Plant Growth Regul (2015) 77:57–65
DOI 10.1007/s10725-015-0035-0

ORIGINAL PAPER

Requirement of R-SNAREs VAMP721 and VAMP722 for the gametophyte activity, embryogenesis and seedling root development in *Arabidopsis*

Liang Zhang · Wanchang Li · Tianqi Wang · Fengxia Zheng · Jingyuan Li

Received: 3 December 2014 / Accepted: 29 January 2015 / Published online: 4 February 2015

Abstract Membrane fusion between transport vesicles and target membranes is mediated by SNARE complex, a key regulator of vesicular traffic. A functional SNARE complex consists of four coiled-coil helical bundles supplied by Q-SNARE and R-SNARE. Here, we analyze the *Arabidopsis* R-SNAREs VAMP721 and VAMP722. Reciprocal crosses indicated that the transmission of *vamp721vamp722* allele was slightly reduced through gametophytes of $VAMP721^{-/-}$-$VAMP722^{+/-}$ plants and obviously blocked through pollen of $VAMP721^{+/-}VAMP722^{-/-}$ plants. The observation of embryogenesis showed that *vamp721vamp722* mutations resulted in abnormal embryo morphology, such as embryos with asymmetric developing cotyledons, three developing cotyledons, unfolded cotyledons and roots, and partial arrested embryo development at globular stage. Moreover, double mutant seedlings grew rudimentary roots displaying reduced meristem zone, disorganized QC cells, and disordered cell layer pattern and cell file alignment. Confocal images revealed that VAMP721 and VAMP722 were expressed throughout whole root. Taken together, our results suggest that VAMP721 and VAMP722 are involved in gametophyte transmission, embryo development and seedling root growth in *Arabidopsis*.

Keywords R-SNARE · Gametophyte transmission · Embryogenesis · Root growth · *Arabidopsis*

Abbreviations

DIC	Differential interference contrast
KN	KNOLLE
PVC	Pre-vacuolar compartment
QC	Quiescent center
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
TE	Transmission efficiency
TGN	*trans*-Golgi network
VAMP	Vesicle-associated membrane protein

Introduction

The shuttling of vesicles within different membrane-delimited compartments is a common feature in eukaryotes, which maintains the endomembrane system and delivers proteins to their sites of action in the cell. Generally, the vesicle transport can be divided into four essential steps including vesicle budding, transport, tethering, and fusion (Cai et al. 2007). These steps are tightly regulated by many critical proteins, such as coat proteins, motor proteins, and tethering factors (Cai et al. 2007). The specifity of vesicle targeting and correction of cargo delivery are required for the various physiologic processes during plant growth and development (Pratelli et al. 2004). An understanding of the importance of vesicle traffic in plants is rapidly developing, but its molecular mechanism is mostly unknown.

The fusion of vesicles with specific target compartment, a process mediated by the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex, is the

Electronic supplementary material The online version of this article (doi:10.1007/s10725-015-0035-0) contains supplementary material, which is available to authorized users.

L. Zhang · W. Li · T. Wang · F. Zheng · J. Li (✉)
College of Life Science, Henan Normal University, Xinxiang 453007, China
e-mail: ljy041026@htu.cn

L. Zhang · W. Li · J. Li
Engineering Laboratory of Green Medicinal Material Biotechnology, Xinxiang 453007, Henan Province, China

final step in vesicle-mediated transport (Jahn and Scheller 2006). The SNARE protein family is highly conserved in eukaryotic cells, and all SNARE proteins are characterized by the coiled-coil helices, named SNARE motif, adjacent to a transmembrane domain (Sollner et al. 1993). Based on the conserved amino acids in SNARE motif, SNARE proteins can be classified into four groups: (1) Qa-SNAREs, (2) Qb and Qc-SNAREs, (3) SNAP25-like containing Qb and Qc tandem SNARE motifs and (4) R-SNAREs (Fasshauer et al. 1998). Q-SNAREs, also called t-SNAREs, are localized to target organelle membranes, while R-SNARE, also called v-SNAREs, is localized to vesicle membranes. One set of Qa-, Qb-, Qc- and R-SNAREs constitutes a functional SNARE complex with a four-helical bundle assembled by four SNARE motifs (Hong 2005). The correct combinations of cognate SNAREs drive specific membrane fusions.

R-SNAREs can be further subdivided into short VAMPs (vesicle-associated membrane proteins) or brevins and long VAMPs or longins within the animal and fungi lineage (Rossi et al. 2004). In plants, only longin type R-SNAREs exist (Sanderfoot 2007). The *Arabidopsis* genome encodes 15 R-SNAREs including two Sec22-like, two Ykt6-like and 11 VAMP7-like longin R-SNAREs (Lipka et al. 2007). The VAMP7-like proteins in higher plants consist of two major groups: VAMP71 and VAMP72 groups (Sanderfoot 2007). The VAMP72 group appears to be specific to the green plant lineage and likely represents the R-SNARE components for secretion (Sanderfoot 2007). Recently, the significance of R-SNAREs in plant growth has received considerable attention.

Previously it has been reported that *Arabidopsis* R-SNAREs VAMP721 and VAMP722 are involved in immune responses to pathogens (Kwon et al. 2008). Our previous work showed that loss function of VAMP721 and VAMP722 resulted in a dwarf seedling phenotype characterized with rudimentary roots, cotyledons and hypocotyls (Zhang et al. 2011). Recently, Yi et al. (2013) found that ABA treatment enhanced growth inhibition and early depletion of the amount of VAMP721/722 protein level in haploinsufficient $VAMP721^{+/-}$ $VAMP722^{-/-}$ and $VAMP721^{-/-}VAMP722^{+/-}$ plants (Yi et al. 2013). In this study, we further investigated the function of VAMP721 and VAMP722 in plant growth by analyzing mutant phenotypes. Our results suggest that VAMP721 and VAMP722 are essential for embryo development and normal cell growth in seedling roots.

