidopsis*. Loss of function of either AtVPS34 or AtVPS30 leads to defective pollen development and germination (Fujiki et al., 2007; Qin et al., 2007; Harrison-Lowe and Olsen, 2008; Lee et al., 2008). AtVPS30 colocalizes with the pre-autophagosomal structure marker protein AtAtg8. Pollen germination is disrupted in *atvps30* mutants, in which deficiency of AtVPS30 affects genes related to autophagy, VPS and the glycosylphosphatidylinositol anchor system. The *atvps34* mutant shows reduced numbers of nuclei in pollen grains. In addition, *atvps34* pollen grains contain large vacuoles, whereas many small vacuoles are present in wild-type pollen grains. These results suggest that vacuole fission in *atvps34* pollen is abnormal, and vacuolar reorganization is affected by loss of function of *AtVPS34*. However, the molecular mechanisms by which the PI3-kinase complex controls pollen germination are still largely unknown.

Here, we describe the analysis of *Arabidopsis AtVPS15* mutants that produced pollen grains morphologically identical to those of wild-type plants, but displayed obvious pollen germination defects. The *AtVPS15* gene was mainly expressed in pollen. Yeast two-hybrid analysis revealed that AtVPS15 might interact directly with AtVPS34. Our work suggests that AtVPS15 and AtVPS34 might work together in pollen germination in *Arabidopsis*.

2. MATERIALS AND METHODS

2.1. Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used throughout experiments. All the SALK mutants used in this study (i.e., *SALK_006558*, *SALK_004719*, *SALK_002285*) were requested from the Arabidopsis Biological Resource Center (ABRC) (Alonso and Stepanova, 2003). The seeds of mutants were grown on solid half-strength Murashige and Skoog (1/2MS) media containing 10 μg/mL kanamycin for about 10 days at 22°C and segregation ratios for kanamycin-resistance were calculated for identifying T-DNA transmission ratios. *atvps15-3/+ qrt1/qrt1* double mutants were obtained by crossing *atvps15-3/+* with *qrt1* mutants. F_2 progeny were screened on half-strength MS medium with 10 μg/mL of kanamycin and pollens from resistant plants were examined under the microscope for the *qrt1* phenotype.

2.2. Gene cloning, vector construction, and transformation

The open reading frame of *AtVPS15* was amplified from *Arabidopsis* cDNA by PCR and cloned into the gateway vector pB7WGF4, which was modified from the gateway vector pB7WGF2 by replacing *CaMV35S* promoter in pB7WGF2 with *LAT52* promoter (Walhout et al., 2000; Earley et al., 2006; Liu et al., 2008). The construct was sequenced to confirm a correct reading frame. The *AtVPS15* promoter was amplified from *Arabidopsis* genomic DNA. The construct proAtVPS15:GUS was generated by ligating *Sal* I-*Sma* I pBI101 fragment with *Sal* I-*Sma* I pBluescript SK^+ (pBS) harboring *AtVPS15* promoter. Amino acid sequences were aligned using the Needle (v6.0.1) on line program (Needleman and Wunsch, 1970).

Constructs were transformed into *Agrobacterium tumefaciens* GV3101/pMP90, using a freeze-thaw procedure (Holsters et al., 1978). *Arabidopsis* transformation and transgenic plant screening were carried out as previously described (Qin et al., 2005).

2.3. Pollen germination analysis and microscopy

For cytochemical analysis of pollen, mature anthers or mature pollens were soaked in Alexander's stain or 4,6-diamidino-2-phenylindole (DAPI) solution as described previously (Alexander, 1969; Regan and Moffatt, 1990; Weigel and Glazebrook, 2002). Anthers stained by Alexander's solution were observed under an Olympus BX51 microscope (Olympus, Tokyo, Japan). Pollens stained by DAPI were observed under confocal laser scanning microscope (CLSM, LEICA TCS SPE Confocal Microscope, Japan).

For scanning electron microscopy (SEM), mature tetrads of *atvps15/+ qrt1/qrt1* were dehydrated for one day using silicon dioxide and then mounted and coated as described (Weigel and Glazebrook, 2002). SEM analysis was examined under a scanning electron microscope (JEOL, JSM-6610LV, Japan).

For *in vitro* pollen germination assay, pollens and tetrads were assayed on solid germination medium. The germination medium was used as described previously (Li et al., 1999). Pollen grains were incubated at 22°C and 100% humidity for 6 h. For statistical analysis of germination ratios, each assay was repeated three times, and pollen grains were collected from more than 10 different plants. Pollen tubes and pollen grains were counted using SPOT software (Spot Imaging Solutions, USA).