Materials and methods

Plant materials and growth conditions

All plants used for experiments were Arabidopsis Col-0. The homozygous *vamp721* (At1g04750), *vamp722* (At2g33120) mutants, SALK_037273, SALK_119149 and $vamp721^{-/-}$ $vamp722^{+/-}$, $vamp721^{+/-}vamp722^{-/-}$ heterozygous double mutants were described previously (Kwon et al. 2008). According to the aberrant seedling morphology, *vamp721-vamp722* double mutant seedlings were isolated from progeny of either heterozygous double mutants (Zhang et al. 2011). Plants expressing GFP-VAMP721 and GFP-VAMP722 (Zhang et al. 2011) were used for confocal analysis. For growing seedlings on agar-containing plates, *Arabidopsis* seeds were pretreated with 70 % ethanol for 1 min, surface-sterilized in 2.5 % bleach for 10 min, and washed with distilled water. The seeds were planted on 1/2 MS medium (Sigma) supplemented with 1 % (w/v) sucrose, 1 % (w/v) agar (pH 5.8), and placed at 4 °C in the dark for 48 h before germination. Growth conditions were at 23 °C with a 16-h-light/8-h-dark cycle.

Progeny analysis and genotyping

For the progeny analysis of self-pollinated heterozygous double mutant plants, the *Arabidopsis* seeds were planted horizontally on 1/2 MS solid medium. The *vamp721-vamp722* seedlings were counted based on its obviously abnormal morphology within 7 days after germination. The remaining seedlings were identified after 25-days growth in medium. For the progeny analysis of reciprocal crosses, 25-days-old seedlings cultured on 1/2 MS solid medium were used for the genotyping. The transmission efficiency (TE) = (observed number of mutant alleles/observed number of wild-type alleles) × 100, as described previously (Ebel et al. 2004). The genotypes of T-DNA insertion lines were determined by a PCR-based method. The following primers were used: LBb1 (5′-GCGTGGACCGCTTGCTGCAACTCTC-3′), LP1 (5′-CCCCCGTCCATTAAGAATTAAG-3′), RP1 (5′-ATTGAGGACAGAAAGGGTCAGATTC-3′) for *vamp721*, and LBb1 (5′-GCGTGGACCGCTTGCTGCAACTCTC-3′), LP2 (5′-CTCTGAGATCGGTCCCGTAAAATCGG-3′), RP2 (5′-AACTATGCCCATGAATCATAGAC-3′) for *vamp722*.

Complementation of the vamp721vamp722 double mutant

For rescuing the *vamp721vamp722* mutant, 1.8 kb *VAMP721* promoter and 2.0 kb *VAMP722* promoter before the start codon of each gene were amplified from genomic DNA of wild-type *Arabidopsis thaliana* ecotype Columbia plants and cloned into the pCAMBIA1300 binary expression vector with *Hind*III and *Sal*I respectively. The genomic sequence of *VAMP721* plus 1.0 kb 3′UTR was PCR amplified and cloned into the pCAMBIA1300 with *Sal*I and *Kpn*I under *VAMP721* promoter. The genomic sequence of *VAMP722* plus 1.0 kb 3′UTR was PCR

amplified and cloned into the pCAMBIA1300 with *BamH*I and *EcoR*I under *VAMP722* promoter. All The sequences cloned above were checked by sequencing. The rescue constructs were transformed into *Arabidopsis tumefaciens* strain GV3101. The resulting *A. tumefaciens* transformants were used to transform *vamp721*$^{-/-}$ *vamp722*$^{+/-}$ and *vamp721*$^{+/-}$*vamp722*$^{-/-}$ plants, respectively using the floral dip method (Clough and Bent 1998). The selection of transgenic lines was performed on 1/2 MS solid medium containing 3 % sucrose with 25 μg/ml hygromycin. The homozygous identity of T-DNA insertion of the rescued plants was confirmed by PCR assay in the T2 plants.

Clearing of *Arabidopsis* embryos

For clearing of Arabidopsis embryos, growing siliques were harvested from soil-grown plants and dissected under a stereo-microscope. Ovules from individual siliques were collected and fixed for 1–4 h in ethanol/acetic acid (6:1) at room temperature. Then, ovules were washed three times for 5 min in 100 % ethanol and one time in 70 % ethanol. In turn, the ovules were incubated in a clearing solution (chloralhydrate/glycerol/water 8:1:2 v/v) for 24 h, mounted on slide with 30 % glycerol, and observed by Olymplus BX51 with Nomarsky Differential Interference Contrast (DIC) optics.

Cross-section analysis of wild-type and vamp721vamp722 mutant roots

The root tissue from 4-day-old seedling was cut and immediately vacuum infiltrated and fixed with 2.5 % (v/v) glutaraldehyde and 2 % (v/v) paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.2) at room temperature for 4 h, followed by postfixation in 1 % OsO4 buffer at 4 °C overnight. Samples were subsequently rinsed with 0.1 M phosphate buffer and dehydrated through a graded ethanol series (30–100 %). Then, samples were embedded in LR White (EMS) and polymerized at 60 °C for 24 h. Semithin (1 mm) sections were cut using an ultracut microtome (EM UC6; Leica). Semthin sections were stained with toluidine blue O before observation.

Confocal microscopy

For confocal analysis, seedlings mounted in half-strength MS liquid were analyzed with Leica SP5 confocal microscope. For imaging of GFP, the signals were visualized by excitation with an Argon laser at 488 nm and detected with a 500- to 550-nm emission filter. The images were edited using Image J software and Adobe Photoshop CS2.

Statistical analysis

The segregation ratios of progeny genotypes recovered from self-pollinated heterozygous double mutant plants were analyzed with χ^2 test using SPSS software.

Results

vamp721 shows synthetic lethality with *vamp722*

We previously identified the *vamp721vamp722* homozygous double mutant seedlings from the progeny of heterozygous double mutants cultured on the 1/2 MS solid medium (Zhang et al. 2011). In this study, we analyzed the progeny segregation in detail. The results showed that the percentage of *vamp721vamp722* seedlings segregated from *VAMP721*$^{-/-}$*VAMP722*$^{+/-}$ mutants was 17 %, less than the expected 25 %. Similar results were obtained from *VAMP721*$^{+/-}$*VAMP722*$^{-/-}$ mutants (Table 1). Moreover, the segregation ratios of progeny genotypes recovered from the two parental lines *VAMP721*$^{-/-}$*VAMP722*$^{+/-}$ and *VAMP721*$^{+/-}$*VAMP722*$^{-/-}$ indicated a deviation from the expected 1:2 segregation, but this was statistically significant only for the latter (Table S1), suggesting that the gametophytic activity was disturbed at some extent.

The analysis of gametophyte transmission

To determine whether VAMP721 and VAMP722 play a role in gametophyte development, we analyzed the gametophyte transmission between heterozygous double mutant and wild-type plants (Table 2). The results showed that 86.3 and 87.8 % of both *vamp721* and *vamp722* alleles were transmitted via the female gametophytes of *VAMP721*$^{-/-}$*VAMP722*$^{+/-}$ and

Table 1 Progeny analysis of self-pollinated *Arabidopsis* seedlings segregating the *vamp721vamp722* mutants

Parental lines	*VAMP721*$^{-/-}$*VAMP722*$^{+/-}$			*VAMP721*$^{+/-}$*VAMP722*$^{-/-}$		
Progeny genotypes	*VAMP721*$^{-/-}$ *VAMP722*$^{+/+}$	*VAMP721*$^{-/-}$ *VAMP722*$^{+/-}$	*vamp721 vamp722*	*VAMP721*$^{+/+}$ *VAMP722*$^{-/-}$	*VAMP721*$^{+/-}$ *VAMP722*$^{-/-}$	*vamp721 vamp722*
Expected	25 %	50 %	25 %	25 %	50 %	25 %
Observed	64	116	36	88	112	31
	30 %	53 %	17 %	38 %	49 %	13 %

Table 2 Transmission efficiency (TE) of *vamp721vamp722* allele in reciprocal crosses

Parental genotypes (Female × male)	Progeny genotypes				
	VAMP721$^{+/-}$ *VAMP722*$^{+/-}$	*VAMP721*$^{+/-}$ *VAMP722*$^{+/+}$	*VAMP721*$^{+/-}$ *VAMP722*$^{+/-}$	*VAMP721*$^{+/+}$ *VAMP722*$^{+/-}$	TE[a] (%)
VAMP721$^{-/-}$*VAMP722*$^{+/-}$ × WT	88	102			86.3
WT × *VAMP72*$^{1-/-}$*VAMP722*$^{+/-}$	80	89			89.9
VAMP721$^{+/-}$*VAMP722*$^{-/-}$ × WT			101	115	87.8
WT × *VAMP721*$^{+/-}$*VAMP722*$^{-/-}$			82	152	53.9

[a] The genetic transmission of *vamp721vamp722* allele was analyzed by genotyping F1 progeny of the denoted crosses. The TE was calculated as described in "Materials and methods"

VAMP721$^{+/-}$*VAMP722*$^{-/-}$, respectively. When double heterozygous mutant plants were used as the pollen donors, 89.9 % of *vamp721vamp722* gametes were inherited from the male parental line *VAMP721*$^{-/-}$*VAMP722*$^{+/-}$. While, 53.9 % of the double mutant gametes were transmitted for parent *VAMP721*$^{+/-}$*VAMP722*$^{-/-}$, indicating that the TE of both mutant alleles was reduced through male gametophytes of *VAMP721*$^{+/-}$*VAMP722*$^{-/-}$.

The *vamp721vamp722* mutant shows embryo development defects

The segregation ratio of *vamp721vamp722* seedlings derived from the progenies of self-pollinated *VAMP721*$^{-/-}$*VAMP722*$^{+/-}$ plants suggested that the double mutant was partially embryonic lethal; thus, we examined the premature seeds in the seedpods of *VAMP721*$^{-/-}$*VAMP722*$^{+/-}$ plants. The results showed that some of the seeds from *VAMP721*$^{-/-}$*VAMP722*$^{+/-}$ plants exhibited a yellowish appearance (green:yellow = 629:167); however, almost all the seeds from single mutant *vamp721*, *vamp722*, or wild-type plants showed normal appearance (Fig. S1). We also observed the abnormal appearance of seeds from *VAMP721*$^{+/-}$*VAMP722*$^{-/-}$ plants (Fig. S2).

We then examined the embryogenesis in more detail, observing embryos in cleared seedpods from the *VAMP721*$^{-/-}$*VAMP722*$^{+/-}$ mutant plants at different development stages. We observed the development of control embryos including globular, heart, torpedo, early cotyledon, and late cotyledon stage (Fig. 1a–e). The examination of yellowish seeds in mutant siliques showed that abnormal embryos were observed from late heart to cotyledon stage. As shown in Fig. 1f, one embryo at late heart stage grew two asymmetric developing cotyledons. Similar appearance was observed at the torpedo stage embryo which also exhibited increased lateral proliferation of root compared with the elongated root in control (Fig. 1g). Figure 2h showed that one torpedo stage embryo of mutant plants has three developing cotyledons instead of two in the control. Moreover, cotyledon-stage embryos from mutant plants developed rudimentary cotyledons and roots, and did not fold, or only partially folded, compared to the fully folded embryonic root and cotyledons in the control (Fig. 1i, j). Interestingly, the development of part embryos was arrested at globular-like stage (Fig. 1k), while the control embryos from the same seedpods were at cotyledon stage. We verified that these abnormal embryos were the *vamp721vamp722* double homozygous mutants by PCR-based genotyping (Fig. S3).

The incorporation of a genomic fragment containing the *VAMP721* gene into *VAMP721*$^{-/-}$*VAMP722*$^{+/-}$ plants fully rescued the defective embryo phenotype in the double homozygous mutant (Fig. S1). Similarly, the incorporation of a genomic fragment containing the *VAMP722* gene into *VAMP721*$^{+/-}$*VAMP722*$^{-/-}$ plants also rescued the defective embryo phenotype (Fig. S2).

vamp721vamp722 mutations cause defects in root development

The analysis of embryo development suggested that *vamp721vamp722* mutations likely affect postembryonic root growth. In order to confirm the function of VAMP721 and VAMP722 in root development, we analyzed and compared the root cell morphology in wild-type and double mutant seedlings. In contrast to wild-type seedlings, 4-days-old *vamp721vamp722* seedlings showed severely retarded growth characterized with stunted cotyledon, hypocotyl and root (Fig. 2a). The meristem zone in *vamp721vamp722* roots was smaller than that in wild-type siblings. Moreover, *vamp721vamp722* roots exhibited disorganized columella cells and no obvious transition from elongation to differentiation zone compared with control roots (Fig. 2b, c). In comparison with wild-type roots, the root longitudinal sections of *vamp721vamp722* showed disordered cell layer pattern for epidermis, cortex, and stele, which displayed abnormal cell files, such as expanded cells in epidermis and cortex. Particularly, *vamp721vamp722* roots failed to specify the quiescent center (QC)

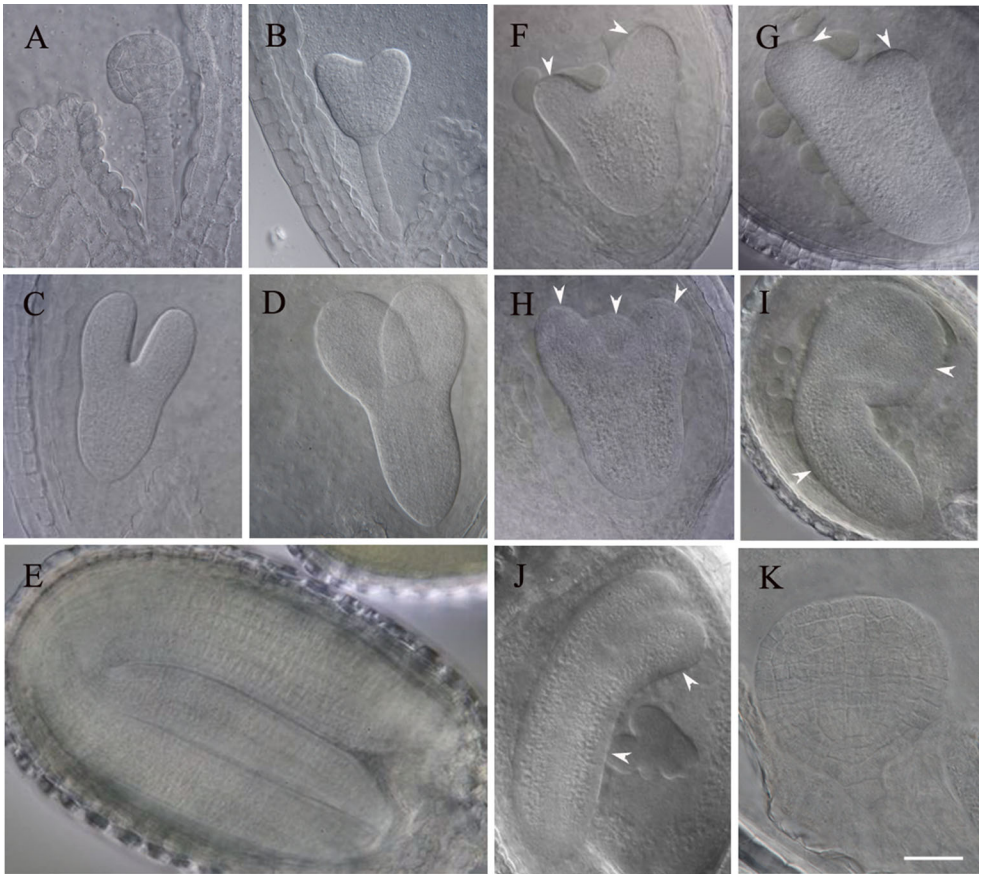

Fig. 1 Embryo development of *vamp721vamp722* double mutant. (**a**–**e**) The wild type-looking (control) embryos at globular (**a**), heart (**b**), torpedo (**c**), and cotyledon stage (**d**, **e**) dissected from seedpods of $VAMP721^{+/-}VAMP722^{-/-}$ plants. (**f**–**j**) *vamp721vamp722* double mutant embryos exhibited abnormal morphology at late heart (**f** *arrowheads* indicate asymmetric developing cotyledons), torpedo (**g**, **h** *arrowheads* indicate asymmetric and three developing cotyledons, respectively), and cotyledon stage (**i**, **j** *arrowheads* indicate rudimentary cotyledons and roots). **k** Embryogenesis of part double mutant was arrested at globular-like stage in the mature seedpods. *Bars* = 50 μm

cells compared with that in wild-type roots (Fig. 2d, e). The disordered cell pattern and cell file alignment were also observed in *vamp721vamp722* root cross sections compared with wild-type roots (Fig. 2f, g).

Early data have shown that VAMP721 and VAMP722 were localized at plasma membrane and unknown organelles in *Arabidopsis* protoplasts by transient assays (Uemura et al. 2004, 2005). Our previous work confirmed the plasma membrane localization of VAMP721 and VAMP722 in transgenic *Arabidopsis* seedings and showed that VAMP721 and VAMP722 were localized to *trans*-Golgi network (TGN) in root cells (Zhang et al. 2011). However, the expression pattern of VAMP721 and VAMP722 in root tissue is still unclear. Thus, we examined the expression of VAMP721 and VAMP722 in root cells using seedlings expressing GFP-VAMP721 or GFP-VAMP722 under control of their native promoters. The results showed that GFP-VAMP721 was expressed in root cap, columella cells, stele cells, epidermis and cortex cells in meristem zone, and cells in elongation zone (Fig. 3a, b). In the differentiation zone, VAMP721 showed strong expression in xylem, root hairs, epidermis and cortex cells (Fig. 3c). The signals of GFP-VAMP722 were also detected in whole root, including meristem, elongation and differentiation zone (Fig. 3d–f), similar to the expression pattern of GFP-VAMP721.

Discussion

Recent studies indicate that SNARE proteins are not restricted to the housekeeping function in membrane fusion, but play important roles in plant shoot morphogenesis (Ohtomo et al. 2005), vascular network formation (Shirakawa et al. 2009), plant defense (Assaad et al. 2004), vacuole biogenesis (Ebine et al. 2008), and abiotic stress responses (Leshem et al. 2006). However, the knowledge about the function of R-SNAREs in plant development is still limited. Based on the sequence information from available

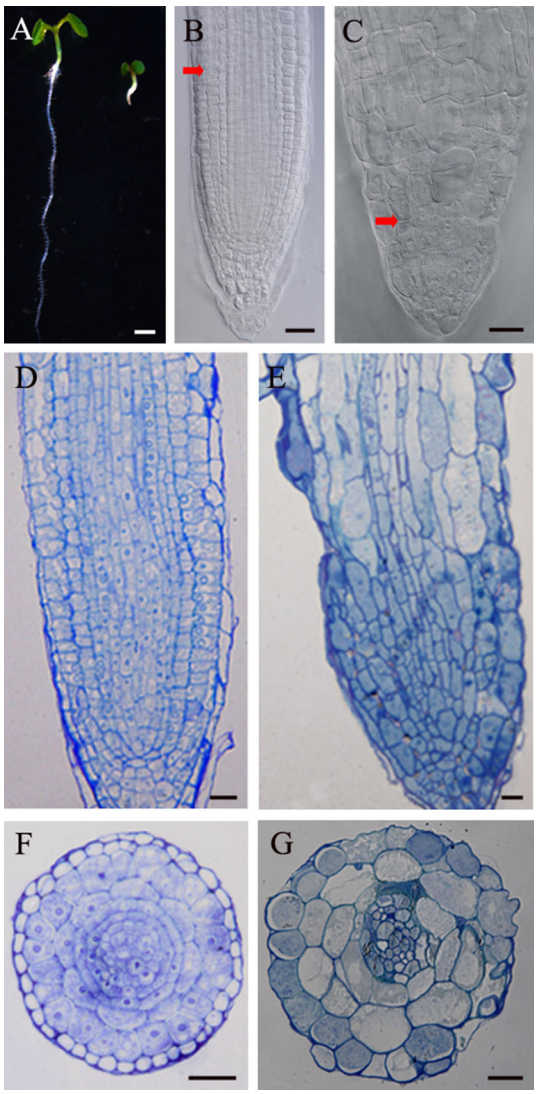

Fig. 2 Phenotypic analysis of *vamp721vamp722* double mutant roots. **a** 4-days-old *vamp721vamp722* seedlings displayed a dwarf stature compared with wild-type seedlings. **b**, **c** The meristem zone in *vamp721vamp722* roots (**b**) was much smaller than in wild type (**c**). The *red arrow* indicates the uppermost cell in meristem zone. **d**, **e** The longitudinal section in wild-type (**d**) and double mutant (**e**) roots. **f**, **g** The cross section in wild-type (**d**) and double mutant (**e**) roots. *Bars* = 1 mm in (**a**); 50 μm in (**b**–**g**). (Color figure online)

Arabidopsis genome assemblies, *VAMP721* and *VAMP722* are classified into R-SNARE members of the VAMP72 group (Sanderfoot 2007). The T-DNA insertion lines of *VAMP721* and *VAMP722* and their heterozygous double mutants were indistinguishable from wild-type plants. Moreover, no homozygous double mutant plants can be isolated from the progeny of heterozygous double mutants (Kwon et al. 2008), suggesting that VAMP721 and VAMP722 have redundant as well as essential functions in plant development. We previously identified the *vamp721-vamp722* double mutant seedlings in 1/2 MS medium (Zhang et al. 2011). In the present study, we analyzed the progeny segregation of heterozygous double mutants and found that $VAMP721^{-/-}VAMP722^{+/-}$ and $VAMP721^{+/-}$-$VAMP722^{-/-}$ plants exhibited a non-mendelian segregation ratio, but this was statistically significant only for the latter, consistent with other reports (Kwon et al. 2008). Particularly, the percentage of *vamp721vamp722* seedlings segregated from either heterozygous double mutant was less than the expected ratio. These results suggest that loss function of VAMP721 and VAMP722 leads to a gametophytic defect and/or portion of embryo lethality.

Assuming random segregation during meiosis and the absence of post-meiotic selection, the genotypes of the gametes should be equally distributed (Howden et al. 1998). In our study, we assessed the TE of double mutant alleles through male and female gametes. Reciprocal crosses between $VAMP721^{-/-}VAMP722^{+/-}$ and wild-type plants revealed that *vamp721vamp722* gametes could be inherited from the male or female parent, but at a slightly reduced frequency. Moreover, the transmission of *vamp721vamp722* allele was partially reduced through the pollen, not the female gametophyte of $VAMP721^{+/-}$-$VAMP722^{-/-}$, suggesting that $VAMP721^{+/-}VAMP722^{-/-}$ plants produce partial either non-viable or non-functional pollen. However, the reduced male gametophyte had no obvious effects on the fertilization of ovules. Recent studies indicated that *Arabidopsis* R-SNARE SEC22 plays an essential role in vesicle trafficking between ER and Golgi, and *sec22* mutation highly reduced male and female gametophyte transmission (El-Kasmi et al. 2011), suggesting that early secretory pathway is required for the gametophyte development. *Arabidopsis* Qa-SNAREs SYP21 and SYP22 mediate the protein trafficking between pre-vacuolar compartments (PVC) and vacuoles and are essential for male gametophytic viability (Shirakawa et al. 2011). In addition, TGN-localized Qa-SNAREs SYP41 and SYP42, which regulate the secretory and vacuolar transport pathways in the post-Golgi network, also play important roles in pollen function (Sanderfoot et al. 2001; Uemura et al. 2012). We previously found that VAMP721 and VAMP722 were localized at TGN and mediated the secretory trafficking to plasma membrane (Zhang et al. 2011). Based on our results together with recent publications, we speculate that the protein traffic mediated by VAMP721 and VAMP722 is required for the gametophyte activity in *Arabidopsis*.

The segregation ratio of *vamp721vamp722* seedlings implied the possibility of partial embryo development defects. The examination of premature seeds revealed that $VAMP721^{-/-}VAMP722^{+/-}$ or $VAMP721^{+/-}VAMP722^{-/-}$ plants developed part of seeds which displayed yellowish appearance. The clearing of embryos from these abnormal seeds showed defective embryo development from late

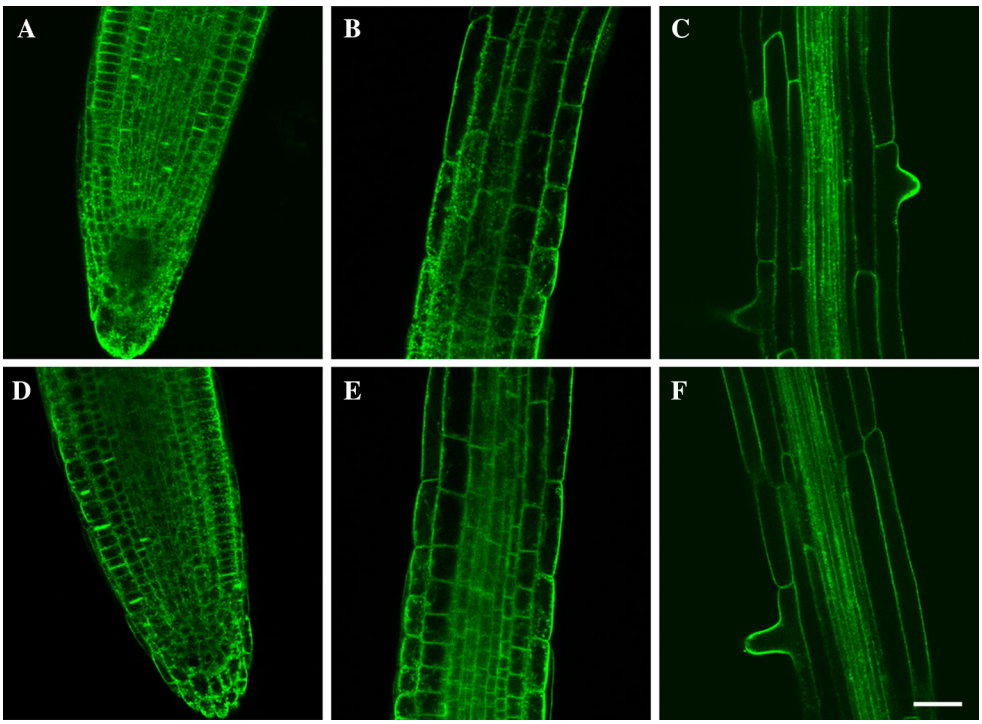

Fig. 3 The expression of VAMP721 and VAMP722 in roots. **a–c** GFP-VAMP721 were expressed in whole root, including root cap, meristem zone (**a**), elongation zone (**b**), and differentiation zone (**c**). **d–f** The expression of GFP-VAMP722 in root cells. *Bars* = 20 μm

heart to cotyledon stage, such as embryos with asymmetric developing cotyledons, expanded roots, three developing cotyledons, and unfolded cotyledons and roots. In addition, the arrested embryo development at globular-like stage was frequently observed from the abnormal seeds, which likely accounts for the reduced segregation ratio of *vamp721-vamp722* seedlings. We confirmed that these abnormal embryos contained both *vamp721* and *vamp722* T-DNA insertions by PCR-based genotyping. The complementation of the *vamp721vamp722* double mutant rescued the defective embryo phenotype, confirming that the embryo alterations were due to the T-DNA insertions in the *VAMP721* and *VAMP722* genes. The microarray data from *Arabidopsis* eFP Browser show that *VAMP721* and *VAMP722* genes are expressed throughout embryo development, and *VAMP721* exhibits a higher expression level than that of *VAMP722* (Fig. S4) (Winter et al. 2007), this might suggest that VAMP721 and VAMP722 play independent functions in embryogenesis. It was reported that *Arabidopsis* v-SNAREs VTI11 and VTI12, two members of VTI1 family, regulated vesicle trafficking to lytic and storage vacuoles, respectively, and loss function of *VTI11* and *VTI12* caused embryo lethality (Surpin et al. 2003; Sanmartin et al. 2007), indicating that the vacuolar trafficking mediated by SNARE proteins is required for embryo viability. The *Arabidopsis* cytokinesis-specific Qa-SNARE KNOLLE (KN) is also essential for embryo development, and mutations in the *KN* gene disrupt the regular pattern of embryogenesis by altering the rate and plane of cell division as well as cell morphology (Lukowitz et al. 1996). Recent studies indicated two distinct types of KNOLLE-containing SNARE complexes appear to jointly medi-ate membrane fusion in *Arabidopsis* cytokinesis and either complex includes VAMP721 and VAMP722 as the R-SNARE components (El Kasmi et al. 2013). Given that VAMP721 and VAMP722 are involved in the cell plate formation during cytokinesis (Zhang et al. 2011), our results suggest that the membrane fusion mediated by VAMP721 and VAMP722 is required for the cell division and cell morphology in embryo development.

vamp721vamp722 seedlings exhibited a dwarf stature characterized with much smaller organs (Zhang et al. 2011). In this study, we focus on the roles of VAMP721 and VAMP722 in root development. Our results showed that *vamp721vamp722* mutations led to severe reduction of meristem zone and mixed elongation and differentiation zone in roots. In addition, disordered cell layer pattern and cell file alignment were observed in root longitudinal and cross sections. Particularly, *vamp721vamp722* mutations impaired the QC patterning. Furthermore, VAMP721 and VAMP722 were expressed in all root cells, confirming their essential function in root growth. Our previous work showed that *vamp721vamp722* mutant root cells frequently exhibited cell wall stubs or gaps and incomplete cytokinesis

characterized with a high incidence of binucleate cells. Moreover, abnormal and asymmetric cell plate assemblies were scored highly in *vamp721vamp722* cytokinetic root cells labeled with cell plate-specific marker GFP-KNOLLE (Zhang et al. 2011). Concerning the significant roles of VAMP721 and VAMP722 in cytokinesis (Zhang et al. 2011), it is reasonable to deduce that the rudimentary roots of *vamp721vamp722* mutants are likely due to the disruption of cell division activity. Furthermore, VAMP721 and VAMP722 were shown to localize at plasma membrane and TGN compartment in non-dividing cells and were required for the secretory trafficking to plasma membrane, since the GFP-tagged PM proteins aquaporin PIP2a and low temperature responsive protein LTI6A were severely accumulated in the cytoplasm of root cells (Zhang et al. 2011). Our new findings indicate that VAMP721 and VAMP722 are involved in the polar PM localization of auxin transporters (our unpublished data). Aquaporins are ubiquitous channel proteins that facilitate root water uptake (Javot et al. 2003). Auxin transporters are essential for the directional auxin transport and cellular auxin homeostasis which plays important roles in root development, such as root meristem patterning and tropic growth (Rahman et al. 2010; Cazzonelli et al. 2013). Based on the analysis above, we speculate that VAMP721 and VAMP722 probably play a general role in the secretory trafficking of PM proteins, such as channel proteins and transporters which are essential for root growth. Taken together, our results suggest that the protein trafficking mediated by VAMP721 and VAMP722 is essential for root development.

In conclusion, R-SNARE proteins play essential roles in plant growth and development in addition to vesicle trafficking. Our results indicated that R-SNAREs VAMP721 and VAMP722 are required for the gametophyte activity in *Arabidopsis*. Furthermore, *vamp721vamp722* mutations resulted in abnormal embryo and seedling root development. Based on the function of SNARE protein in membrane traffic, we propose that VAMP721 and VAMP722 are involved in plant development through the regulation of protein trafficking related to specific physiologic process.

Acknowledgments This work was funded by the National Natural Science Foundation of China (31300163, 31370219 and 31270225) and Research Start-up Funds (qd12133).

References

Assaad FF, Qiu JL, Youngs H, Ehrhardt D, Zimmerli L, Kalde M, Wanner G, Peck SC, Edwards H, Ramonell K, Somerville CR, Thordal-Christensen H (2004) The PEN1 syntaxin defines a novel cellular compartment upon fungal attack and is required for the timely assembly of papillae. Mol Biol Cell 15(11):5118–5129

Cai H, Reinisch K, Ferro-Novick S (2007) Coats, tethers, Rabs, and SNAREs work together to mediate the intracellular destination of a transport vesicle. Dev Cell 12(5):671–682

Cazzonelli CI, Vanstraelen M, Simon S, Yin K, Carron-Arthur A, Nisar N, Tarle G, Cuttriss AJ, Searle IR, Benkova E, Mathesius U, Masle J, Friml J, Pogson BJ (2013) Role of the *Arabidopsis* PIN6 auxin transporter in auxin homeostasis and auxin-mediated development. PLoS One 8(7):e70069

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium- mediated transformation of *Arabidopsis thaliana*. Plant J 16(6):735–743

Ebel C, Mariconti L, Gruissem W (2004) Plant retinoblastoma homologues control nuclear proliferation in the female gametophyte. Nature 429(6993):776–780

Ebine K, Okatani Y, Uemura T, Goh T, Shoda K, Niihama M, Morita MT, Spitzer C, Otegui MS, Nakano A, Ueda T (2008) A SNARE complex unique to seed plants is required for protein storage vacuole biogenesis and seed development of *Arabidopsis thaliana*. Plant Cell 20(11):3006–3021

El Kasmi F, Krause C, Hiller U, Stierhof YD, Mayer U, Conner L, Kong L, Reichardt I, Sanderfoot AA, Jurgens G (2013) SNARE complexes of different composition jointly mediate membrane fusion in *Arabidopsis* cytokinesis. Mol Biol Cell 24(10):1593–1601

El-Kasmi F, Pacher T, Strompen G, Stierhof YD, Muller LM, Koncz C, Mayer U, Jurgens G (2011) *Arabidopsis* SNARE protein SEC22 is essential for gametophyte development and maintenance of Golgi-stack integrity. Plant J 66(2):268–279

Fasshauer D, Sutton RB, Brunger AT, Jahn R (1998) Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. Proc Natl Acad Sci USA 95(26):15781–15786

Hong W (2005) SNAREs and traffic. Biochim Biophys Acta 1744(2):120–144

Howden R, Park SK, Moore JM, Orme J, Grossniklaus U, Twell D (1998) Selection of T-DNA-tagged male and female gametophytic mutants by segregation distortion in *Arabidopsis*. Genetics 149(2):621–631

Jahn R, Scheller RH (2006) SNAREs–engines for membrane fusion. Nature 7(9):631–643

Javot H, Lauvergeat V, Santoni V, Martin-Laurent F, Guclu J, Vinh J, Heyes J, Franck KI, Schaffner AR, Bouchez D, Maurel C (2003) Role of a single aquaporin isoform in root water uptake. Plant Cell 15(2):509–522

Kwon C, Neu C, Pajonk S, Yun HS, Lipka U, Humphry M, Bau S, Straus M, Kwaaitaal M, Rampelt H, El Kasmi F, Jurgens G, Parker J, Panstruga R, Lipka V, Schulze-Lefert P (2008) Co-option of a default secretory pathway for plant immune responses. Nature 451(7180):835–840

Leshem Y, Melamed-Book N, Cagnac O, Ronen G, Nishri Y, Solomon M, Cohen G, Levine A (2006) Suppression of *Arabidopsis* vesicle-SNARE expression inhibited fusion of H_2O_2-containing vesicles with tonoplast and increased salt tolerance. Proc Natl Acad Sci USA 103(47):18008–18013

Lipka V, Kwon C, Panstruga R (2007) SNARE-ware: the role of SNARE-domain proteins in plant biology. Annu Rev Cell Dev Biol 23:147–174

Lukowitz W, Mayer U, Jurgens G (1996) Cytokinesis in the *Arabidopsis* embryo involves the syntaxin-related KNOLLE gene product. Cell 84(1):61–71

Ohtomo I, Ueda H, Shimada T, Nishiyama C, Komoto Y, Hara-Nishimura I, Takahashi T (2005) Identification of an allele of VAM3/SYP22 that confers a semi-dwarf phenotype in *Arabidopsis thaliana*. Plant Cell Physiol 46(8):1358–1365

Pratelli R, Sutter JU, Blatt MR (2004) A new catch in the SNARE. Trends Plant Sci 9(4):187–195

Rahman A, Takahashi M, Shibasaki K, Wu S, Inaba T, Tsurumi S, Baskin TI (2010) Gravitropism of *Arabidopsis thaliana* roots requires the polarization of PIN2 toward the root tip in meristematic cortical cells. Plant Cell 22(6):1762–1776

Rossi V, Banfield DK, Vacca M, Dietrich LE, Ungermann C, D'Esposito M, Galli T, Filippini F (2004) Longins and their longin domains: regulated SNAREs and multifunctional SNARE regulators. Trends Biochem Sci 29(12):682–688

Sanderfoot A (2007) Increases in the number of SNARE genes parallels the rise of multicellularity among the green plants. Plant Physiol 144(1):6–17

Sanderfoot AA, Pilgrim M, Adam L, Raikhel NV (2001) Disruption of individual members of *Arabidopsis* syntaxin gene families indicates each has essential functions. Plant Cell 13(3):659–666

Sanmartin M, Ordonez A, Sohn EJ, Robert S, Sanchez-Serrano JJ, Surpin MA, Raikhel NV, Rojo E (2007) Divergent functions of VTI12 and VTI11 in trafficking to storage and lytic vacuoles in *Arabidopsis*. Proc Natl Acad Sci USA 104(9):3645–3650

Shirakawa M, Ueda H, Shimada T, Nishiyama C, Hara-Nishimura I (2009) Vacuolar SNAREs function in the formation of the leaf vascular network by regulating auxin distribution. Plant Cell Physiol 50(7):1319–1328

Shirakawa M, Ueda H, Shimada T, Koumoto Y, Shimada TL, Kondo M, Takahashi T, Okuyama Y, Nishimura M, Hara-Nishimura I (2011) *Arabidopsis* Qa-SNARE SYP2 proteins localized to different subcellular regions function redundantly in vacuolar protein sorting and plant development. Plant J 64(6):924–935

Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE (1993) SNAP receptors implicated in vesicle targeting and fusion. Nature 362(6418):318–324

Surpin M, Zheng H, Morita MT, Saito C, Avila E, Blakeslee JJ, Bandyopadhyay A, Kovaleva V, Carter D, Murphy A, Tasaka M, Raikhel N (2003) The VTI family of SNARE proteins is necessary for plant viability and mediates different protein transport pathways. Plant Cell 15(12):2885–2899

Uemura T, Ueda T, Ohniwa RL, Nakano A, Takeyasu K, Sato MH (2004) Systematic analysis of SNARE molecules in *Arabidopsis*: dissection of the post-Golgi network in plant cells. Cell Struct Funct 29(2):49–65

Uemura T, Sato MH, Takeyasu K (2005) The longin domain regulates subcellular targeting of VAMP7 in *Arabidopsis thaliana*. FEBS Lett 579(13):2842–2846

Uemura T, Kim H, Saito C, Ebine K, Ueda T, Schulze-Lefert P, Nakano A (2012) Qa-SNAREs localized to the trans-Golgi network regulate multiple transport pathways and extracellular disease resistance in plants. Proc Natl Acad Sci USA 109(5):1784–1789

Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ (2007) An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS One 2(8):e718

Yi C, Park S, Yun HS, Kwon C (2013) Vesicle-associated membrane proteins 721 and 722 are required for unimpeded growth of *Arabidopsis* under ABA application. J Plant Physiol 170(5):529–533

Zhang L, Zhang H, Liu P, Hao H, Jin JB, Lin J (2011) *Arabidopsis* R-SNARE proteins VAMP721 and VAMP722 are required for cell plate formation. PLoS ONE 6(10):e26129